IN SEARCH OF BETTER MANAGEMENT OPTIONS FOR POTATO (*Solanum tuberosum* L.) VIRUSES: IMPROVING METHODS OF DETECTION, AND REVEALING NEW SOURCES OF RESISTANCE

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Authorization to Submit Thesis

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Abstract

Traditionally, *Potato virus* Y (PVY) strains have been defined based on genetic reactions in potato indicators expressing hypersensitive reaction (HR) response due to the presence of different *N* genes. Currently, there are five known PVY isolates: PVY^{O}, PVY^{C}, PVY^{Z}, PVY^{N}, and PVY^{E}, with PVY^{C} and PVY^{E} being uncommon in North America. The genetic background of North American potato cultivars, thus far, has been poorly characterized for the presence of N genes producing HR response to various PVY strains. I studied HR response in eight potato cultivars, (Russet Norkotah, Ranger Russet, Russet Burbank, Alturas, Western Russet, Yukon Gem, Rio Grande, and Shepody) induced by five strains of PVY commonly circulating in North America. A number of these cultivars were found to contain *N* genes – including a several new putative *N* genes. In order to support my findings of a putative gene Nz_{tbr} (that was proposed to confer resistance against the distinct strain PVY^{Z}), I report on a genetic study of the Nz_{tbr} inheritance in crosses between three cultivars. The cultivars chosen were Maris Bard (*Ny:Nz*), King Edward (*ny:nz*), and Russet Norkotah (*ny:nz*). These crosses subsequently proved the existence of Nz_{tbr} – which is a nuclear gene. For our research, as well as for potato seed certification programs, significant amounts of specific antibodies for large-scale screenings are required. Sandwich ELISA assays, the most convenient method, require antibodies from different animal species. Unfortunately, this adds complexity to reagent production. Here, I describe a biotechnological approach to circumvent the problem of host protein contamination of the antigen for potato viruses. I developed detection systems for six potato viruses, PVY, PVS, PVX, PVA and PLRV. This document, as a whole, improves detection methods for potato viruses and describes a new way of looking at resistance.

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Dedication

I would like to thank my husband, Daniel Rowley, for his outstanding love and support during my studies at the University of Idaho, and for his extra help and understanding during my pregnancy. My parents are always there to listen to what I am doing with my research, and they have always been my biggest fans. Thank you!

Table of Contents

List of Figures

List of Tables

Chapter 1

Interactions between strains of Potato virus Y circulating in North America and N genes in potato (*Solanum Tuberosum* L.)

1.1 Abstract

Traditionally, *Potato virus Y* (PVY) strains have been defined based on genetic reactions in potato indicators expressing a hypersensitive reaction (HR) response due to the presence of different *N* genes. Five PVY strains are known currently, PVY^{O}, PVY^{C}, PVY^{Z}, PVY^{N}, and PVY^{E}, with PVY^{C} and PVY^{E} rarely found in North America (Blanchard et al, 2008). The genetic background of the majority of North American potato cultivars has so far been poorly characterized for the presence of *N* genes inducing HR towards different PVY strains. Here, I studied the HR response in eight potato cultivars (Russet Norkotah, Ranger Russet, Russet Burbank, Alturas, Western Russet, Yukon Gem, Rio Grande, Shepody) induced by five strains of PVY circulating in North America. These PVY isolates included representatives of PVY^{N-Wi}, PVY^{NA-N}, PVY^{O}, PVY^{Z}-NTN, and PVY^{N} strains. Potato cultivars tested included most common cultivars in the U.S., and standard indicators Desiree and Maris Bard that have known genetic backgrounds. Virus-free potato plants were mechanically inoculated with PVY inoculum, and local and systemic foliar symptoms were observed for 8 weeks post-inoculation under climate-controlled conditions. Virus status of the inoculated plants was tested starting at 3 weeks post-inoculation, by serotype-specific ELISA and RT-PCR, in order to monitor successful infections and confirm the identity of the inoculated PVY isolate. This systematic approach allowed me to identify *Ny* and *Nz* genes present in several North American cultivars. Three more new, putative *N* genes have been postulated as being expressed in the cultivar Yukon Gem. These *N* genes could represent a valuable source of resistance to PVY isolates from eight strains of PVY.

1.2 Introduction

Potato virus Y (PVY) exists as a complex of strains which are currently classified using two different systems, molecular and genetic or biological (see Karasev and Gray, 2013a,b for a

recent review). The original classification of PVY strains was based on the biological reactions of PVY isolates in two hosts, potato and tobacco, and strains of PVY were defined based on reactions to a set of potato cultivars with a known genetic backgrounds (Cockerham, 1970; deBokx and Huttinga, 1981; Singh et al., 2008; Karasev and Gray, 2013a,b). The strain designated as PVY^O comprises isolates inducing hypersensitive resistance (HR) in the presence of the Ny_{tbr} gene, while the strain designated as PVY^C comprises isolates inducing HR in the presence of the Nc_{tbr} gene. The strain designated as PVY^N was defined as comprising PVY isolates that failed to induce HR in the presence of either Ny_{tbr} or Nc_{tbr} genes. Jones (1990) reported on a new strain of PVY, PVY^Z, which failed to elicit HR in the presence of Ny_{tbr} and Nc_{tbr}, but induced HR in certain potato cultivars like Maris Bard and Pentland Ivory. These cultivars were postulated to harbor a new resistance gene Nz_{tbr}. This new PVY^Z strain was different from the PVY^N strain that failed to induce HR in Maris Bard and Pentland Ivory. Of these four strains of PVY, PVY^N was able to induce vein necrosis in tobacco, whereas PVY^O, PVY^C, and PVY^Z induced only mosaic and vein clearing (see Singh et al., 2008). Kerlan et al. (1999) reported on another strain of PVY, later named PVY^E, which could overcome all three genes Ny_{tbr}, Nc_{tbr}, and Nz_{tbr}, but, unlike PVY^N, did not induce vein necrosis in tobacco (Singh et al., 2008). Isolates from both PVY^Z and PVY^E strains proved to be very rare (Kerlan et. al., 2011; Galvino-Costa et al., 2012) in contemporary potato production areas, which delayed the characterization of the Nz_{tbr} gene.

Currently, complete genome sequences are known for three non-recombinant strains of PVY, i.e. PVY^O, PVY^C, and PVY^N (Robaglia et al., 1989; Turpen, 1989; Singh and Singh, 1996; Lorenzen et al., 2006a; Fomicheva et al., 2009; Dullemans et al., 2011; Karasev et al., 2011). In addition to these main, parental genomes, various recombinant PVY genomes have been sequenced, built mainly of segments of PVY^O and PVY^N sequences spliced in various combinations (Thole et al. 1993; Jakab et al. 1997; Glais et al., 1998, 2002b; Boonham et al., 2002; Nie and Singh, 2003b; Lorenzen et al., 2006a; Chikh Ali et al., 2007, 2008; Schubert et

al., 2007; Hu et al., 2009b; Hu et al., 2011; Karasev et al., 2011; Galvino-Costa et al., 2012). At least nine recombination patterns were revealed when PVY^{O} and PVY^{N} were defined as parents, based on sequence analysis of multiple PVY genomes (Hu et al., 2009a). These recombinant patterns had between one to four recombinant junctions (RJs), most of which were relatively conserved in their position in the PVY genome, especially in the P1, HC-Pro, and VPg-NIa regions (Hu et al., 2009a). The three most common recombinant patterns are characteristic of PVY^{NTN}, $PVY^{N:O}$, and PVY^{N-Wi} strains. Other non-recombinant, PVY^{NA-N} (Nie and Singh, 2003b), and recombinant genomes, e.g., NE-11 (Lorenzen et al., 2008), were significantly different from PVY^{O} and PVY^{N}, had also been found. Interestingly, almost all PVY recombinants induced vein necrosis in tobacco. The recombinant PVY^{NTN} strain attracted the most attention, because a distinct necrotic tuber reaction, called potato tuber necrotic ringspot disease (PTNRD) syndrome, was associated with this recombinant type (Beczner et al., 1984; Le Romancer et al., 1994; Glais et al., 2002). Notably, very few of the recombinant isolates were tested on potato indicators (see Table 1.1), to determine their genetic type (for exceptions see Kerlan et al., 2011; Galvino-Costa et al., 2012), and consequently these two classifications, genetic and molecular, have yet to merge.

The genetic classification of PVY strains relies on reactions conferred by the resistance genes in potato, and thus has a practical dimension. Therefore it would be useful to look at the genetics of PVY resistance in potato. Two types of genes confer resistance against PVY in potato (*Solanum tuberosum* L.). Extreme resistance or immunity is conferred by *R* genes providing a broad, resistance against PVY that is not strain specific. *R* genes are found in wild relatives of potato, and at least three *R* genes have been identified and mapped – Ry_{adg}, Ry_{sto}, and Ry_{chc}. A hypersensitive response against PVY is conferred by strain specific *N* genes that are common in cultivated potato varieties. Two genes inducing HR have been identified, Ny_{tbr} conferring resistance to PVY^{O} strain, and Nc_{tbr} conferring resistance to the PVY^{C} strain. In 1990, Jones (1990) postulated existence of a new, putative HR-inducing gene, Nz_{tbr}, triggered by a

PVY strain named PVY^{Z}. This putative Nz_{tbr} was expressed in some potato cultivars, like Maris Bard and Pentland Ivory, which also harbored two other genes, Ny_{tbr} and Nc_{tbr} (Jones, 1990; Kerlan et al., 1999; Singh et al., 2008), or Yukon Gold (Kerlan et al, 2010). However, PVY^{Z} isolates turned out to be very rare (Jones, 1990; Kerlan et al., 1999; Singh et al., 2008; Kerlan et al., 2010; Kehoe and Jones, 2011; Quintero-Ferrer et al., 2014), and up until now a formal genetic proof of the Nz_{tbr} existence and its monogenic nature was not available. In our experiments, we obtained evidence of the monogenic nature of the Nz gene in potato and thus proved its existence (see Chapter 2).

N genes have been extensively studied in Europe, at least until the early 1990s (see Cockerham, 1970; deBokx and Huttinga, 1981; Jones, 1990; Kerlan et al., 1999; Singh et al., 2008; Kerlan et al., 2010; Karasev and Gray, 2013a,b). In Europe, *N* genes were viewed as valuable sources of resistance against two strains of PVY: PVY^{C} and PVY^{O} (Cockerham, 1970). In North America, however, most of the efforts were invested in the introgression of the *R* genes into commercially acceptable cultivars, and *N* genes were essentially neglected (Whitworth et al., 2009), and, hence, almost no information is available on the presence of the *N* genes in major potato cultivars grown in North America. Here, I embarked on a screening project, focusing on testing most popular potato cultivars grown in Idaho for their resistance response to five main strains of PVY circulating in North America, $PVY^{N\text{-}Wi}$, PVY^{NTN}, PVY^{O}, PVY^{N}, and $PVY^{NA\text{-}N}$ (Gray et al., 2010). In the case of one particular cultivar, Yukon Gem, we expanded the panel of PVY strains for testing to include an additional three strains, $PVY^{N:O}$, PVY-NE-11, and PVY^{E}.

1.3 Materials and methods

1.3.1 Selection and sources of potato cultivars

Selection of potato cultivars for testing was largely based on seed potato acreage for each cultivar as reported in the official Potato Seed Directories of Idaho and Montana (ICIA, 2013; MSU, 2013)(Table 1). Cultivar Maris Bard was originally received from the national potato

germplasm collection in Sturgeon Bay, WI, as tissue culture plantlets. All other cultivars, Desiree, Russet Burbank, Russet Norkotah, Ranger Russet, Alturas, Western Russet, Yukon Gem, Rio Grande, and Shepody, were supplied from the University of Idaho Potato Tissue Culture Laboratory (kindly provided by Lorie Ewing). All cultivars were maintained in tissue culture in a clean growth room under grow lights 16 hour day 8 hour night 20-22°C. Plantlets were cut and transferred to new media (Murashige & Skoog Basal Salt Mixture and 15 g/liter sucrose pH 5.6) every 8 weeks and, after the transfer, plantlets were planted in soil (Sunshine Mix #2) and kept in a Conviron growth chamber or greenhouse bay (21-22°C 16 hour day 8 hour night, location depended on season) at any time from 2 to 8 weeks. While in-vitro, the plantlets were periodically subjected to RT-PCR (Lorenzen et al, 2006b) tests against the main potato viruses to confirm their virus-free status.

1.3.2 Plant maintenance

Virus-free tissue culture plants from each cultivar were planted in 4-inch pots in a greenhouse bay at ~21-25^{o}C containing sunshine potting soil mix (Sunshine #2) and a slow release fertilizer (Osmocote™ 14-14-14). While in the greenhouse, natural lighting was supplemented with incandescent lamps to provide 16 hour days and 8 hour nights. Water soluble fertilizer (Miracle Gro™ water soluble all-purpose plant food, mixed according to manufacturer's instructions) was added one time per week throughout the growing period. Plants were allowed to grow in Conviron growth chambers under artificial light provided by fluorescent and incandescent lamps with a 16 hour day/8 hour night cycle and maintained at 20 to 22°C.

1.3.3 Reference isolates of PVY, inoculations, phenotype screening, and laboratory testing

The five isolates of PVY, N1 (PVY^{N-Wi}), L26 (PVY^{Z}-NTN), Oz (PVY^{O}), Mont (PVY^{N}), and RRA-1 (PVY^{NA-N}), were from Dr. Alexander Karasev's laboratory collection at the University of Idaho. Main features of these five isolates are listed in Table 1.2. All five isolates have been described (Table 1.2) and have been previously subjected to whole genome sequencing, with

their corresponding GenBank accession numbers (Table 1.2). PVY isolates were maintained in tobacco cv. Burley in an insect-free, climate-controlled growth chamber. Infected tobacco tissue was used as an inoculum source. Tissues were homogenized in phosphate buffer (50 mM sodium phosphate, pH 7.0 plus 50 mM Na Dieca) at a dilution rate of 1:10 using a mortar and pestle chilled on ice. Potato plants were mechanically inoculated by sprinkling a small amount of carborundum onto the leaf and gently rubbing over the surface with the homogenized tobacco tissue using a gloved finger. The inoculum was gently rubbed over the surface of three leaves: one lower, one middle and one upper leaf. Healthy control plants were mock- inoculated with buffer and carborundum only. Plants were inoculated at the six to ten leaf stage, and grown in the same growth chambers mentioned above, with a 16 hour day/8 hour night cycle and maintained at 20 to 22°C. Plants were arranged according to cultivar and the isolate in which they were inoculated. Three plants from each cultivar were inoculated with a single isolate, and grouped together. I used one healthy control plant per cultivar.

Symptom observations started 4 to 5 days after inoculation and were carried out for 6 to 8 weeks. Cultivars Maris Bard and Desiree, with defined genetic background relative to main strains of PVY, were used as controls in each experiment. Three plants of each cultivar were used per an inoculation with a specific PVY isolate, and, routinely, three cultivars plus two control cultivars (Maris Bard and Desiree) were analyzed at a time in a single experiment to fit the temperature-controlled room. Each experiment was repeated three times.

All inoculated plants were tested for the development of a systemic PVY infection at 3 to 4 weeks post-inoculation using a triple antibody sandwich (TAS) ELISA (Karasev et al., 2010), and IC-RT-PCR (Chikh Ali et al., 2013). Three strain-specific monoclonal antibodies were used in addition to a polyclonal antiserum, MAb2 which recognizes PVY^{O}, $PVY^{N\text{-}Wi}$, $PVY^{N:O}$, and PVY^{C} strains (McDonald and Kristjansson, 1993, Agdia, Elkhart, IN), 1F5 which recognizes PVY^{N}, PVY^{O}-O5, PVY-NE11, $PVY^{NA\text{-}N}$, and PVY^{NTN} (Ellis et al., 1996; Karasev et al., 2010, Nikolaeva et al., 2012; Agdia, Elkhart, IN), and SASA-N (Scottish Agriculture Science Agency,

Edinburgh, Scotland) which recognizes PVY^{N}, PVY-NE11, PVY^{NA-N}, and PVY^{NTN} (Karasev et al., 2010, Nikolaeva et al., 2012;).

1.4 Results

1.4.1 Symptom observation and the HR phenotype

In my observations of the PVY infection in potato cultivars tested I focused on two types of symptoms: a) local and systemic necrotic reactions which indicate the induction of the hypersensitive resistance reaction (HR) and typically reveal the presence of a corresponding *N* gene in a cultivar (For example, see Fig. 4 and 8); and b) the presence or absence of other systemic symptoms, not associated with HR. These symptoms are usually various degrees of mosaic (For example, see Fig. 5 and 6) and crinkling (For example, see Fig. 7), sometimes associated with stunting or growth retardation. To confirm or disprove that a particular strain of PVY infected a particular cultivar systemically, we tested each inoculated plant 3 to 4 weeks post inoculation (wpi) using two laboratory methods, TAS-ELISA and IC-RT-PCR as described in Materials and Methods.

1.4.1.1 Control cultivars, Maris Bard and Desiree.

In agreement with previous publications (Cockerham, 1970; deBokx and Huttinga, 1981; Jones, 1990; Singh et al., 2008; Karasev et al., 2010; Kerlan et al., 2010), both PVY-L26 (PVY^{Z}) and PVY-Tb60 (PVY^{O}) induced typical a HR in cv. Maris Bard, following mechanical inoculation. This HR started as necrotic rings developing into local lesions on inoculated leaves at about 7 days post-inoculation (dpi), with the lesions subsequently expanding along the veins which became necrotic a few days later (Fig. 1.1). After 10-14 days, inoculated leaves were fully necrotized and often detached from the plant (For example, see Fig. 4). By 3 wpi, symptoms of systemic necrotic reaction and leaf-drop became apparent (Fig. 1.1), with necrotic streaks visible on the stem, and mosaic (For example, see Fig. 5) and crinkling (For example, see Fig. 7) of the top young leaves. In cv. Desiree, a very similar HR was observed upon inoculation with PVY-Tb60 (PVY^{O}) but not with PVY-L26 (PVY^{Z}). Instead, PVY-L26 infection of cv. Desiree

resulted in the lack of a local necrotic reaction on inoculated leaves and a mild systemic mosaic of upper non-inoculated leaves which developed in 2-3 weeks. Infection of PVY-Mont (PVY^{N}) in cvs. Maris Bard and Desiree resulted in a mild to very mild systemic mosaic, with crinkling visible only in the case of cultivar Maris Bard; no local lesions were observed in either cultivar in response to infection with PVY-Mont (PVY^{N}). The systemic PVY-Mont infection in these cases was easily confirmed at 3 to 4 wpi by laboratory methods, both ELISA and RT-PCR. Isolates PVY-N1 (PVY^{N-Wi}) and PVY-RRA1 (PVY^{NA-N}) induced reactions similar to PVY^{N} in both cvs Maris Bard and Desiree, namely mild mosaic (For example, see Fig. 6) in upper non-inoculated leaves and no local lesions in inoculated leaves. In addition to mild mosaic, PVY-N1 (PVY^{N-Wi}) induced crinkling in systemically infected leaves and stunting in cv Maris Bard (see Table 1.3). Once again, systemic PVY-N1 and PVY-RRA1 infections in all these cases were easily confirmed at 3-4 wpi by laboratory methods, both ELISA and IC-RT-PCR (Chikh Ali et al., 2013).

1.4.1.2 Cultivars Russet Burbank and Shepody.

Three of the five tested PVY isolates, N1 (PVY^{N-Wi}), L26 (PVY^{NTN}), and Tb60 (PVY^{O}), induced a very mild mosaic (For example, see Fig. 6) in systemically infected, non-inoculated upper leaves of Russet Burbank and Shepody, clearly visible by about 2-3 wpi., but no other local or systemic symptoms (Table 1.3). Two other isolates tested Mont (PVY^{N}) and RRA-1 (PVY^{NA-N}), induced no visible local or systemic symptoms during the course of infection, up to 8 weeks, in both Russet Burbank and in Shepody (see Table 1.3). However, systemic PVY-Mont and PVY-RRA1 infections in these cases were easily confirmed at 3-4 wpi by laboratory methods, both ELISA and IC-RT-PCR.

1.4.1.3 Cultivar Russet Norkotah.

In Russet Norkotah, only a very mild systemic mosaic (For example, see Fig. 6) could be transiently observed on upper, non-inoculated leaves around 3 wpi, and as plants grew they seemed to recover and stayed essentially symptomless until the end of the experiment, 8 wpi. I did not see compelling differences between plants infected with any of the five isolates tested,

PVY-N1 (PVY^{N-Wi}), PVY-L26 (PVY^{Z}-NTN), PVY-Tb60 (PVY^{O}), PVY-Mont (PVY^{N}), and PVY-RRA1 (PVY^{NA-N}) (Fig. 1.2). These inoculated plants looked similar to the healthy, non-inoculated plants providing virtually no clue of the PVY infection. Nevertheless, systemic PVY infection was detected at 3 wpi in all cases, regardless of the symptoms observed.

1.4.1.4 Cultivars Ranger Russet, Alturas, and Western Russet.

One of the tested isolates, Tb60 (PVY^{O}), induced a clear local and systemic HR reaction in all three cultivars, Ranger Russet, Alturas, and Western Russet (Figs. 1.3-1.5). This included formation of local lesions which later expanded and led to completely necrotized inoculated leaves by 12-14 dpi. The local HR was followed by a distinct systemic HR (For example, see Fig. 4), which in the case of Ranger Russet was so quick and severe that the inoculated plant usually died between 3 and 4 wpi (Fig. 1.3). In the case of Alturas (Fig. 1.4) and Western Russet (Fig. 1.5), upon infection with Tb60 (PVY^{O}) the systemic HR developed slightly slower and did not kill the plant as in Ranger Russet; infected plants stayed alive until the end of the experiment at 8 wpi, but displayed a leaf-drop syndrome or “palm-tree” appearance (Figs 1.4, 1.5, 4). ‘Palm tree’ in that the infected plant has a long bare main stem with leaves only at the stem tip. Two other isolates, N1 (PVY^{N-Wi}) and L26 (PVY^{Z}-NTN), induced mosaic and crinkling in all three cultivars, Ranger Russet, Alturas, and Western Russet (see Table 1.3), and in the case of Ranger Russet both isolates led to visible stunting of the infected plants (Fig. 1.3). Two isolates, Mont (PVY^{N}), and RRA1 (PVY^{NA-N}) induced a mild mosaic (For example, see Fig. 6) in all three cultivars, Ranger Russet, Alturas, and Western Russet, and occasionally produced virtually no symptoms (Table 1.3). The systemic infection in all of these cases was easily confirmed at 3-4 wpi by laboratory methods, both TAS-ELISA and IC-RT-PCR (Chikh Ali et al, 2013).

1.4.1.5 Cultivar Rio Grande.

Three isolates, L26 (PVY^{Z}-NTN), Tb60 (PVY^{O}), and RRA1 (PVY^{NA-N}), induced a local HR in inoculated leaves of Rio Grande visible by 12 dpi (Fig. 1.6). These local HR symptoms varied

from necrotic rings (L26, PVY^{NTN}), to simple necrotic lesions (RRA1, PVY^{NA-N}), to diffuse necrotic lesions expanding along the veins (Tb60, PVY^{O}) (see Fig. 1.6). In the case of L26 (PVY^{NTN}) and Tb60 (PVY^{O}), this local HR reaction was followed by the systemic HR which led to a leaf-drop syndrome (Fig. 4), while Rio Grande plants inoculated with RRA1 (PVY^{NA-N}) stayed symptomless (Table 1.3). Isolate N1 (PVY^{N-Wi}) induced mosaic (Fig. 5) and crinkling (Fig. 7) in upper, non-inoculated leaves, by about 2-3 wpi, but no local or systemic HR reaction (Table 1.3). Isolate Mont (PVY^{N}) induced no local or systemic reaction in inoculated Rio Grande plants throughout the duration of the experiment, 8 wpi. All plants were tested at 3 wpi by TAS-ELISA and IC-RT-PCR to confirm systemic infection with the isolate/strain indicated; interestingly, Rio Grande plants inoculated with RRA1 (PVY^{NA-N}) lacked systemic infection.

1.4.1.6 Cultivar Yukon Gem.

The symptoms expressed by cv. Yukon Gem upon inoculation with the five PVY isolates, N1 (PVY^{N-Wi}), L26 (PVY^{Z}-NTN), Tb60 (PVY^{O}), Mont (PVY^{N}), and RRA1 (PVY^{NA-N}), were the most diverse among the cultivars tested (Fig. 1.7). Four of the isolates tested, N1 (PVY^{N-Wi}), L26 (PVY^{Z}-NTN), Tb60 (PVY^{O}), and RRA1 (PVY^{NA-N}), induced local HR in Yukon Gem, clearly visible as local lesions later spreading along the veins and leading to a complete necrotization of the inoculated leaves (Table 1.3). In the case of N1 (PVY^{N-Wi}) and RRA1 (PVY^{NA-N}), this local HR reaction was followed by the pronounced systemic HR that was visible as leaf-drop syndrome (Fig. 1.7); plants infected with RRA1 (PVY^{NA-N}) died between 5 to 6 wpi (Fig. 1.7). The systemic infection in these two cases was easily confirmed at 3-4 wpi by laboratory methods, both ELISA and IC-RT-PCR. In the case of L26 (PVY^{Z}-NTN) and Tb60 (PVY^{O}), however, the local HR apparently confined inhibited virus replication, and no systemic symptoms were observed through the duration of the experiment, 8 wpi. Consistent with these visual observations, Yukon Gem plants inoculated with L26 (PVY^{Z}-NTN) or Tb60 (PVY^{O}) were found not to be systemically infected after laboratory testing by TAS-ELISA and IC-RT-PCR. The most unusual reaction was observed when isolate Mont (PVY^{N}) was inoculated in Yukon Gem plants - Mont (PVY^{N})

induced no local or systemic reaction in Yukon Gem throughout the duration of the experiment, 8 wpi (see Table 1.3, Fig. 1.7). Interestingly, these Yukon Gem plants inoculated with Mont (PVY^{N}) were found not to be systemically infected after laboratory testing, both by TAS-ELISA and IC- RT-PCR. Control cultivars, Maris Bard and Desiree, inoculated with the same Mont (PVY^{N}) inoculum and maintained in the same room did become systemically infected.

Given this unusual spectrum of reactions to different strains of PVY exhibited by Yukon Gem, an expanded set of isolates was tested for this cultivar, including those from strains PVY^{O}-O5 (one isolate), PVY^{N-Wi} (one additional isolate), $PVY^{N:O}$ (two isolates), PVY-NE11 (two isolates), and PVY^{E} (two isolates) (see Table 1.2 for a complete list). The experiment was done the same way as the other isolate testing, which is described in detail in Materials and Methods. Isolate ID269 (PVY^{O}-O5) induced the same reactions in Yukon Gem as Tb60 (PVY^{O}), namely a distinct local HR with no subsequent systemic infection (Table 1.4, Fig. 1.8). Isolate N3 (PVY^{N-Wi}) induced reactions very similar to another PVY^{N-Wi} isolate, N1, namely local HR by 10-12 dpi that was followed by pronounced systemic HR leading to a leaf-drop syndrome (Table 1.4). Two isolates tested, NE-11 and ID20 (both PVY-NE11), induced typical necrotic rings on the inoculated leaves of Yukon Gem (Fig. 1.8, B), which was followed by local HR leading to fully necrotized inoculated leaves. However, no systemic symptoms were observed in Yukon Gem plants inoculated with NE-11 and ID20 (both PVY-NE11); Yukon Gem plants inoculated with NE-11 and ID20 (both PVY-NE11) were found to be not systemically infected after laboratory testing by TAS-ELISA and IC-RT-PCR. Two isolates tested, AGA and MON (both PVY^{E}), induced a typical local HR in Yukon Gem resulting in necrotization of inoculated leaves (Fig. 1.8, C and D), but no systemic symptoms; Yukon Gem plants inoculated with AGA and MON (both PVY^{E}) were found to be not systemically infected after laboratory testing by TAS-ELISA and IC-RT-PCR.

1.5 Discussion

Unlike *R* genes, which provide broad, stable, and strain non-specific resistance, *N* genes confer strain specific resistance to pathogens, and are sensitive to environmental conditions (especially temperature). Hence, they are often ignored by breeders as less desirable sources of resistance, not worth pursuing as valuable traits. This is especially true for the potato-PVY pathosystem, where for many years the main emphasis of the breeding for resistance was focused on *R* genes (Whitworth et al., 2009), and *N* genes were essentially neglected. However, *R* genes are difficult to introduce into commercially significant cultivars, and the process of introgression of various *R* genes conferring resistance to PVY into commercial potato cultivars has been slow. On the other hand, *N* genes are quite common in commercial cultivars, and may be used immediately for at least partial protection, provided their presence and strain specificity in potato cultivars is known. Considerable efforts were undertaken in the past in Europe, (Kerlan et al, 1999) on identifying *N* genes conferring resistance to PVY^{O}, PVY^{C}, and, later, to PVY^{Z}. However, no similar screening efforts were undertaken in North America so far, to identify *N* genes specific to PVY strains circulating in the field, and this work represents the first attempt to close this knowledge gap.

Necrotic reactions, both local and systemic, were observed in multiple combinations of PVY strains and potato cultivars tested in this work (summarized in Table 1.3). These necrotic reactions indicated specific HR responses that revealed the presence of corresponding *N* genes in the tested cultivars, namely Ny_{tbr} and Nz_{tbr} (Table 1.5). For the first time, a sub-set of North American cultivars was screened for the presence of two *N* genes conferring resistance to two strains of PVY, PVY^{O} and PVY^{Z}-NTN (Tables 1.3 and 1.5). Interestingly, both *Ny* and *Nz* genes seem to be widely available in several commercially acceptable cultivars, *Ny* being particularly common – it was found in five of the eight tested cultivars, Ranger Russet, Alturas, Western Russet, Yukon Gem, and Rio Grande. The *Nz* gene was found only in two of the eight tested cultivars, but in both cultivars, Yukon Gem and Rio Grande, it was in combination with the *Ny*

gene (see Table 1.5). Both these cultivars, Rio Grande and Yukon Gem, produced an HR response against one other strain of PVY, PVY^{NA-N}, indicating an additional source of resistance against PVY present, not described previously (Table 1.3, Fig. 1.6). In the case of Rio Grande, only a local HR was produced by the isolate RRA1 (PVY^{NA-N}), and no systemic infection was detected for the duration of the experiment, 8 weeks after inoculation (Table 1.3, Fig. 1.6). Based on these data, we propose an existence of a new, putative resistance gene *Nna*, conferring resistance against PVY^{NA-N}, and expressed in both Yukon Gem and Rio Grande. Apparently, this *Nna* gene is able to provide complete protection against infection with the isolate RRA1 (PVY^{NA-N}) in Rio Grande under the conditions used for the screening (Table 1.3, Fig. 1.6).

Yukon Gem responded with HR to at least four of the initial PVY strains tested (Table 1.3) and was subjected to a more detailed study, using an expanded set of PVY isolates representing eight strains of PVY in total (Table 1.4). Of the 13 tested isolates of PVY representing 8 strains of PVY, at least 12 isolates induced a local HR in Yukon Gem (Table 1.4, Figs 1.7 and 1.8). Of these 12 isolates, 5 isolates that belonged to 3 strains of PVY, induced a systemic HR which was concomitant with the full systemic infection in upper non-inoculated leaves easily detectable by ELISA and IC-RT-PCR. However, 7 isolates representing 4 strains of PVY were unable to establish systemic infection following the induction of the local HR (see Table 1.4, Figs 1.7 and 1.8). Taken together, these data suggest that Yukon Gem may harbor additional *N* genes, besides *Ny*, *Nz*, and the putative *Nna* genes. We propose these new, putative genes be named *Nw* (resistance against PVY^{N-Wi}/$PVY^{N:O}$), *Nne* (resistance against PVY-NE11), and *Ne* (resistance against PVY^{E}). The single isolate from the PVY^{N} strain, PVY-Mont, was unable to infect Yukon Gem, producing no local HR or any other local or systemic symptoms; no virus could be detected by ELISA or IC-RT-PCR in upper, non-inoculated leaves (Tables 1.3 and 1.5). We hypothesize that this may be due to the presence of another *N* gene, tentatively named *Nn* (Kerlan et al., 2010), that confers resistance against PVY^{N}. Thus, Yukon

Gem appears to harbor a large set of known and putative new *N* genes conferring partial or complete resistance against 13 isolates of PVY that belong to 8 different strains of the virus (Table 1.5). Due to this unique set of resistance genes providing partial protection against multiple strains of PVY, Yukon Gem could be considered a good source of a broad resistance against PVY in potato breeding programs. Of course, to confirm existence of these new *N* genes, genetic evidence will be needed, confirmed through genetic crosses and segregation analysis.

Yukon Gem was previously reported to express local and systemic necrotic reactions to several uncharacterized isolates from three strains of PVY - PVY^{NTN}, PVY^{O}, and $PVY^{N\text{-}Wi}$ (Whitworth et al, 2010), which is consistent with the data obtained by us (Table 1.3, Fig. 1.7). In our case, however, we used fully-sequenced and thus fully defined isolates from these same three strains of PVY, plus several additional isolates representing five more PVY strains (Table 1.5, Fig. 1.8). Yukon Gem appears to express a very broad resistance to essentially all strains tested in our experiments and consequently is identified as a prospective source of a broad resistance against most of the known strains of PVY. Cultivar Yukon Gem represents a product of a cross between cvs. Brodick and Yukon Gold, cultivars displaying significant level of PVY-resistance; it was released in 2006 (Whitworth et al., 2010). It is worth noting that Brodick has Pentland Ivory in its pedigree, which harbors three known *N* genes, *Ny*, *Nc*, and *Nz*, conferring resistance to three strains of PVY, PVY^{O}, PVY^{C}, and PVY^{Z}, respectively (Cockerham, 1970; deBokx and Huttinga, 1981; Jones, 190; Singh et al., 2008). Yukon Gold, on the other hand, has both Katahdin and *S. phureja* in its pedigree, and perhaps these are sources of the *Ny* and *Nz* genes identified in Yukon Gold previously (Valkonen, 1997; Kerlan et al, 2011).

References

1. Blanchard, A., Rolland, M., Lacroix, C., Kerlan, C., and Jacquot, J. 2008. *Potato virus* Y: a century of evolution. Current Topics in Virology 7: 21-32.
2. Chikh Ali, M., Gray, S., and Karasev, A.V. 2013 An improved multiplex IC-RT-PCR assay distinguishes nine strains of Potato virus Y. Plant Disease 97:1370-1374.
3. Ellis P, Stace-Smith R, Bowler G, and Mackenzie DJ. 1996. Production of monoclonal antibodies for detection and identification of strains of potato virus Y. Can. J. Plant Pathology. 18: 64-70.
4. Chikh Ali, M., T. Maoka, and K.T. Natsuaki. 2007. The occurrence and characterization of new recombinant isolates of PVY displaying shared properties of PVYNW and PVYNTN. Journal of Phytopathology 155: 409-415.
5. Chikh Ali, M., Maoka, T., and Natsuaki, K.T. 2008. Whole genome sequence and characterization of a novel isolate of PVY inducing tuber necrotic ringspot in potato and leaf mosaic in tobacco. Journal of Phytopathology 156: 413-418.
6. Cockerham, G. 1970. Genetic studies on resistance to potato viruses X and Y. Heredity 25:309-348.
7. De Bokx, J.A., Huttinga, H. 1981. Potato virus Y. Descriptions of plant viruses, No. 242. Common Mycol Institution/Association of Applied Biology, New England
8. Dullemans, A.M., Cuperus, C., et al. 2011. Complete nucleotide sequence of a potato isolate of strain group C of Potato virus Y from 1938. Archives Virology. 156 (3): 473-7.
9. Fomicheva, V.W., Fletcher J.D., and Schubert J. 2009. Potato virus Y strain spectrum in New Zealand - absence of recombinant N:O Strains. Journal of Phytopathology 157: 507–510.
10. Galvino-Costa, S.B.F., Figueira, A., Camargos, V.V., Geraldino, P.S., Hu, X., Nikolaeva, O.V., Kerlan, C., and Karasev, A.V. 2012. A novel type of *Potato virus Y* recombinant genome, determined for the genetic strain PVY^{E}. Plant Pathology 61: 388-398.
11. Glais, L., M., Tribodet, and C. Kerlan. 2002. Genomic variability in Potato potyvirus Y (PVY): evidence that PVYN-W and PVYNTN variants are single to multiple recombinants between PVYO and PVYN isolates. Archives of Virology 147: 363-378.
12. Glais, L., Tribodet, M., and Kerlan, C. 2002. Genomic variability in Potato potyvirus Y (PVY): evidence that $PVY^{N}W$ and PVY^{NTN} variants are single or multiple recombinants between PVY^{O} and PVY^{N} isolates. Archives of Virology. 147: 363–378.

13. Gray, S.M., DeBoer, S.H., Lorenzen, J., Karasev, A.V., Whitworth, J., Nolte, P., Singh, R.P., Boucher, A., and Xu, H. 2010. *Potato virus Y*: a significant and evolving threat to potato crops in the United States and Canada – Feature Article. Plant Disease 94: 1384-1397.
14. Hu, X., Karasev, A.V., Brown, C.J., and Lorenzen, J.H. 2009a. Sequence characteristics of potato virus Y recombinants. Journal of General Virology 90: 3033–41.
15. Hu, X., Meacham, T., Ewing, L., Gray, S. M., and Karasev, A. V. 2009b. A novel recombinant strain of *Potato virus Y* suggests a new viral genetic determinant of vein necrosis in tobacco. Virus Research 143: 68-76.
16. Hu, X., X. Nie, C. He, and X. Xiong. 2011. Differential pathogenicity of two different recombinant PVYNTN isolates in Physalis floridana is likely determined by the coat protein gene. Virology Journal 8: 207.
17. Jakab, G., Droz E., Brigneti G., Baulcombe D.C., and Malnoë P. 1997. Infectious in vivo and in vitro transcripts from full-length cDNA clone of PVY-N605, a Swiss necrotic isolate of potato virus Y. Journal of General Virology 78: 3141–3145.
18. Jones, R.A.C. 1990. Strain group specific and virus specific hypersensitive reactions to infection with potyviruses in potato cultivars. Annual Applied Biology. 117: 93–105.
19. Karasev, A.V. and Gray, S.M. 2013a. Genetic diversity of *Potato virus Y* complex. American Journal of Potato Research 90: 7-13.
20. Karasev, A.V. and Gray, S.M. 2013b. Continuous and emerging challenges of *Potato virus Y* in potato. Annual Review of Phytopathology 51: 571-586.
21. Karasev, A.V., Nikolaeva, O.V., Hu, X. *et al*., 2010. Serological properties of ordinary and necrotic isolates of Potato virus Y: a case study of PVY^N misidentification. American Journal of Potato Research 87: 1–9.
22. Kerlan, C., Nikolaeva, O.V., Hu, X., Meacham, T., Gray, S.M.,and Karasev, A.V. 2011. Identification of the molecular make-up of the *Potato virus Y* strain PVY^Z: Genetic typing of PVY^Z-NTN. Phytopathology 101: 1052-1060.
23. Kerlan, C., Tribodet, M., Glais, L., and Guillet, M. 1999 Variability of *Potato virus* Y in potato crops in France. Journal of Phytopathology. 147: 643–651.
24. Le Romancer, Kerlan C.M. and Nedellec, M., 1994 Biological characterization of various geographical isolates of *potato virus* Y inducing superficial necrosis on potato tubers Plant Pathology 43, 138-144.
25. Lorenzen, J. H., Meacham, T., Berger, P. H., Shiel, P. J., Crosslin, J. M., Hamm, P. B., and Kopp H. 2006a. Whole genome characterization of Potato virus Y isolates collected in the

western USA and their comparison to isolates from Europe and Canada. Archives of Virology 151: 1055-1074.

26. Lorenzen, J., P. Nolte, D. Martin, J. Pasche, and N. Gudmestad. 2008. NE-11 represents a new strain variant class of Potato virus Y. Archives of Virology 153: 517-525.
27. Lorenzen, J.H., Piche, L.M., Gudmestad, N.C., Meacham, T., and Shiel, P. 2006b. A multiplex PCR assay to characterize Potato virus Y isolates and identify strain mixtures. Plant Disease 90: 935–40.
28. Nie, X., and R.P. Singh. 2003. Evolution of North American PVYNTN strain Tu660 from local PVYN by mutation rather than recombination. Virus Genes 26: 39–47.
29. Nikolaeva, O.V., Roop, D., Galvino-Costa, S.F.B., Figueira, A.R., Gray, S.M., and Karasev, A.V. 2012. Epitope mapping for monoclonal antibodies recognizing tuber necrotic strains of *Potato virus Y*. American Journal of Potato Research 89: 121-128.
30. Robaglia, C., Durand-Tardif, M., Boudazin, G., Astier-Manifacier, S. & Casse-Delbart, F. 1989. Nucleotide sequence of *Potato Virus* Y (N strain) genomic RNA. Journal of General Virology 70, 935-947.
31. Schubert, J., V. Fomitcheva, and J. Sztangret-Wisniewska. 2007. Differentiation of *Potato virus* Y using improved sets of diagnostic PCR-primers. Journal of Virological Methods 140: 66–74.
32. Singh, M., and R.P. Singh. 1996. Nucleotide sequence and genome organization of a Canadian isolate of the common strain of potato virus Y (PVYO). Canadian Journal of Plant Pathology 18: 209-214.
33. Singh, R.P., Valkonen, J.P.T., Gray, S.M., Boonham, N., Jones, R.A.C., Kerlan, C., and Schubert, J. 2008. Discussion Paper: The naming of Potato virus Y strains infecting potato. Archives of Virology 153, 1-13.
34. Thole, V., T. Dalmay, J. Burgyán, and E. Balazs. 1993. Cloning and sequencing of potato virus Y (Hungarian isolate) genomic RNA. Gene 123: 149–156.
35. Turpen, T. 1989. Molecular cloning of a *Potato virus* Y genome: nucleotide sequence homology in non-coding regions of potyviruses. Journal of General Virology 70: 1951-1960.
36. Valkonen, J. P. T. 1997. Novel resistances to potyviruses in tuber-bearing potato species, and temperature-sensitive expression of hypersensitive resistance to *Potato virus* Y. Annual Applied Biology 130: 91-104.
37. Whitworth J.L., Novy R.G., Hall D.G., Crowlin J.M., Brown C.R. 2009. Characterization of broad spectrum *Potato virus Y* resistance in a Solanum tuberosum ssp. Andigena- derived

population and select breeding clones using molecular markers, grafting, and field inoculations. American Journal of Potato Research. 86:286-296.

38. Whitworth, J.L., Novy, R.G., Stark, J.C., Pavek, J.J., Corsini, D.L., Love, S.L., Miller, J.S., Vales, M.I., Mosley, A.R., Yilma, S., James, S.R., Hane, D.C., Charlton, B.A., Brown, C.R., Knowles, N.R., and Pavek, M.J. 2010. Yukon Gem: A yellow-fleshed potato cultivar suitable for fresh-pack and processing with resistances to PVYO and late blight. Amer. J. Potato Research 87: 327-336.

Chapter 2

Establishing a monogenic nature of the *Nz* gene conferring resistance against *Potato virus Y* strain Z (PVY^{Z}) in potato (*Solanum Tuberosum* L.)

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2.1 Abstract

Hypersensitive resistance (HR) to *Potato virus Y* (PVY) in potato (*Solanum tuberosum L.)* is conferred by strain-specific *N* genes. Two such genes have been identified in potato so far, Ny_{tbr} conferring resistance to PVY^{O}, and Nc_{tbr} conferring resistance to PVY^{C}. A third, putative gene Nz_{tbr} was proposed to confer resistance against a distinct strain PVY^{Z}. However, due to the scarcity of the PVY^{Z} isolates of PVY, no formal proof of the monogenic nature of this new gene, Nz_{tbr}, was available until now. Here, I report on a genetic study of Nz_{tbr} inheritance in three crosses between cultivars Maris Bard (*Ny:Nz*), King Edward (*ny:nz*), and Russet Norkotah (*ny:nz*). A fully-sequenced PVY isolate, L26 representing the PVY^{Z}, strain was used to screen the parents and the progeny for the virus-induced HR phenotype in foliage. Based on the phenotypic analysis of the 203 progenies of crosses between Maris Bard and Russet Norkotah, and Maris Bard and King Edward, segregation of the HR phenotype in PVY^{Z}-infected plants was found to be close to 1:1, indicating a monogenic, dominant nature of the Nz_{tbr} gene. Since the PVY^{Z} strain comprises PVY^{NTN} isolates associated with tuber necrotic ringspot disease (PTNRD) in susceptible potato cultivars, the Nz_{tbr} gene could represent a valuable source of resistance against PTNRD-inducing PVY isolates. This is the first demonstration that Nz_{tbr} is a single, dominant *N* gene in potato conferring resistance to the PVY^{Z}-NTN strain.

2.2 Introduction

Two types of genes confer resistance against *Potato virus Y* (PVY) in potato (*Solanum tuberosum* L.). Extreme resistance or immunity is conferred by *R* genes providing a broad, strain non-specific resistance against PVY. *R* genes are found in wild relatives of potato, and at

least three *R* genes have been identified and mapped – Ry_{adg}, Ry_{sto}, and Ry_{chc} (Gebhardt and Valkonen, 2001) Hypersensitive resistance (HR) against PVY is conferred by strain specific *N* genes that are common in cultivated potato varieties. Two genes inducing, HR have been identified Ny_{tbr} conferring resistance to PVY^O strain, and Nc_{tbr} conferring resistance to PVY^C strain. In 1990, Jones (1990) postulated the existence of a new, putative HR-inducing gene, Nz_{tbr}, triggered by a PVY strain designated as PVY^Z. This putative Nz_{tbr} was expressed in some potato cultivars, like Maris Bard and Pentland Ivory, which also harbored two other genes, Ny_{tbr} and Nc_{tbr} (Jones, 1990; Kerlan et al., 1999; Singh et al., 2008), or Yukon Gold (Kerlan et al, 2011). However, PVY^Z isolates turned out to be very rare (Jones, 1990; Kerlan et al., 1999; Singh et al., 2008; Kerlan et al., 2011; Kehoe and Jones, 2011; Quintero-Ferrer et al., 2014), and up until now a formal genetic proof of the Nz_{tbr} existence and its monogenic nature was not available.

PVY exists as a complex of strains defined based on reactions to a set of potato cultivars with a defined genetic background (Cockerham, 1970; deBokx and Huttinga, 1981; Singh et al., 2008; Karasev and Gray, 2013a,b; Blanchard et al, 2008). Strain PVY^O comprises isolates inducing HR in the presence of the Ny_{tbr} gene, and strain PVY^C comprises isolates inducing HR in the presence of the Nc_{tbr} gene. Strain PVY^N was defined as comprising PVY isolates that did not induce HR in the presence of either Ny_{tbr} or Nc_{tbr} genes. In late 1980s, a new strain of PVY was found, PVY^Z, which did not elicit HR in the presence of Ny_{tbr} and Nc_{tbr}, but induced HR in certain potato cultivars like Maris Bard and Pentland Ivory postulated to harbor a new resistance gene Nz_{tbr} (Jones, 1990). This new PVY^Z strain was clearly different from the PVY^N strain in that it did not induce HR in Maris Bard and Pentland Ivory. Of these four strains of PVY, only PVY^N was able to induce vein necrosis in tobacco, whereas PVY^O, PVY^C, and PVY^Z induced only mosaic and vein clearing (see Singh et al., 2008). In 1999, another strain of PVY was found, later named PVY^E, which could overcome all three genes Ny_{tbr}, Nc_{tbr}, and Nz_{tbr}, but, unlike PVY^N, did not induce vein necrosis in tobacco (Kerlan et al., 1999; Singh et

al., 2008). Isolates from both PVY^Z and PVY^E strains proved to be very rare (Kerlan et. al., 2011; Galvino-Costa et al., 2012) in contemporary potato production areas, which delayed the characterization of the Nz_{tbr} gene.

Recently, Kerlan et al. (2011) demonstrated that a PVY isolate, designated as L26 and previously found in Idaho, belonged to the PVY^Z strain, and identified the molecular structure of the PVY^Z strain as identical to typical PVY^{NTN} recombinants. Since PVY^{NTN} recombinants were found to be involved in the induction of the potato tuber necrotic ringspot disease (PTNRD) (Beczner et al., 1984; Le Romancer et al., 1994; Singh et al., 2008), it immediately revived interest in the Nz_{tbr} gene as a potential new source of resistance against PTNRD-inducing isolates of PVY. Having this PVY^Z isolate L26 in our laboratory collection (Hu et al., 2009a; Kerlan et al., 2011; Quintero-Ferrer et al., 2014) I embarked on a genetic study of the Nz_{tbr} inheritance to check: 1) if this HR-inducing gene exists; 2) if this gene is associated with the nucleus or an organelle (maternal inheritance); and 3) if this is a single dominant gene similar to other *N* genes conferring strain specific resistance against PVY.

2.3 Materials and methods

2.3.1 Sources of potato cultivars

Cultivars Maris Bard and King Edward were originally received from the national potato germplasm collection in Sturgeon Bay, WI, as tissue culture plantlets. Cultivar Russet Norkotah was from the University of Idaho Potato Tissue Culture Laboratory. All three cultivars were maintained in tissue culture and periodically subjected to ELISA (Karasev et al, 2010) and RT-PCR (Lorenzen et al, 2006a) tests against all of the main potato viruses to confirm their virus-free status.

2.3.2 Crosses and plant maintenance

Virus-free tissue culture plants from each cultivar were planted in gallon containers in a greenhouse bay at the University of Idaho at ~21 to 25 degrees C in sunshine potting soil mix (Sunshine #2) with slow release fertilizer (Osmocote™ (N)14-(P_2O_5)14-(K_2O)14). A 45% shade

cloth was used to keep the plants cool. Lighting was 16 hour days and 8 hour nights. A water soluble fertilizer (Miracle Gro™ all-purpose plant food, mixed according to manufacturer's instructions) was added 2 times per week to all plants throughout the growing period. At flowering, pollen was collected by emasculation of the buds. Pollen was then stored in gelatin capsules on ice and subsequently stored at -20° C. Newly collected pollen was added to the same gelatin capsules each time pollinations were made. Open flowers were pollinated after emasculation, and each flower was labeled with the date and the nature of the cross made. Cheesecloth was used to cover the seeds while they matured. Fruits were removed from the plants at maturation and then seeds were extracted from the fruits. Seeds were removed by cutting the fruits in half and scooping them out with a scalpel. Using cheesecloth, the seeds were washed and gently 'scrubbed' under plain water to remove the clear coating. They were allowed to dry in the cheesecloth for one week before storing in paper seed packets. Before sowing, the seeds were soaked for 24 hours in deionized water and then placed on top of filter paper inside a petri dish. The filter paper was saturated with water and was continually monitored to avoid desiccation. The petri dishes were maintained in a growth chamber set to ~21 degrees C. The seeds were kept in the dark until they began to sprout. Once sprouting occurred, they were transferred directly to potting mix in a plug tray, and covered in moistened medium grade vermiculite. At the 2 to 3 true-leaf stage, plantlets were transplanted into a 4 inch pots containing Sunshine Mix #2 mixed with slow release fertilizer (Osmocote™ (N)14-(P_2O_5)14-(K_2O)14) per manufacturer's instructions. These plants were allowed to grow in Conviron growth chambers under artificial light provided by fluorescent and incandescent lamps with a 16 hour day and 8 hour night cycle and maintained at 20 to 22°C.

2.3.3 Reference isolates of PVY, inoculations, phenotype screening, and laboratory testing

The isolates L26 (PVY^Z-NTN) and Oz (PVY^O) were from Dr. Karasev's laboratory collection at the University of Idaho. PVY isolates were maintained in tobacco plants cv. Burley in an insect-free, climate-controlled Conviron growth chamber. Infected tobacco tissue

homogenized in phosphate buffer (50 mM sodium phosphate, pH 7.0 plus 50 mM Na Dieca) at dilution rate 1:10 with a mortar and pestle on ice was used as an inoculum source. Potato plants were mechanically inoculated at the six to ten leaf stage, and grown in the same growth chambers mentioned above with a 16 hour day and 8 hour night cycle and maintained at 20-22°C. Symptom observations started 4-5 days after inoculation and were carried out for 6 to 8 weeks.

All inoculated plants were tested for the development of systemic PVY infection at three weeks post-inoculation using a triple antibody sandwich (TAS) ELISA (Karasev et al., 2010), and immunocapture IC-RT-PCR (Chikh-Ali et al., 2013). Three strain-specific monoclonal antibodies were used in addition to a polyclonal antiserum, MAb2 which recognizes PVY^{O}, PVY^{N-Wi}, $PVY^{N:O}$, and PVY^{C} strains (McDonald and Kristjansson, 1993, Agdia, Elkhart, IN), 1F5 which recognizes PVY^{N}, PVY^{O}-O5, and PVY^{NTN} (Ellis et al., 1996; Karasev et al., 2010, Agdia, Elkhart, IN), and SASA-N (Scottish Agriculture Science Agency, Edinburgh, Scotland). which recognizes PVY^{N}, and PVY^{NTN} IC-RT-PCR typing was performed using the same ELISA plant extracts according to the protocol of Chikh-Ali et al. (2013).

2.4 Results

2.4.1 Crosses and seed production

Pollination was successful for four of the crosses attempted. ‘Maris Bard’ X ‘Russet Norkotah’ produced four fruits, with a total of 461 seeds (Table 2.1). ‘Russet Norkotah’ X ‘Maris Bard’ produced three fruits with a total of 350 seeds (Table 2.1). ‘King Edward’ X ‘Maris Bard’ produced two fruits with 112 seeds. ‘King Edward’ X ‘Russet Norkotah’ produced three fruits with 220 seeds (Table 2.1). King Edward appeared to be male sterile, producing no pollen, and was used in crosses only as a female parent.

2.4.2 HR phenotype

In parental cv. Maris Bard, both PVY-L26 (PVY^{Z}) and PVY-Oz (PVY^{O}) induced HR symptoms following mechanical inoculation. This HR started as necrotic rings developing into

local lesions on inoculated leaves at about 7 days post-inoculation (dpi), and subsequently expanded along the veins which became necrotic a few days later. In 10-14 days, inoculated leaves were fully necrotized and often separated from the plant. By 3 weeks p.i., symptoms of systemic necrotic reaction and leaf-drop became apparent, with necrotic streaks visible on the stem, and severe mosaic and crinkling of the top young leaves.

In parental cvs. King Edward and Russet Norkotah, no HR was observed upon inoculation either with PVY-L26 or PVY-Oz. Instead, distinct systemic mosaic and crinkling of upper non-inoculated leaves developed in King Edward. In Russet Norkotah, only a mild systemic mosaic could be observed on upper, non-inoculated leaves by 3 weeks p.i., and as plants grew they seemed to recover and stayed essentially symptomless until the end of the experiment, 8 weeks p.i. Nevertheless, systemic PVY infection was detected at 3 weeks p.i. in all cases, regardless of the HR, or mosaic and crinkling symptoms observed. In the progeny of crosses inoculated with PVY-L26, we observed the same local HR symptoms, and scored the HR reaction as the expression of the putative *Nz* gene. Inoculated leaves of the progeny of the crosses were either symptomless or showed a local HR presented as necrotic rings, and necrotic lesions of various sizes (Fig. 2.1).

2.4.3 Segregation of the HR phenotypes in crosses

Five separate experiments were conducted challenging the progenies of three different crosses that included Maris Bard as a parent providing the putative gene *Nz* with PVY-L26 (PVY^Z), testing 203 individual plants in total (Table 2.2). Two of these crosses were reciprocal, involving Maris Bard and Russet Norkotah as male and female parents, while only a single cross involving King Edward as a female parent with a male Maris Bard was used due to the encountered male sterility of King Edward. Of the 203 inoculated plants, 100 displayed typical HR symptoms on inoculated leaves while 103 were susceptible to PVY-L26 (Table 2.2) resulting in a ratio of necrotic to susceptible reaction segregation of an almost perfect 1:1. This ratio

suggested the presence of a single copy of a dominant *Nz* gene in the parental cv Maris Bard, according to the expected ratio in the tetraploid potato genome (Cockerham, 1970).

2.5 Discussion

Single, dominant *N* genes confer strain specific resistance to PVY in potato, and so far two genes, Ny_{tbr} and Nc_{tbr}, conferring resistance to PVY^{O} and PVY^{C} strains, respectively, were identified and mapped to potato chromosome IV (Celebi-Toprak et al., 2002; Moury et al., 2011). However, no genes conferring resistance to new, recombinant strains of PVY, especially to the PVY^{NTN} recombinant associated with the PTNRD reaction in susceptible potato cultivars have been identified up to now.

Here, I established that a previously postulated gene Nz_{tbr} (Jones, 1990) segregated in crosses between potato cultivars bearing this gene and those without it (Table 2.2). The segregation ratio confirmed that Nz_{tbr} was indeed a single dominant gene present in the genome of cv Maris Bard. Based on the patterns of segregation in reciprocal crosses between male and female parents, it can be concluded that *Nz* is a nuclear gene. What is even more important is this Nz_{tbr} gene confers resistance to the PVY^{Z} strain of PVY (Jones, 1990; Singh et al., 2008), which was demonstrated to be synonymous to the recombinant strain PVY^{NTN} (Kerlan et al., 2011; Karasev and Gray, 2013a,b; Quintero-Ferrer et al., 2014). PVY^{NTN} was previously shown to predominantly associate with the PTNRD reaction in potato tubers (Beczner et al., 1984; Le Romancer et al., 1994; Singh et al., 2008). Consequently, this Nz_{tbr} gene may provide a new, valuable source of resistance against tuber-necrotic strain of PVY, PVY^{Z}-NTN.

Nz_{tbr} represents the first example of an *N* gene conferring resistance to a recombinant strain of PVY, and to the one associated with a very damaging PTNRD trait. There are at least four other types of recombinant PVY strains (see Karasev and Gray, 2013a,b) for which no corresponding *N* genes are identified. It is clear that a search for these new *N* genes conferring resistance to other recombinant PVY strains is necessary.

References

1. Beczner, L., Horvath, J., Romhanyi, I., and Forster, H. 1984. Studies on the etiology of tuber necrotic ringspot disease in potato. Potato Research 27: 339-352.
2. Blanchard, A., Rolland, M., Lacroix, C., Kerlan, C., and Jacquot, J. 2008. *Potato virus* Y: a century of evolution. Current Topics in Virology 7: 21-32.
3. Celebi-Toprak F., Slack S.A., and Jahn M.M. 2002. A new gene,Nytbr , for hypersensitivity to *Potato virus* Y from Solanum tuberosum Maps to Chromosome IV.Theoretical and Applied Genetics 104: 669–674.
4. Chikh Ali, M., Gray, S., and Karasev, A.V. 2013 An improved multiplex IC-RT-PCR assay distinguishes nine strains of Potato virus Y. Plant Disease 97:1370-1374.
5. Cockerham, G. 1970. Genetic studies on resistance to potato viruses X and Y. Heredity 25:309-348
6. Ellis, P., Stace-Smith, R., Bowler, G, and Mackenzie, D.J. 1996. Production of monoclonal antibodies for detection and identification of strains of *Potato virus* Y. Canadian Journal of Plant Pathology. 18: 64-70.
7. Galvino-Costa, S.B.F., Figueira, A., Camargos, V.V., Geraldino, P.S., Hu, X., Nikolaeva, O.V., Kerlan, C., and Karasev, A.V. 2012. A novel type of *Potato virus Y* recombinant genome, determined for the genetic strain PVY^{E}. *Plant Pathology* 61: 388-398.
8. Hu, X., Karasev, A.V., Brown, C.J., and Lorenzen, J.H. 2009a. Sequence characteristics of *Potato virus* Y recombinants. Journal of General Virology 90: 3033–41.
9. Jones, R.A.C. 1990. Strain group specific and virus specific hypersensitive reactions to infection with potyviruses in potato cultivars. Annual Review of Applied Biology. 117: 93–105.
10. Karasev, A.V. and Gray, S.M. 2013a. Genetic diversity of *Potato virus Y* complex. American Journal of Potato Research 90: 7-13.
11. Karasev, A.V. and Gray, S.M. 2013b. Continuous and emerging challenges of *Potato virus Y* in potato. Annual Review of Phytopathology 51: 571-586.
12. Karasev, A.V., Nikolaeva, O.V., Hu, X. *et al*., 2010. Serological properties of ordinary and necrotic isolates of *Potato virus Y*: a case study of PVY^{N} misidentification. American Journal of Potato Research 87: 1–9.
13. Kehoe, M.A., Jones, R.A. 2011, A proposal to help resolve the disagreement between naming of potato virus Y strain groups defined by resistance phenotypes and those defined by sequencing. Archives of Virology, 156, pp. 2273-2278.

14. Kerlan, C., Nikolaeva, O.V., Hu, X., Meacham, T., Gray, S.M.,and Karasev, A.V. 2011. Identification of the molecular make-up of the *Potato virus Y* strain PVY^{Z}: Genetic typing of PVY^{Z}-NTN. Phytopathology 101: 1052-1060.
15. Kerlan, C., Tribodet, M., Glais, L., and Guillet, M. 1999 Variability of *Potato virus Y* in potato crops in France. Journal of Phytopathology. 147: 643–651.
16. Le Romancer, M, Kerlan, C. and Nedellec, M. 1994. Biological characterization of various geographical isolates of *Potato virus Y* inducing superficial necrosis on potato tubers Plant Pathology 43, 138-144.
17. Lorenzen, J. H., Meacham, T., Berger, P. H., Shiel, P. J., Crosslin, J. M., Hamm, P. B., and Kopp H. 2006a. Whole genome characterization of *Potato virus Y* isolates collected in the western USA and their comparison to isolates from Europe and Canada. Archives of Virology. 151: 1055-1074.
18. Lorenzen, J.H., Piche, L.M., Gudmestad, N.C., Meacham, T., and Shiel, P. 2006b. A multiplex PCR assay to characterize *Potato virus Y* isolates and identify strain mixtures. Plant Disease 90: 935–40.
19. McDonald, J.G., and Kristjansson, G.T. 1993. Properties of strains of *Potato virus Y*N in North America. Plant Disease 77: 87–9.
20. Moury, B., Caromel, B., Johansen, E., Simon, V., Chauvin, L., Jacquot, E., Kerlan, C., and Lefebvre, V. (2011) The helper component proteinase cistron of Potato virus Y induces hypersensitivity and resistance in potato genotypes carrying dominant resistance genes on chromosome IV. Molecular Plant-Microbe Interactions 24: 787-797.
21. Quintero-Ferrer, A., Robles-Hernandez, L., Gonzalez-Franco A.C., Kerlan, C., and Karasev, A.V. 2014. Molecular and biological characterization of a recombinant isolate of *Potato virus Y* from Mexico. Archives of Virology, in press.
22. Singh, R.P., Valkonen, J.P.T., Gray, S.M., Boonham, N., Jones, R.A.C., Kerlan, C., and Schubert, J. 2008. Discussion Paper: The naming of Potato virus Y strains infecting potato. Archives of Virology. 153, 1-13.

Chapter 3

Development of standardized immunodetection systems for six potato viruses using genetic engineering and biotechnology

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3.1 Abstract

Several potato viruses challenge production of clean seed potatoes in Idaho, including PVY, PLRV, PVX, PVA, PVM, and PVS. Some of these viruses, like PVY and PLRV, may cause severe problems in potato production. Potato seed certification programs rely on ELISA-based protocols for detection and monitoring these viruses, and require significant amounts of specific antibodies for large-scale screenings. The most convenient, sandwich ELISA assays, require antibodies from different animal species and thus add complexities for reagent production. All these antibodies need to have a high titer against a specific virus, and produce low background reactions against host proteins. However, many of the viruses, like PVY, PVA, PLRV, PVM, and PVS, are difficult to purify, and host plant contaminants are often present in preparations used as immunogens for antisera production. This virus is purified from a plant, and the samples being tested are homogenized plant material, this results in increased ELISA background reactions, diminishing the sensitivity of the assay. Here, I describe a biotechnological approach to circumvent the problem of host protein contamination of the antigen for potato viruses. Capsid proteins for PVY, PLRV, PVX, PVA, PVM, and PVS were expressed in bacteria, purified, and used as antigens to produce antibodies against six potato viruses essentially free from non-specific reactivity to potato proteins. The successful application of these antibodies in triple antibody-sandwich (TAS) ELISA is described, and applications of this work are discussed.

3.2 Introduction

Potatoes have the potential to be infected by many different viruses reducing yield and tuber quality (Nolte et al, 2004). Symptoms include mosaic patterns on leaves, stunting of the plant, as well as malformed leaves and tubers. (Blanchard et al, 2008). There are cases where symptoms are not always expressed. These reactions can be due to interactions between the virus and the potato plant, growing conditions, and/or age of the plant when infected. Some potato viruses, such as *Potato virus* X, show little to no symptoms and infected plants may be easily overlooked (Burrows and Zitter, 2005). However, potato seed certification is legislatively mandated and based on maintaining acceptably low levels of these viruses. In the U.S., the bulk of potato seed testing is done visually, during winter grow-outs. Winter grow outs are where a certain number of seed potatoes (legislatively mandated) are sent to a warmer climate to be grown and tested. Another method is enzyme-linked immunosorbent assay (ELISA), a good, sensitive method which is widely and successfully used in potato seed production. In the fall 2007, Idaho seed growers made PVY testing by ELISA mandatory for winter grow-outs (Idaho Crop Improvement Association).

Problems in ELISA-based detection may be divided into two main categories: a) low concentration of the viruses in infected foliage or in dormant tubers; and b) lot-to-lot inconsistencies in the quality of virus-specific antisera, (i.e. variable, and often low, titer and often substantial non-specific background). These problems stem from difficulties in purifying a sufficient amount of the virus particle from virus-infected, plant green material, used for the immunization and production of antisera. As a result, in different laboratories, quality of the purified virus antigen varies, and leads to drastically different antisera produced. The result is then low virus-specific titers, and high cross-reactivity with healthy plant tissues. To overcome this inconsistency problem, a novel, biotechnological approach is often used, i.e. production of synthetic or bacterial antigens identical to virus capsid proteins in terms of antigenic specificity.

In this report, I describe production of virus-specific antisera for six common potato viruses. The abundant, bacterially produced Capsid Protein (CP) antigen from each virus was used to immunize several animals. Resulting antisera were found capable of capturing the virus particle when used as coating antibodies in a triple-antibody sandwich (TAS)-ELISA (Karasev et al, 2010) protocol. Hence we describe a complete ELISA-based detection protocol applicable for virus detection in potato and related species.

3.3 Materials and Methods

3.3.1 Virus sources, virus propagation, and purification.

PVY isolate PVY-Oz (strain PVY^{O}; Karasev et al., 2010) and an Idaho isolate of PVM (PVM-ID; Cavileer et al., 1998) were described previously. Other viruses, PVA, PVX, PVS, and PLRV, originally local Idaho potato isolates, were supplied from the laboratory collection of Dr. Alexander Karasev. PVY, PVA, and PVX were propagated in and purified from tobacco leaves (*Nicotiana tabacum* L. cv Burley) (Leiser and Richter, 1979). PVM and PVS were maintained in and purified from potato cv Russet Burbank following the same protocol with slight modifications (Leiser and Richter, 1979). PLRV was maintained in potato plants and purified from *Physalis floridana* Rybd. four weeks after aphid inoculation according to Takanami and Kubo (1979). To exclude cross-contaminations and mixed infections, infected plants were tested for PVY, PVA, PVS, PVX, and PLRV, by using the RT-PCR protocol described by Nie and Singh (2001), and for PVM by using the same protocol and specific primers from Table 1. The purity of the virus preparations was evaluated spectrophotometrically in UV-light between 200 and 320 nm (NanoDrop ND-1000), and electrophoretically, on 4-20% gradient polyacrylamide gels (PAGE) with subsequent staining with Coomassie blue.

3.3.2 Bacterial expression of the virus capsid protein (CP) gene.

The entire CP genes of each of the six viruses were amplified from RNA extracts using a modified Dellaporta protocol (Hu et al., 2009b) and two specific primers; the sequences of these primers are listed in Table 3.1. Each amplified CP gene was cloned into a pMAL expression

vector (New England Biolabs, Ipswich, MA) between the restriction sites (Table 3.1), and the resulting fusion proteins (FP) carrying maltose-binding fragment (MBP) were affinity purified from bacterial extracts using an amylose resin column as described previously (Durrin et al., 2010; Karasev et al., 1998) The purity of the FP antigens for all six viruses was evaluated on 4-20% gradient PAGE with subsequent staining with Coomassie blue. Antigenic specificity of the FP was verified by Western blots probed with virus specific antibodies.

3.3.3 Immunization and antisera testing

Polyclonal antisera against purified virus preparations or affinity-purified, bacterially expressed FPs were produced in rabbits, goats, or guinea pigs after three to five immunizations with Freund's adjuvant (Cocalico Biologicals, Inc). One animal of each species was used for immunization. The specificity of the polyclonal antibodies was initially tested against virus-infected or healthy potato or tobacco tissue in Western blots, and subsequently against virus-infected potato or tobacco tissue in TAS-ELISA. A good antivirus titer of antibodies produced in rabbit, guinea pig, and goat allowed the collection of multiple production bleedings 2 to 4 weeks after a booster immunization.

3.3.4 Plant sample extraction, ELISA procedure, and RT-PCR testing

Plant samples were extracted and subjected to ELISA testing according to the previously published methodology for triple-antibody sandwich (TAS) ELISA (Nikolaeva et al., 1997; Karasev et al., 2010). All samples were loaded in duplicate, and virus positive and negative control samples were included in each ELISA plate. A signal was considered to be positive if it was 3 times higher than the negative control. RT-PCR testing was conducted using the protocol of Nie and Singh (2001) and Lorenzen et al. (2006b).

3.4 Results

3.4.1 Antigen production and purification.

The six amplified CP sequences were cloned in the same bacterial expression vector, pMAL, and after selection of colonies carrying IPTG-inducible expressors, the resulting fusion

proteins were produced in a large-volume liquid medium, up to 1 L. Bacterial cells were washed and then disrupted using extensive sonication as described previously (Nikolaeva et al., 1995; Karasev et al., 1998; Durrin et al., 2010). All six of the bacterially expressed fusion proteins were highly soluble, and thus there was a relatively good yield of each, ranging between 0.5 to 2 mg of purified protein per 1 L of bacterial medium (Nikolaeva et al, 1995; Durrin et al, 2010). This high yield and good solubility of the antigens meant that a single production round in a 1 L flask resulted in a sufficient amount of each virus-specific antigen to immunize two and sometimes three animal species at once, using exactly the same antigen batch, goat and guinea pig, rabbit and guinea pig, or all three at the same time. Because I used the same plasmid construct was used (with the same CP sequenced carried in the plasmid) for antigen expression every time the protein was produced, this approach led to an extremely uniform antigen source which could then be used as a standardized antigen.

3.4.2 Immunization, specificity and sensitivity testing, and an assay development

The immunization procedure followed a typical protocol with 3 to 5 intramuscular and subcutaneous injections as described previously (Karasev et al., 2010). The resulting antisera were tested for virus specificity and their titer was determined using two approaches: Western blot and enzyme linked immunosorbent assay (ELISA). The specificity was determined in Western blot against purified virus preparations (Fig. 3.1). On Fig. 3.1, PLRV is being detected. Fig. 3.1 shows a comparison between the specificities of virus-specific antiserum G-545, a monoclonal antibody specific to the CP, and was compared against a polyclonal produced against bacterially expressed FP. As can be seen from Fig. 3.1, the FP-specific polyclonal antibodies have the specificity of a commercial monoclonal against PLRV (Fig. 3.1, panels C and D).

Both specificity and sensitivity of detection were tested in various ELISA formats, including indirect ELISA and triple-antibody sandwich (TAS) ELISA (Fig. 3.2). Fig 3.2 illustrates the specificity validation in indirect ELISA (Fig. 3.2, PLRV and PVM), or in TAS-ELISA (Fig. 3.2,

PVA and PVY). In this case, I demonstrated that the PLRV-specific antiserum G-545 had the same specificity as the commercial monoclonal from SASA (Fig. 3.2, PLRV), but had a stronger affinity for the purified virus. Once the TAS-ELISA assay was developed, we could evaluate the sensitivity of the assay, roughly corresponding to the titer of the detecting antibody. PVA and PVY titrations of the detecting antibodies were presented as a measure of the system sensitivity (Fig. 3.2). Based on these estimates, we concluded that working dilutions for our antisera in these two cases (PVA and PVY) were between 1:10,000 and 1:50,000; although even the 1:100,000 dilutions provided a robust distinction between virus-infected and healthy plants. Availability of virus-specific antisera from 2 to 3 different animals was the key to the development of TAS-ELISA-based assays for all six viruses, PVY, PLRV, PVX, PVA, PVM, and PVS (Table 3.3).

3.5 Discussion

Bacterially produced antigens can provide an unparalleled uniformity of the antigen used for antibody production due to the high stability of the cloned genes in bacterial plasmids. The lack of uniformity in purified preparations of plant viruses, in addition to inevitable host-derived impurities present in such preparations, is the main reason leading to various lot-to-lot inconsistencies between antisera produced in different laboratories. As a result, the quality of different virus-specific antisera varies dramatically and may significantly affect the outcome of a virus assay. Bacterially produced antigens are produced outside of any plant tissues, hence, cannot carry plant-derived contaminating antigens and thus represent ideal type of antigens for production of high-specificity, low background antisera for the detection of plant viruses. (some of the above statements would benefit greatly from citations supporting them)

Multiple examples of the production of recombinant capsid proteins in bacteria with subsequent development of specific antibodies for immunodetection have been published (e.g., Nikolaeva et al., 1995; Karasev et al., 1998; Ling et al., 2000; Theilmann et al., 2002; Meng et al., 2003). However, successful development of a complete detection system for a plant virus

based on antibodies against recombinant antigens and used in TAS/DAS-ELISA format can be difficult to complete or even predict. In many cases, the use of such a recombinant antigen often solves the problem of an intermediate detecting antibody, usually the capturing antibody still remains a problem (Nikolaeva et al., 1995; Meng et al., 2003). Hence, in many cases antibodies to recombinant proteins fail to bind native virus particles or capsid proteins, a property crucial for capturing the antigen at the first stage in TAS- or DAS-ELISA.

Here, we described the successful development of a complete system for immunodetection of six potato viruses, PVY, PLRV, PVX, PVA, PVM, and PVS, based on antisera against bacterially produced, recombinant CP, and yet suitable not only for Western blots but also for TAS-ELISA format. The ability of the antisera generated to capture virus antigen from plant extracts is especially valuable, since it allows one to use the most advanced, "sandwich" formats of the ELISA protocol, both DAS- and TAS-ELISA for detection of PVY, PLRV, PVX, PVA, PVM, and PVS in field samples. All these antisera (Fig. 3.2) are currently being used for routine large-scale testing for potato viruses at the Virology Laboratory of the University of Idaho and in other laboratories engaged in potato virus testing.

References

1. Blanchard, A., Rolland, M., Lacroix, C., Kerlan, C., and Jacquot, J. 2008. Potato virus Y: a century of evolution. Current Topics in Virology 7: 21-32.
2. Burrows M.E., Zitter T.A., 2005: Plant disease diagnostic clinic. Plant Science Bulletin. 329.
3. Cavileer, T.D., R.C. Clarke, D.L. Corsini, and P.H. Berger. 1998. A new strain of potato carlavirus M. Plant Disease 82: 98-102.
4. Durrin, J.S., O.V. Nikolaeva, C.A. Strausbaugh, and A.V. Karasev. 2010. Immunodetection of two curtoviruses infecting sugar beet. Plant Disease 94: 972-976.
5. Ellis, P., Stace-Smith, R., Bowler, G., and Mackenzie, D.J. 1996. Production of monoclonal antibodies for detection and identification of strains of *potato virus Y*. Canadian Journal of Plant Pathology. 18: 64-70.
6. Hu, X., Meacham, T., Ewing, L., Gray, S.M., and Karasev, A.V. 2009. A novel recombinant strain of *Potato virus Y* suggests a new viral genetic determinant of vein necrosis in tobacco. Virus Research 143: 68-76.
7. Karasev A.V., O.V. Nikolaeva, X. Hu, Z. Sielaff, J. Whitworth, J.H. Lorenzen, and S.M. Gray. 2010. Serological properties of ordinary and necrotic isolates of *Potato virus Y*: a case study of PVY^N misidentification. American Journal of Potato Research 87: 1-9.
8. Karasev, A. V., Nikolaeva, O. V., Lee, R. F., Wisler, G. C., Duffus, J. E., and Dawson, W.O. 1998. Characterization of the beet yellow stunt virus coat protein gene. Phytopathology 88:1040-1045.
9. Leiser, Richter, Coat protein of potyviruses. Archives of Phytopathology. Pfl Schutz. 15: 289, 1979.
10. Ling, K.S., Zhu, H.-Y., Jiang, Z.-Y., and Gonsalves, D. 2000. Effective application of DAS-ELISA for detection of grapevine leafroll associated closterovirus-3 using a polyclonal antiserum developed from recombinant coat protein. European Journal of Plant Pathology. 106:301-309.
11. Meng, B., Credi, R., Petrovic, N., Tomazic, I., and Gonsalves, D. 2003. Antiserum to recombinant virus coat protein detects Rupestris stem pitting associated virus in grapevine. Plant Disease. 87:515-522.
12. Nie, X. and Singh, R.P. 2001. A novel usage of random primers for multiplex RT-PCR detection of virus and viroids in aphids, leaves, and tubers. Journal of Virology Methods 91: 37-49.

13. Nikolaeva O.V., Karasev A.V., Gumpf D.J., Lee R.F., Garnsey S.M., 1995. Production of polyclonal antisera to the coat protein of Citrus tristeza virus expressed in Escherichia coli - application for immunodiagnosis. Phytopathology 85:691-694.
14. Nikolaeva OV, Karasev AV, Powell CA, Garnsey SM, and Lee RF. 1997. Modulation of the antigenic reactivity of the citrus tristeza virus coat protein. Journal of Immunology Methods 206: 97-105.
15. Nolte, P., Whitworth, J.L., Thomton, M.K., McIntosh, C.S. 2004. Effect of seedborne Potato virus Y on performance of Russet Burbank, Russet Norkotah and Shepody potato. Plant Dis. 88:248-252.
16. Takanami, Y. & Kubo, S. I979. Enzyme-assisted purification of two phloem-limited viruses: tobacco necrotic dwarf and potato leafroll. Journal of General Virology 44, 153-I59.
17. Theilmann, J., Mozafari, J., Reade, R., Wu, Z., Xie, W., Jesperson, G., Bernardy, M., Eastwell, K. C., and Rochon, D. 2002. Partial nucleotide sequence and genome organization of a Canadian isolate of Little cherry virus and development of an enzyme-linked immunosorbent assay-based diagnostic test. Phytopathology 92:87-98.

Figures

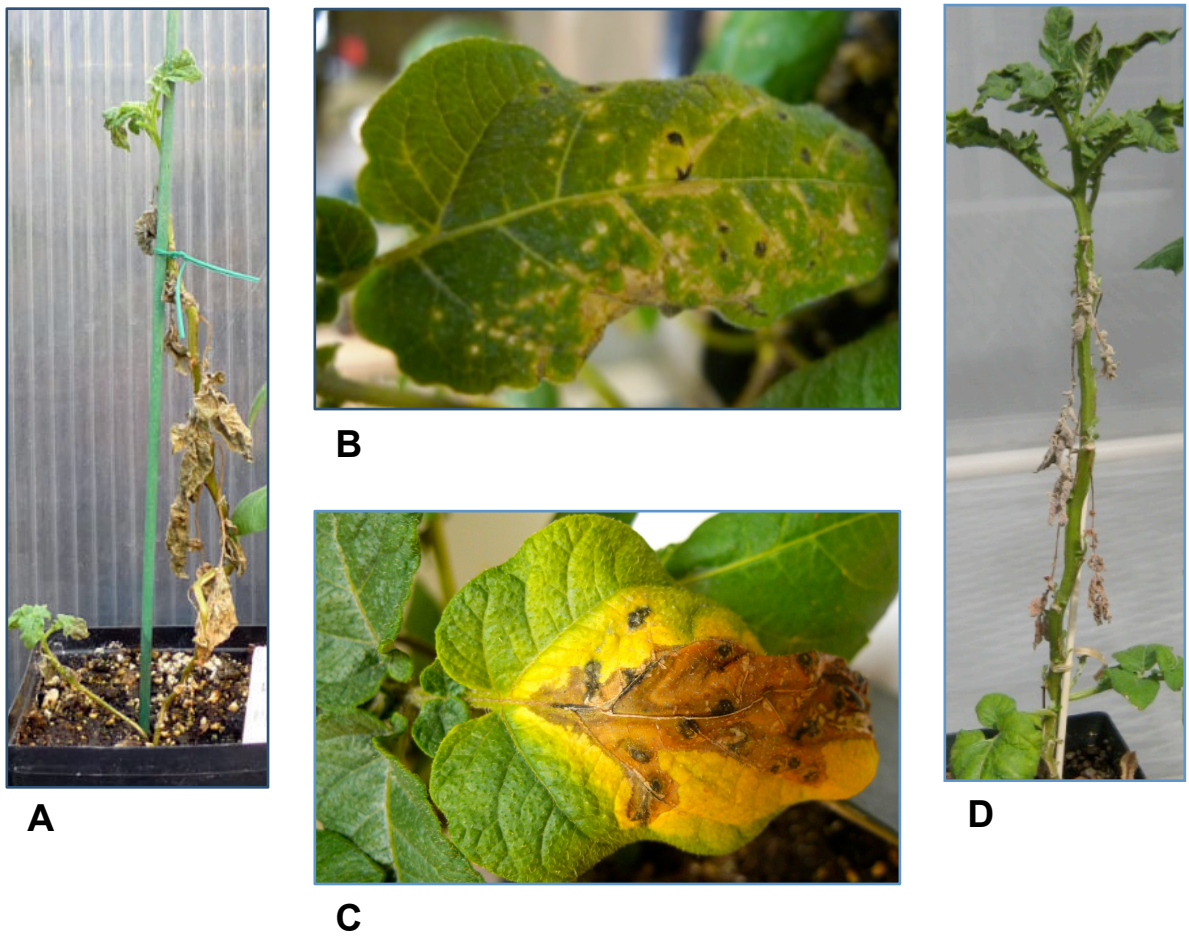

Figure 1.1 Local (B,C) and systemic (A,D) HR induced in cv. Maris Bard upon inoculation with PVY-Tb60 (A and B; PVY^{O}) and PVY-L26 (C and D; PVY^{Z}-NTN). Local HR at 12 days post inoculation (B and C), and systemic HR visible as a characteristic leaf-drop syndrome (A and D) at 5 weeks post inoculation.

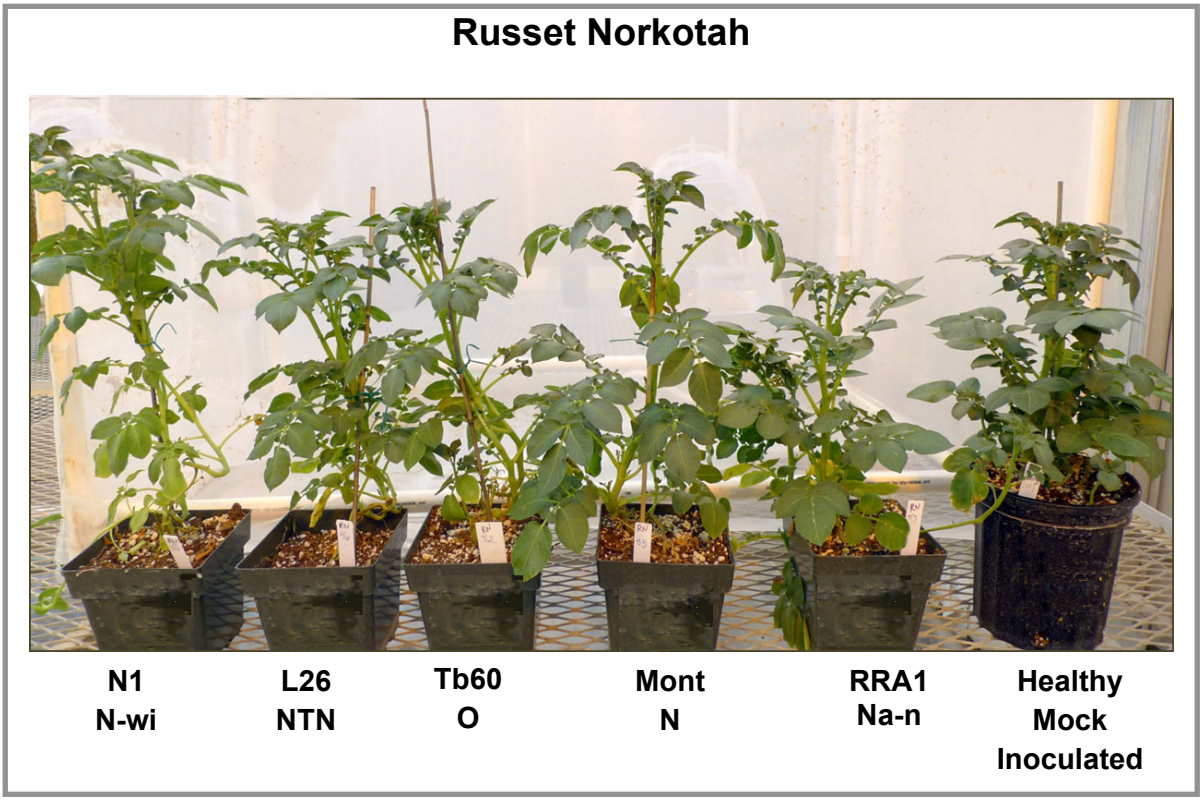

Figure 1.2 Symptoms induced by five virus isolates on cv. Russet Norkotah, as observed at 7 weeks post-inoculation. The five isolates are N1 (PVY^{N-Wi}), Tb60 (PVY^{O}), L26 (PVY^{Z}-NTN), Mont (PVY^{N}), and RRA1 (PVY^{NA-N}). All plants were tested at 3 weeks p.i. by TAS-ELISA and RT-PCR to confirm systemic infection with the isolate/strain indicated.

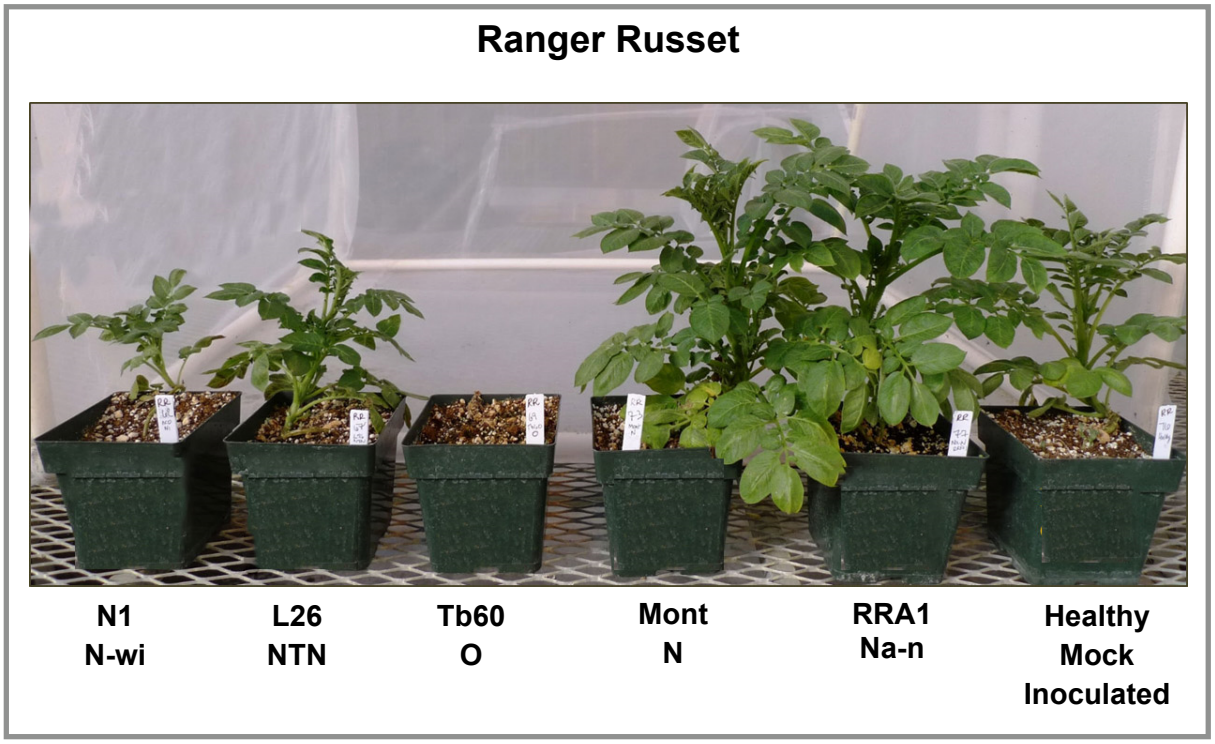

Figure 1.3 Symptoms induced by five virus isolates on cv. Ranger Russet, as observed at 7 weeks post-inoculation. The five isolates were N1 (PVY^{N-Wi}), Tb60 (PVY^{O}), L26 (PVY^{Z}-NTN), Mont (PVY^{N}), and RRA1 (PVY^{NA-N}). All plants were tested by TAS-ELISA and RT-PCR at 3 weeks p.i. to confirm systemic infection with the isolate/strain indicated. The PVY^{O}-infected plant died between 3 and 4 weeks p.i., soon after laboratory confirmation that it was systemically infected with PVY^{O}.

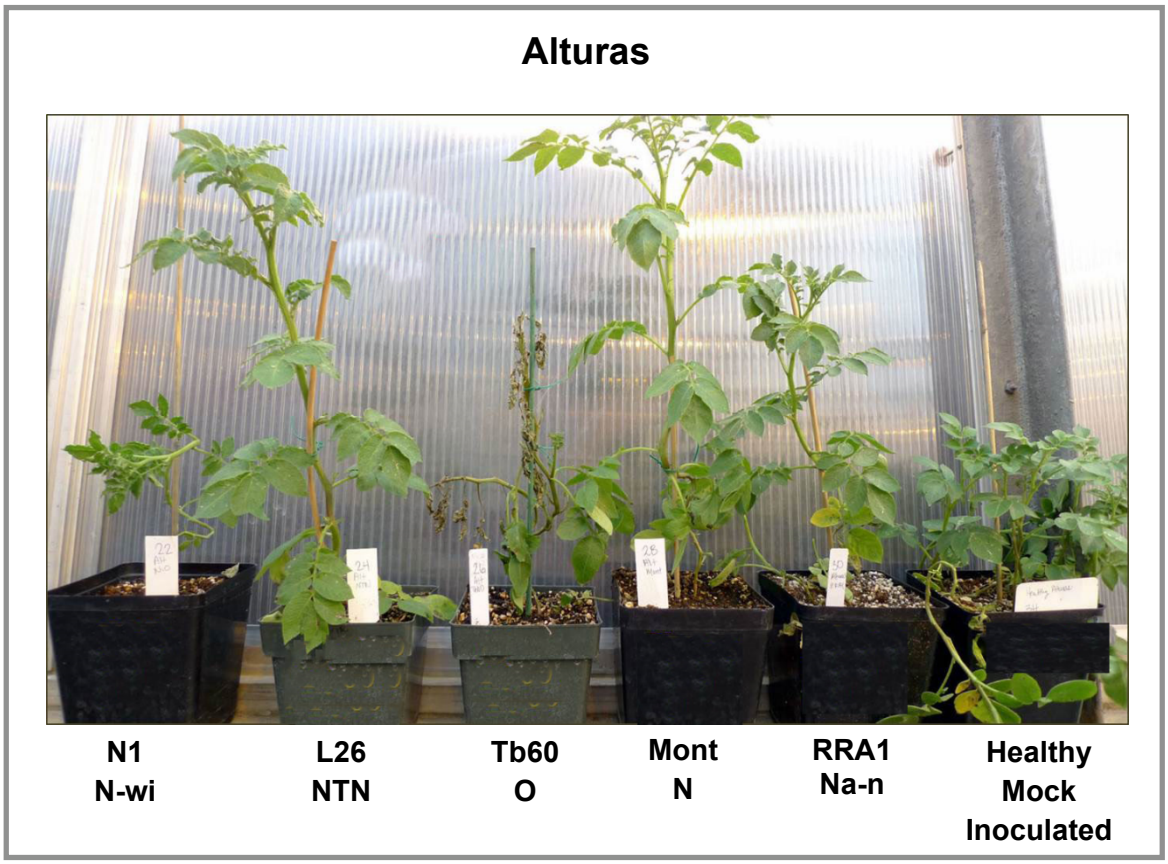

Figure 1.4 Symptoms induced by five virus isolates on cv. Alturas, as observed at 4 weeks post-inoculation. The five isolates are N1 (PVY^{N-Wi}), Tb60 (PVY^{O}), L26 (PVY^{Z}-NTN), Mont (PVY^{N}), and RRA1 (PVY^{NA-N}). All plants were tested at 3 weeks p.i. by TAS-ELISA and RT-PCR to confirm systemic infection with the isolate/strain indicated.

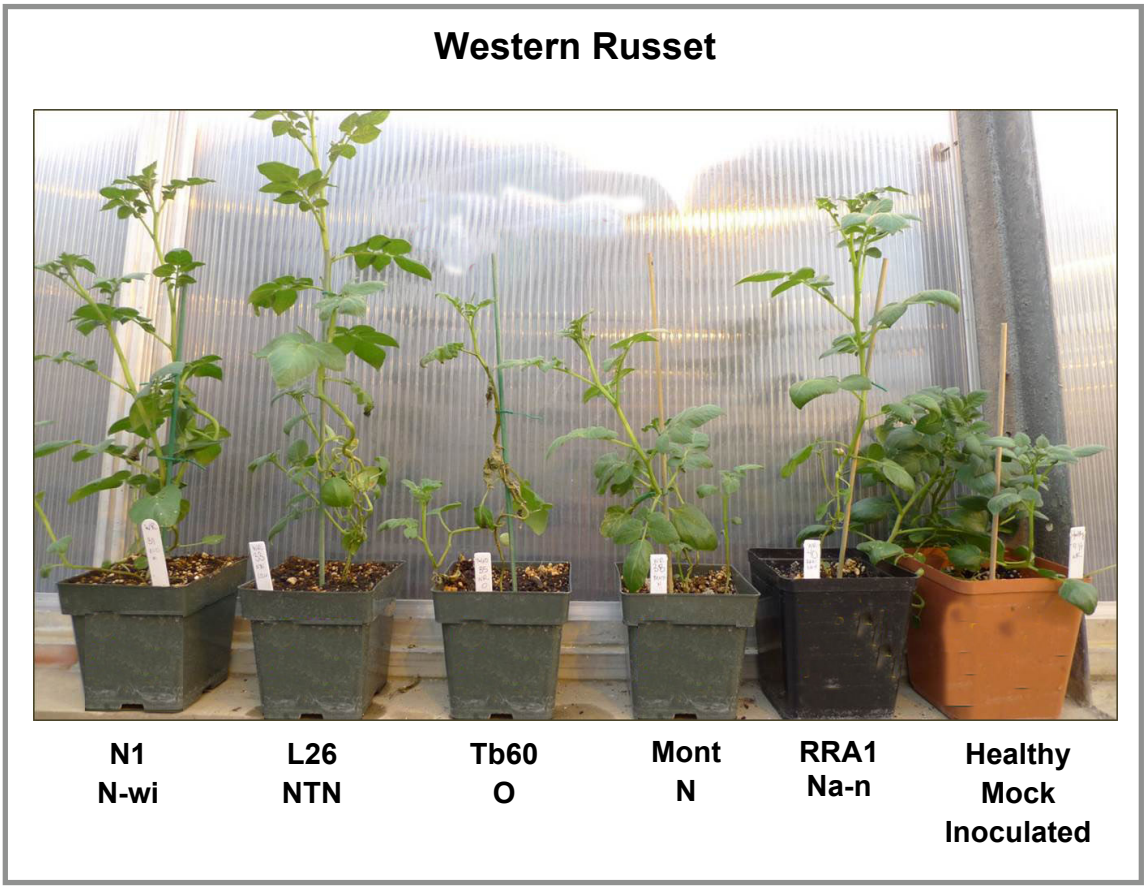

Figure 1.5 Symptoms induced by five virus isolates on cv. Western Russet, as observed at 4 weeks post-inoculation. The five isolates are N1 (PVY^{N-Wi}), Tb60 (PVY^{O}), L26 (PVY^{Z}-NTN), Mont (PVY^{N}), and RRA1 (PVY^{NA-N}). All plants were tested at 3 weeks p.i. by TAS-ELISA and RT-PCR to confirm systemic infection with the isolate/strain indicated.

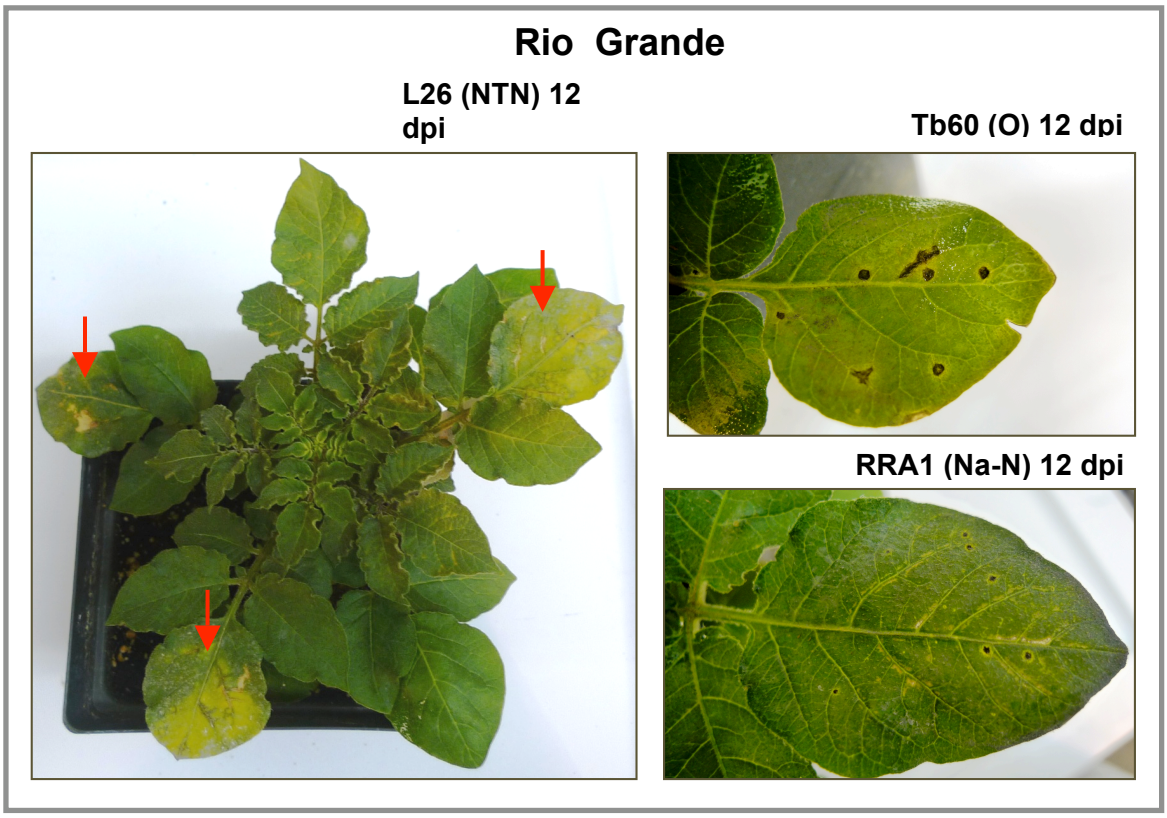

Figure 1.6 Local symptoms on inoculated leaves induced by three isolates in cv. Rio Grande, and RRA1 (PVY^{NA-N}). Red arrows mark the leaves on the plant inoculated with L26 (PVY^{Z}-NTN). The local symptoms varied from necrotic rings (L26, PVY^{NTN}), to simple necrotic lesions (RRA1, PVY^{NA-N}), to diffuse necrotic lesions expanding along the veins (Tb60, PVY^{O}). All plants were tested at 3 weeks p.i. by TAS-ELISA and RT-PCR to confirm systemic infection with the isolate/strain indicated; Rio Grande plants inoculated with RRA1 (PVY^{NA-N}) lacked systemic infection.

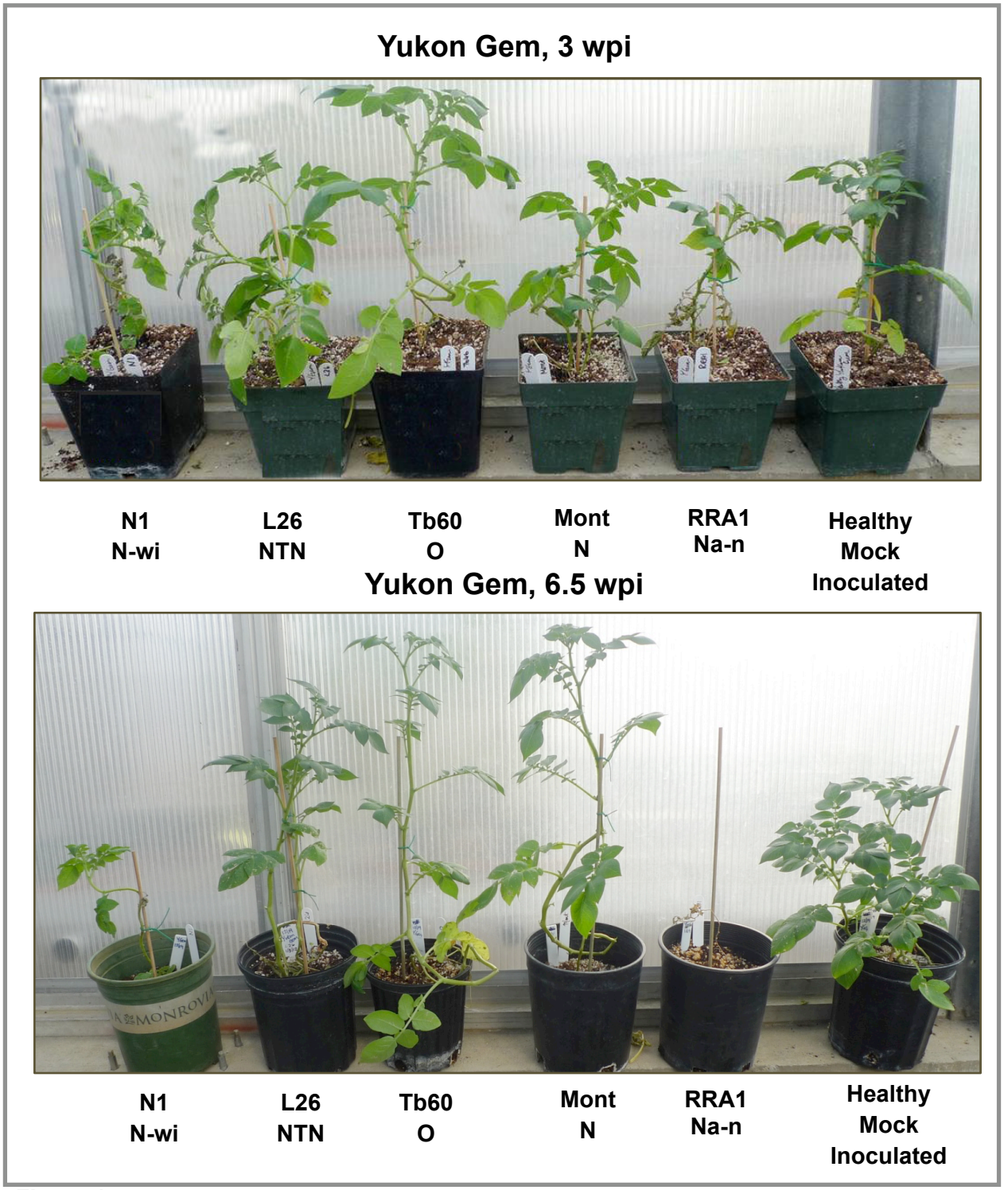

Figure 1.7 Symptoms induced by five virus isolates in cv. Yukon Gem, as observed at 3 and 6.5 weeks post-inoculation. The five isolates were N1 (PVY^{N-Wi}), Tb60 (PVY^{O}), L26 (PVY^{Z}-NTN), Mont (PVY^{N}), and RRA1 (PVY^{NA-N}). All plants were tested at 3 weeks p.i. by TAS-ELISA and RT-PCR to confirm systemic infection with the isolate/strain indicated. Note that these two pictures represented two independent experiments conducted at different times; in both experiments, Yukon Gem plants inoculated with RRA1 (PVY^{NA-N}) died between 5 and 6 weeks post inoculation.

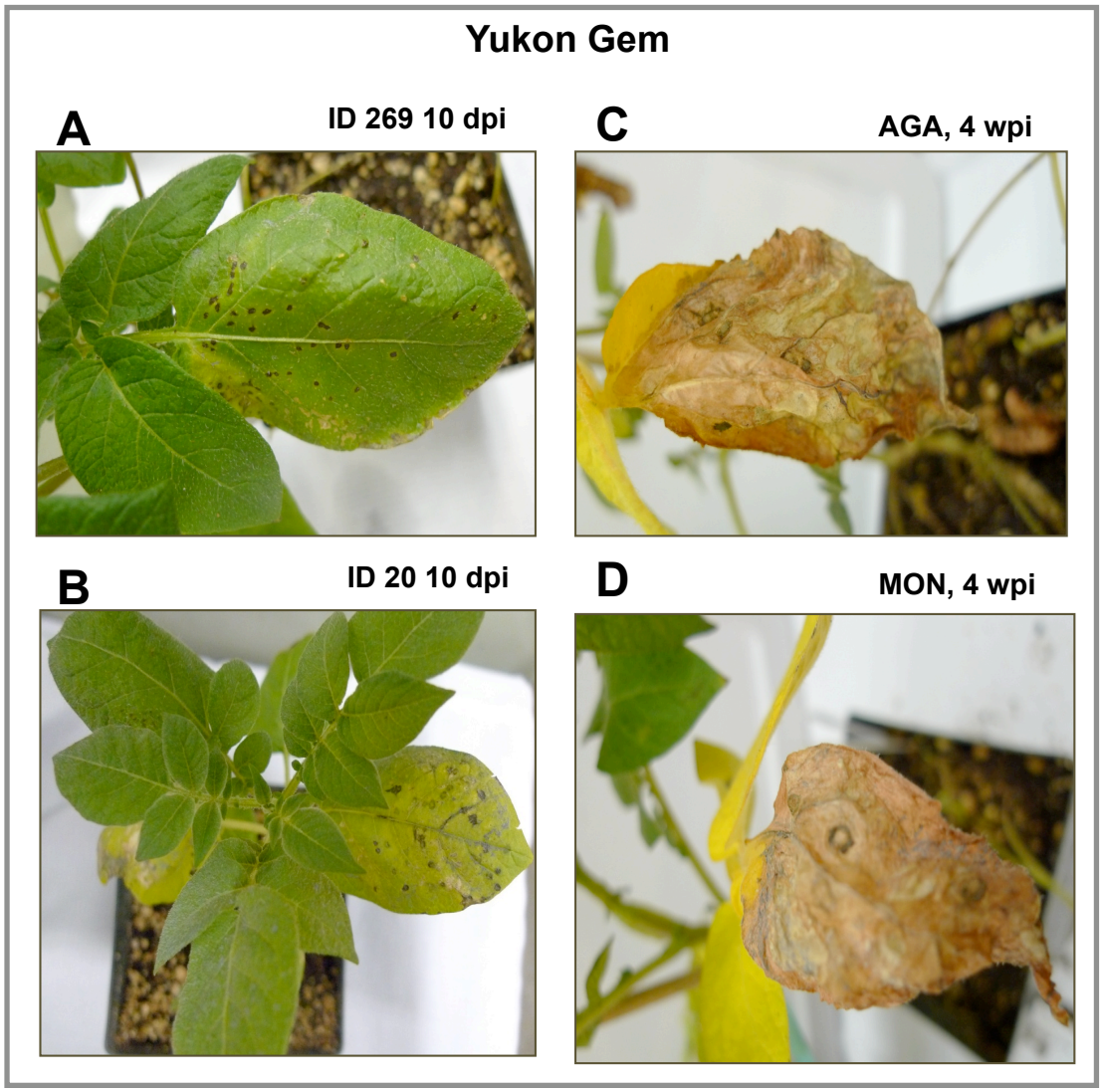

Figure 1.8 Local symptoms on inoculated leaves induced by four isolates in cv. Yukon Gem, as observed at 10 days (A and B) and at 4 weeks (C and D) post-inoculation. The four isolates are PVY-ID269 (A, PVY^{O}-O5), PVY-ID20 (B, PVY-NE11), PVY-AGA (C, PVY^{E}), and PVY-MON (D, PVY^{E}). Local symptoms varied from necrotic rings (ID20, PVY-NE11), to diffuse necrotic lesions expanding along the veins (both ID20, PVY-NE11, and ID269, PVY^{O}-O5), to fully necrotized inoculated leaves (AGA and MON, PVY^{E}). All plants were tested at 3 weeks p.i. by TAS-ELISA and RT-PCR to confirm systemic infection with the isolate/strain indicated; in all cases shown, plants inoculated with PVY-ID269 (**A**, PVY^{O}-O5), PVY-ID20 (**B**, PVY-NE11), PVY-AGA (**C**, PVY^{E}), and PVY-MON (**D**, PVY^{E}) lacked systemic infection.

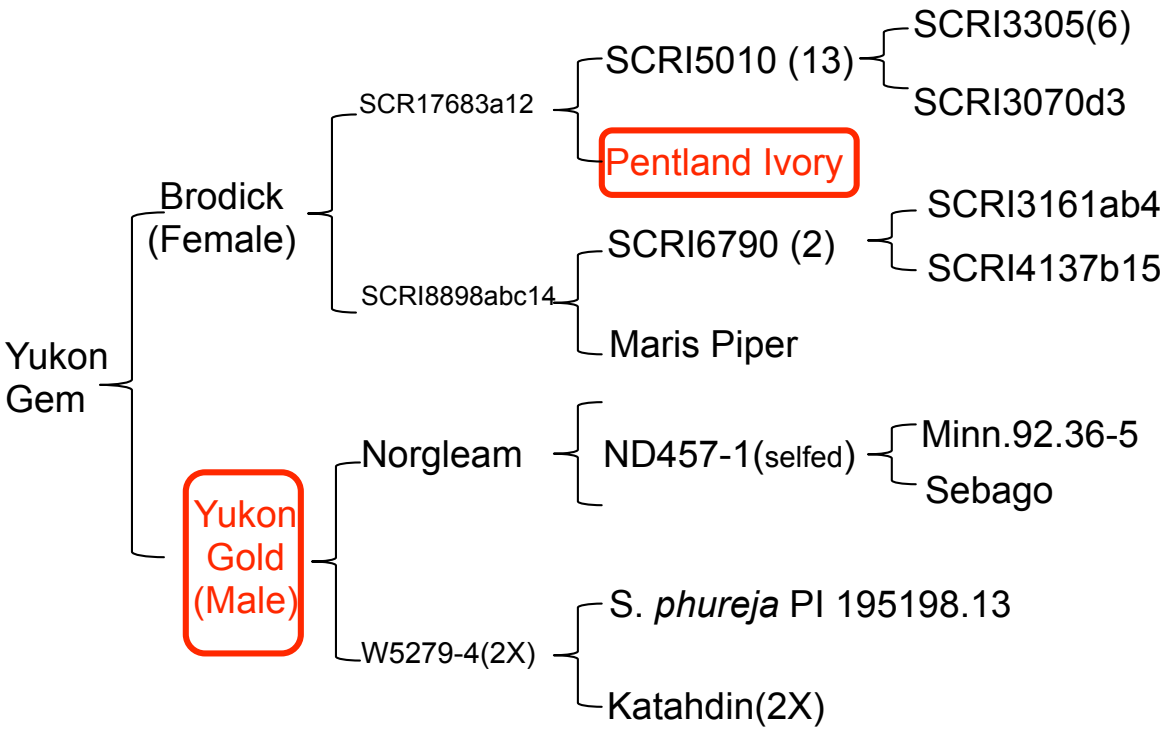

Figure. 1.9 A schematic representation of the pedigree of cv Yukon Gem as described in Whitworth et al (2010). Cultivars Pentland Ivory (***Nc:Ny:Nz***) and Yukon Gold (***Ny:Nz***) used in crosses to produce Yukon Gem were marked on the diagram.

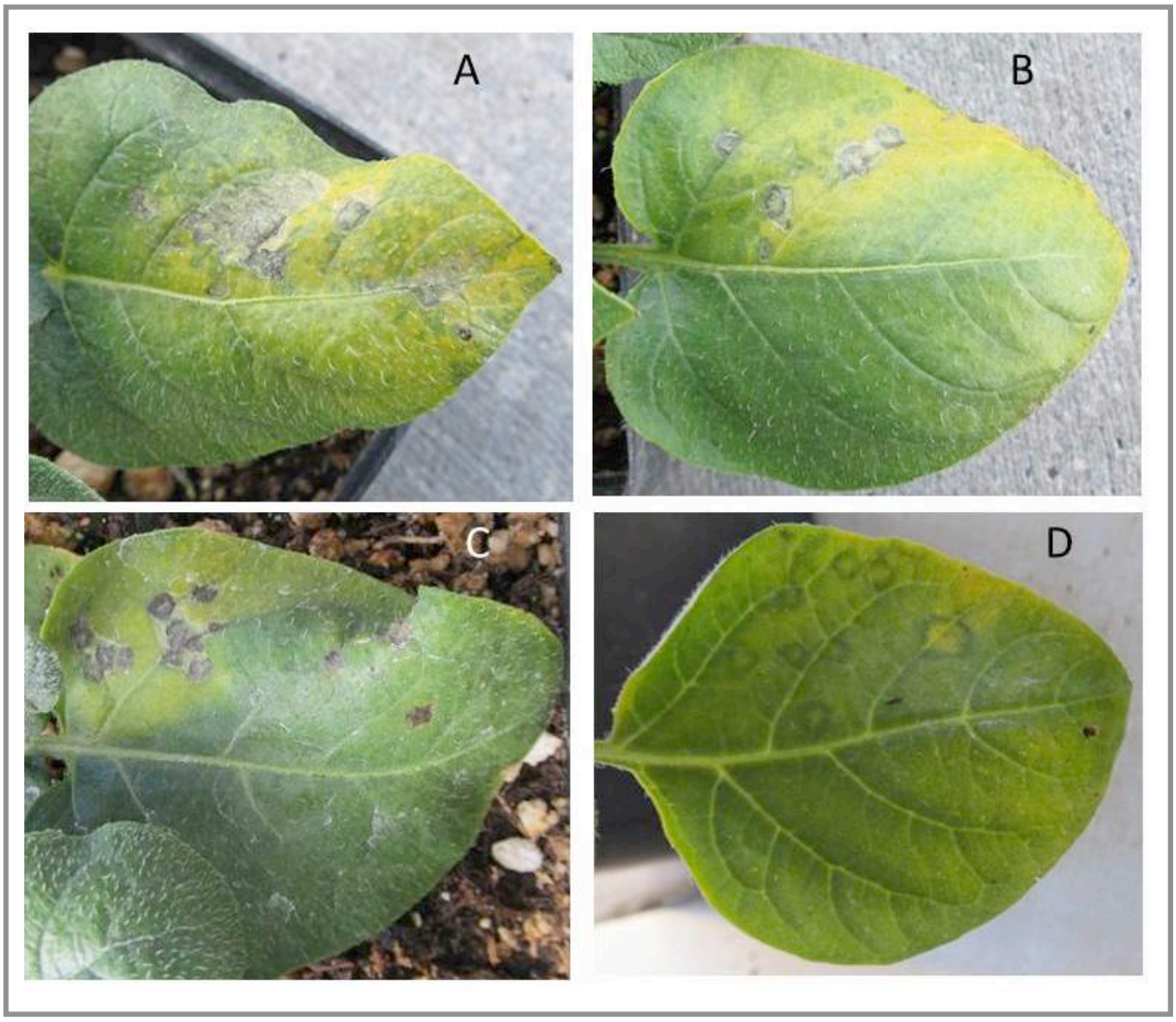

Figure 2.1 Local HR induced by PVY-L26 in progeny of crosses of Maris Bard x Russet Norkotah (A, B), King Edward x Maris Bard (C), and . Russet Norkotah x Maris Bard (D).

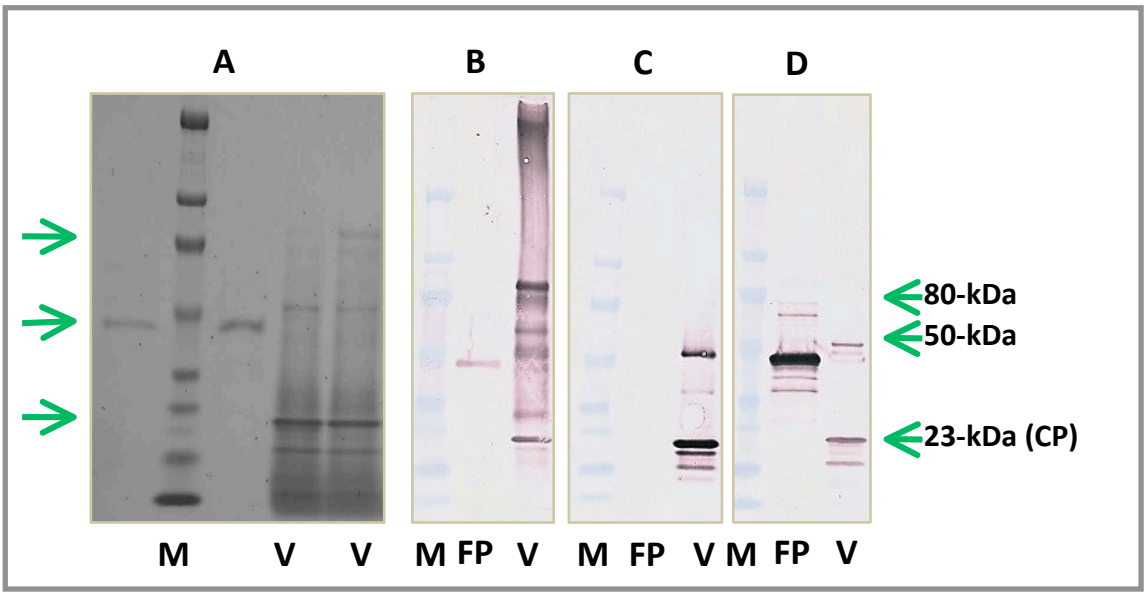

Figure 3.1 An illustration of the PLRV-specific antibodies performance. Polyacrylamide gel electrophoresis (PAGE) analysis of the PLRV structural proteins: (A) Coomassie-stained gel; (B-C) Western blots developed with antiserum UID 10, produced against virus purified from green material (B), MAb2, a monoclonal antibody (C), and G-545, antibody produced against bacterially expressed capsid protein (D). Letters under the panel describe the antigen type – FP=fusion protein (bacterially expressed); V=purified virus (purified from green material); M=marker proteins.

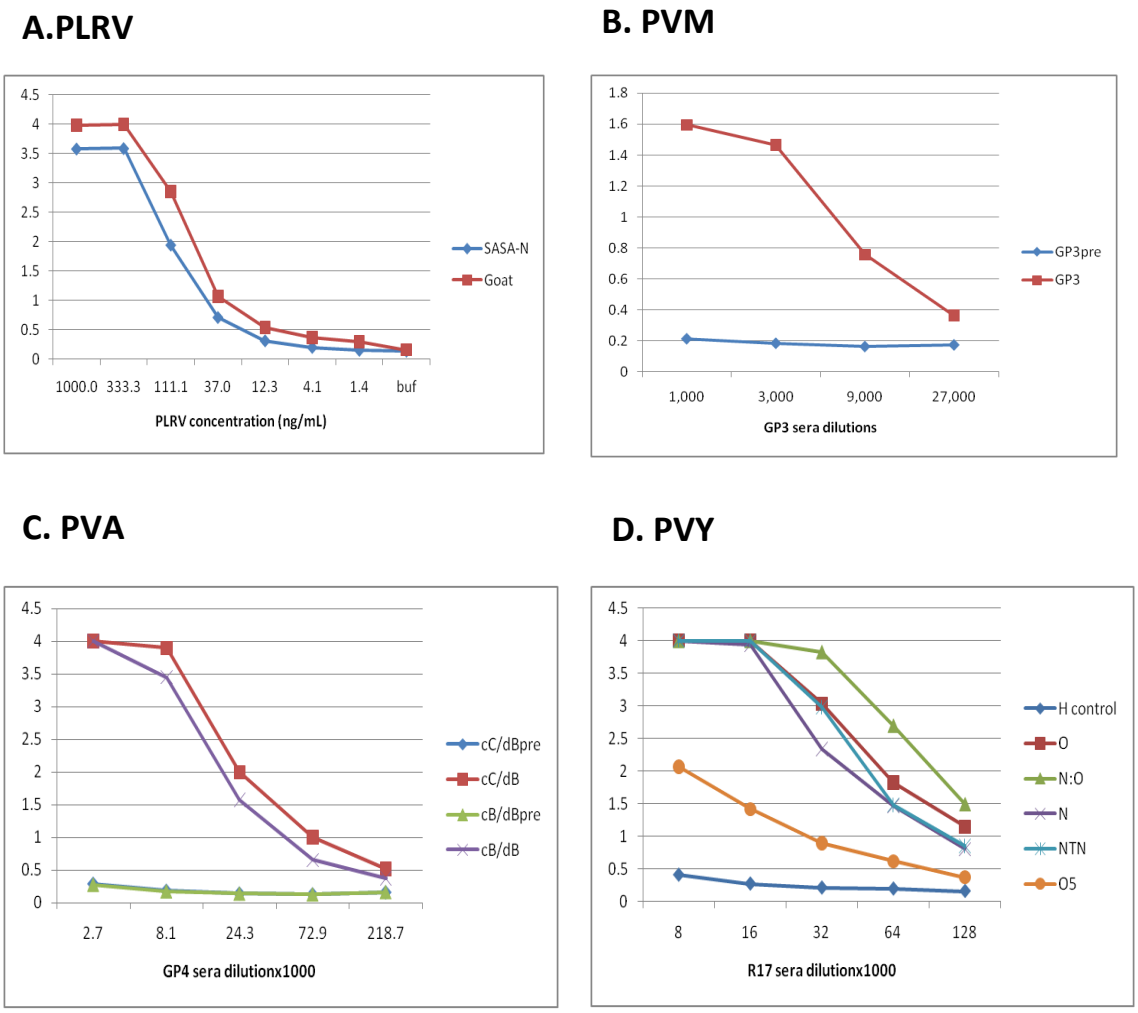

Figure 3.2 Examples of ELISA assays with antibodies produced against bacterially expressed capsid protein (CP). A. PLRV: comparison of the polyclonal G-545 produced to a cloned capsid protein (CP) of PLRV (red line) compared to a commercial monoclonal (blue line). B. PVM: titration of the polyclonal GP3 produced to a cloned CP of PVM as detecting antibody binding plate-trapped purified virus. C. PVA: comparison of the polyclonal antibodies produced in a rabbit to a cloned CP of PVA (red line) compared to a purified virus from green material (purple line) D. PVY: titration of the polyclonal antibody R17 produced to a cloned CP of PVY as detecting antibody binding different strains of PVY from infected plant tissue (tobacco) in the TAS-ELISA format, coating antibody used is polyclonal G-500 produced to the cloned CP of PVY as well.

Figure 4 Examples of systemic necrosis (SN).

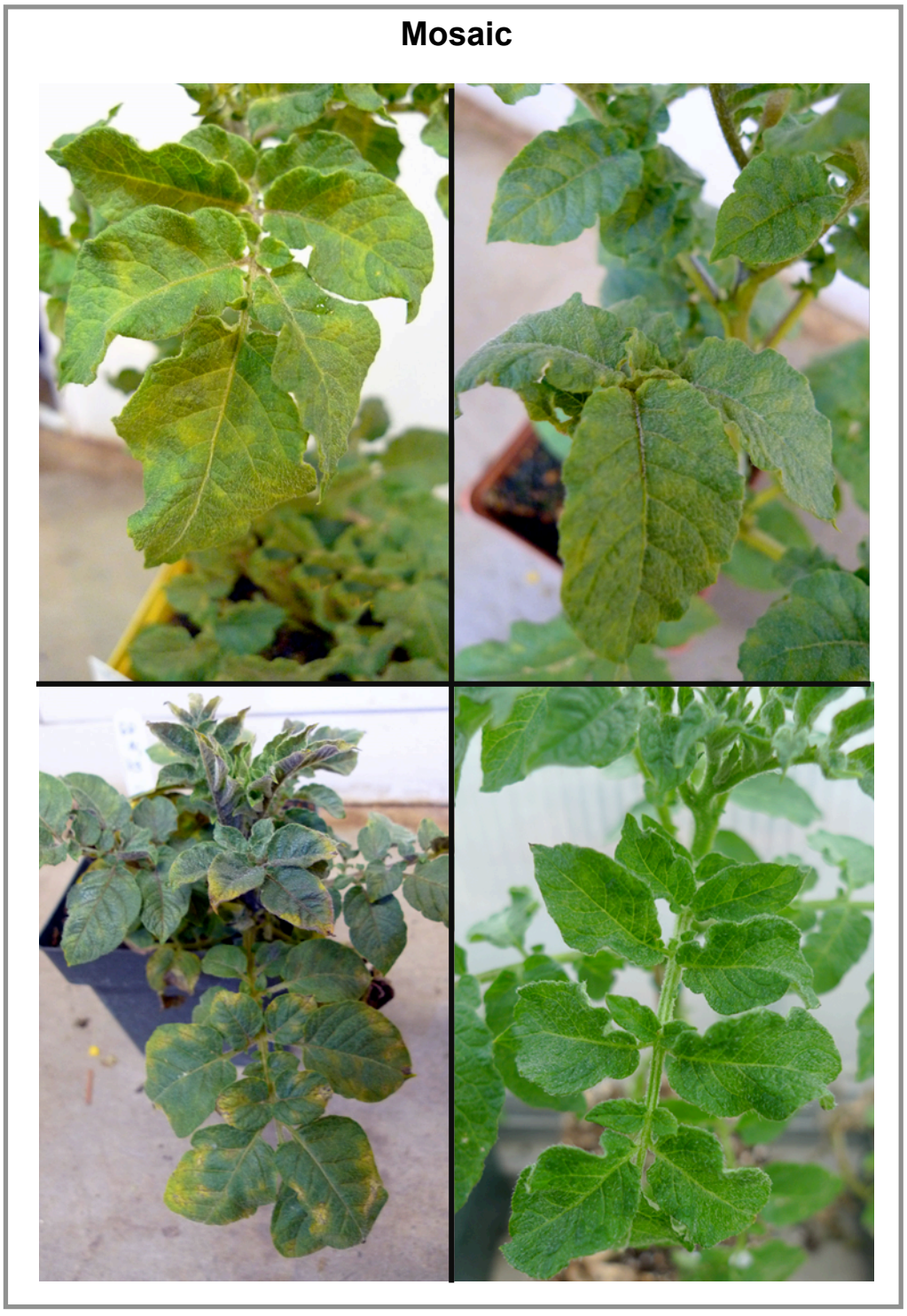

Figure 5 Examples of Mosaic (M).

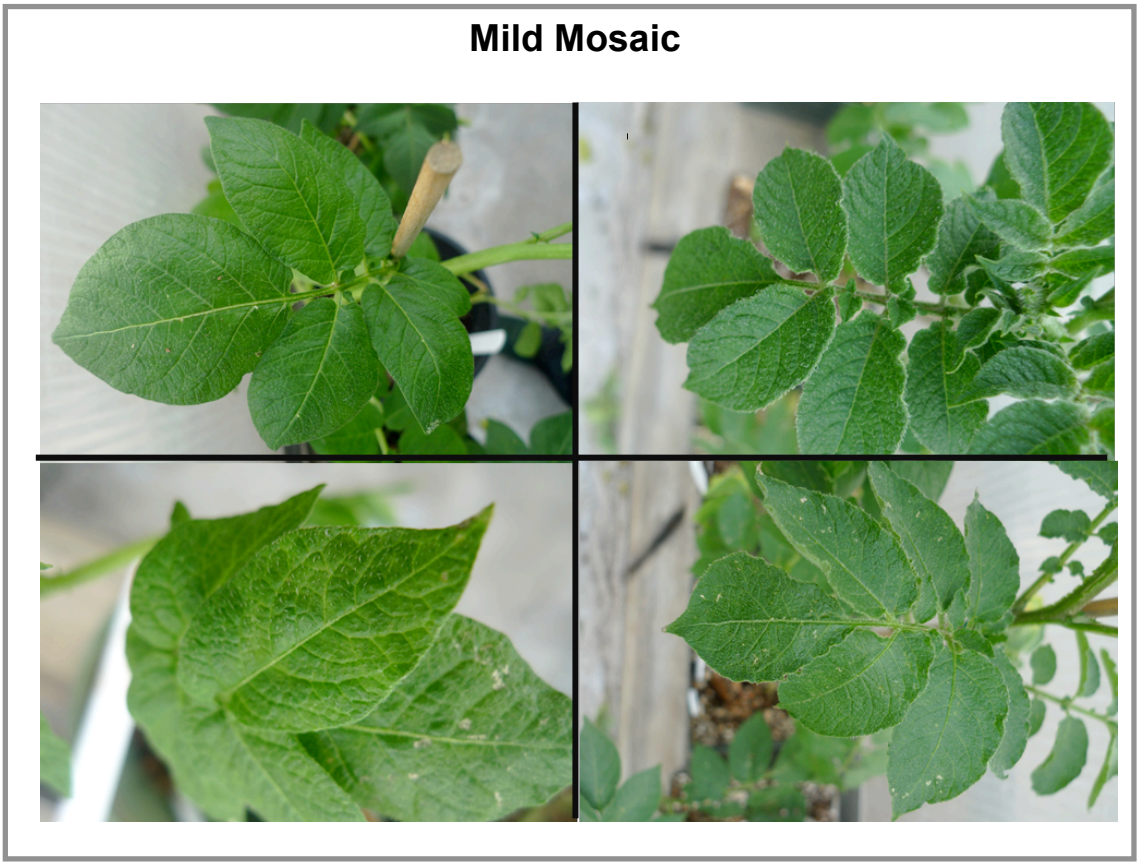

Figure 6 Examples of Mild Mosaic (MM).

Figure 7 Examples of crinkle (Cr).

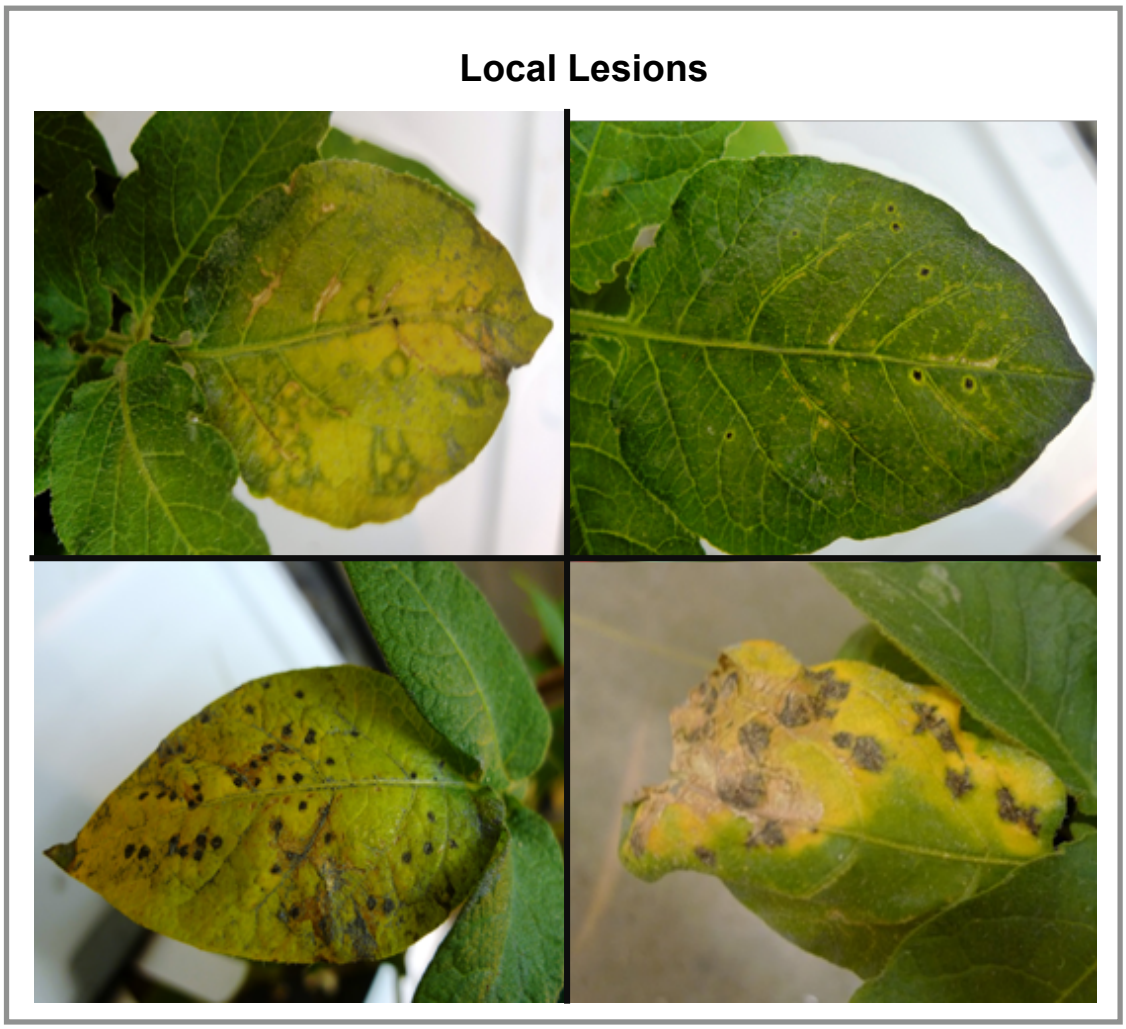

Figure 8 Examples of Local Lesions/Necrosis (LL).

Tables

	2013 Hectacres	
Potato cultivars tested	Idaho	Montana
Russet Norkotah	1,782	506
Ranger Russet	2,026	484
Russet Burbank	5,675	1740
Alturas	625	282
Western Russet	71	10
Yukon Gem	28	4
Rio Grande	0.1	30
Shepody	253	14

Table 1.1 Production of seed potato cultivars in two states selected for testing.

Isolates	Strain	Tobacco bioassay	Serotype	Genome sequence[a]
N1	PVY^{N-Wi}	vein necrosis	O	Recombinant HQ912863
N3	PVY^{N-Wi}	vein necrosis	O	Recombinant HQ912868
ME142	$PVY^{N:O}$	vein necrosis	O	Recombinant HQ912871
ME162	$PVY^{N:O}$	vein necrosis	O	Recombinant HQ912872
L26	PVY^{NTN}	mosaic	N	Recombinant FJ204164
Tb60	PVY^{O}	mosaic	O	O-type sequence EF026074
ID269	PVY^{O}-O5	mosaic	O/O5	Recombinant FJ643477
Mont	PVY^{N}	vein necrosis	N	N-type sequence AY884983
RRA-1	PVY^{NA-N}	vein necrosis	N	NA-N-type sequence AY884984
NE-11	PVY-NE11	vein necrosis	N	Recombinant DQ157180
ID20	PVY-NE11	vein necrosis	N	Recombinant HQ912867
AGA	PVY^{E}	vein necrosis	N/AST	Recombinant JF928459
MON	PVY^{E}	vein necrosis	N	Recombinant JF928458

[a] : sequences deposited in Genbank

Table 1.2 Phenotypic traits on tobacco plants for the PVY isolates used in this study.

Cultivar	Isolate/strain of PVY				
	*N1/**N-Wi***	*L26/**NTN***	*Tb60/**O***	*Mont/**N***	*RRA1/**NA-N***
Maris Bard	M, Cr, St	M, Cr, LL, SN	M, Cr, LL, SN	M, Cr	MM
Desiree	MM	M	M, Cr, LL, SN	MM	MM
Russet Norkotah	NS	NS	NS	NS	NS
Ranger Russet	M, Cr, St	M, Cr, St	M, Cr, LL, SN	M	M
Russet Burbank	MM	MM	MM	NS	NS
Alturas	M, Cr, St	M, Cr, St	M, Cr, LL, SN	M M, or NS	MM
Western Russet	M, Cr	M, Cr	M, Cr, LL, SN	MM, or NS	Very MM
Yukon Gem	M, Cr, St, LL, SN	LL, No infection	LL, No infection	No infection	M, Cr, LL, SN
Rio Grande	M, Cr	M, Cr, LL, SN	M, Cr, LL, SN	NS	LL, No infection
Shepody	MM	MM	MM	NS	NS

Table 1.3 Summary of symptoms expressed by different potato cultivars upon mechanical inoculation with five isolates of PVY representing five distinct PVY strains. MM=Mild Mosaic, Cr=Crinkle, M=Mosaic, LL=Local Lesions, SN=Systemic Necrosis St=Stunting, NS= No symptoms, No infection= No systemic infection. Examples of these symptoms can be seen in Figures 4-7.

PVY strain	*Isolate name*	*Symptoms*
N-Wi	*N1	M, Cr, St, **LL, SN**
	N3	M, Cr, St, **LL, SN**
N:O	Me-142	M, Cr, St, **LL, SN**
	Me-162	M, Cr, St, **LL, SN**
O	*Tb60	**LL**, No infection
O5	ID269	**LL**, No infection
Z-NTN	*L26	**LL**, No infection
N	*Mont	No infection
NA-N	*RRA1	M, Cr, **LL, SN**
NE-11	NE-11	**LL**, No infection
	ID20	**LL**, No infection
E	AGA	**LL**, No infection
	MON	**LL**, No infection

* Asterix marks the isolates tested in initial experiments, see Table 1.3. Necrotic reactions are highlighted in bold.

Table 1.4 Summary of leaf symptoms expressed by cv. Yukon Gem upon inoculation with an expanded set of PVY isolates/strains. M=Mosaic, Cr=Crinkle, St=Stunting, LL=Local Lesions, SN=Systemic Necrosis, No infection=No systemic infection. Examples of these symptoms can be seen in Figures 4-7.

	N genes triggered by strains	
Potato cultivars tested	PVY^{O}	PVY^{Z}
Maris Bard	***Ny***	***Nz***
Desiree	***Ny***	*nz*
Russet Norkotah	*ny*	*nz*
Ranger Russet	***Ny***	*nz*
Russet Burbank	*ny*	*nz*
Alturas	***Ny***	*nz*
Western Russet	***Ny***	*nz*
Yukon Gem	***Ny***	***Nz***
Rio Grande	***Ny***	***Nz***
Shepody	*ny*	*nz*

Table 1.5 Summary of the known and proposed genetic backgrounds for the potato cultivars tested. ***Ny:*** has *N* gene. Ny: Does not have *N* gene.

		Male parent		
		Maris Bard	Russet Norkotah	King Edward
Female parent	Maris Bard	N/A	Fruit 1=111 Fruit 2=130 Fruit 3=93 Fruit 4=127	None
	Russet Norkotah	Fruit 1=120 Fruit 2=113 Fruit 3=117	N/A	None
	King Edward	Fruit 1=60 Fruit 2=52	Fruit 1=78 Fruit 2=92 Fruit3=50	N/A

Table 2.1 Number of seeds per fruit from crosses between different potato cultivar. Used either as male or female parents.

Experiment/Cross	No. of plants	No. of displaying any level of HR	No. of susceptible	Ratio
1. RNxMB	30	15	15	1:1
2. MBxRN	29	10	19	1:2
3. KExMB	28	15	13	1:1
4. RNxMB	56	30	26	1:1
5. MBxRN	60	30	30	1:1

Table 2.2 Segregation of the potato progeny of crosses between cvs Maris Bard and Russet Norkotah, and Maris Bard and King Edward according to their HR reaction against PVY-L26 (PVY^{Z}).

Virus	Primers	Restriction sites
PLRV	OVN-F:GGTGAATTCATGAGTACGGTCGTG R:GGTCTGCAGCTATTTGGGGTTTTG	EcoRI/PstI
PVY	ONpvyNcpFxR F:5'GGTGAATTCGCAAATGACACAATCGAT3' R:5'GGTCTGCAGTTACATGTTCTTVACTCCAAGTAG3'	EcoRI/PstI
PVM	Mcp FxR F:GGTGAATTCATGGGAGATTCA R: GGTGTCGACTCATTTGTTATT	SalI/EcoRI
PVA	PVAcpFxR F:GGTGGATCCGCCGRAACTCTT R:GGTGTCGACTTACACCCCCTT	BamHI/SalI
PVS	PVScpFxR F:GGAGTCGACTCATTGGTTGAT R:GGAGTCGACTCATTGGTTGAT	EcoRI/SalI
PVX	DRpvyXcpFxR F:GGAGAATTCATGGCGCCCAAA R:GGAGTCGACTCATTGGTTGAT	EcoRI/SalI

Table 3.1 List of primers used to amplify capsid proteins of viruses used in this study, with restriction sites used for cloning into the expression vector indicated.

Virus	Classification	Morphology	Vector
Potato virus Y (PVY)	**Potyvirus**	**Filamentous**	**Aphids**
Potato virus A (PVA)	**Potyvirus**	**Filamentous**	**Aphids**
Potato virus X (PVX)	**Potexvirus**	**Filamentous**	**Mechanical**
Potato virus S (PVS)	**Carlavirus**	**Filamentous**	**Aphids**
Potato Virus M (PVM)	**Carlavirus**	**Filamentous**	**Aphids**
Potato leafroll virus (PLRV	**Luteovirus**	**Spherical**	**Aphids**

Table 3.2 A summary of properties for the six main potato viruses examined in this research.

	Bacterial Antigen			***Conventional Antigen***		
	Rabbit	**Guinea Pig**	**Goat**	**Rabbit**	**Guinea Pig**	**Goat**
***Potato Virus* Y- PVY**	+	+	-	+	+	+
***Potato Virus* A-PVA**	+	+	-	-	+	-
***Potato leafroll virus*- PLRV**	+	+	+	-	+	+
***Potato virus* M-PVM**	+	+	-	-	+	-
***Potato virus* X- PVX**	+	+	-	-	+	-
***Potato virus* S- PVS**	+	+	-	-	+	-

Table 3.3 Summary list of antibodies produced. '**+**' means I produced the antibody successfully, A '**-**' means I did not attempt to produce antibodies in that animal. "bacterial antigen" denotes antibodies produced against the bacterially expressed fusion protein, and 'conventional antigen' denotes antibodies produced again purified virus from green material. It is beneficial to have a wide variety of animal species so TAS-ELISA can be used.