Development of Laboratory Detection Protocols for Two Soil-Borne Potato Viruses, Potato Mop-Top Virus and Tobacco Rattle Virus

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AUTHORIZATION TO SUMBIT THESIS

This thesis of Thanh D. Tran, submitted for the degree of Master of Science with a Major in Plant Science and titled "Development of Laboratory Detection Protocols for Two Soil-Borne Potato Viruses, Potato Mop-Top Virus and Tobacco Rattle Virus," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

Potato mop-top virus (PMTV) and *Tobacco rattle virus* (TRV) are emerging potato pathogens in the United States. PMTV (Pomovirus: Virgaviridae) is a tripartite rod-shaped virus having single-stranded, positive-sense RNA genome encapsidated by a 19.7-kDa capsid protein. The virus is soil-transmitted by plasmodiophoromycete *Spongospora subterranea*. TRV is a bipartite rod-shaped, single-stranded, positive-sense RNA virus encapsidated by a 23.6-kDa capsid protein. TRV is unique in that RNA-1 can replicate and establish systemic infection in some hosts independent of RNA-2, which encodes for CP, resulting in a 'capsidless' virus infection and can present special problems for identification. TRV is also soil-transmitted and vectored by trichodorid nematodes. Both viruses can induce internal necrotic arcs in the flesh of affected tubers, diminishing their quality for processing and fresh market. Visual diagnosis is challenging since both viruses often do not induce symptoms in the foliage, and similar tuber symptoms can be caused by other potato viruses, such as *Potato virus Y*. Two sensitive and specific laboratory methods were developed for fast and reliable detection of PMTV and TRV in foliar and tuber samples from potato: a triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA), and an immunocapture (IC) reverse transcription (RT)-PCR assay. These TAS-ELISA and IC-RT-PCR methodologies were compared to commercially available detection kits from European diagnostic companies and found to be equal or superior in sensitivity and reliability.

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DEDICATION

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CHAPTER 1

INTRODUCTION OF SEED CERTIFICATION PROCESSES

Laboratory diagnosis and production of virus-free potato

Potatoes are vegetatively propagated, in which new plants are grown from tubers produced by the parental plant. This ensures all plants are genetically the same; however, the vegetative crop may be at greater risk of exposure to various pathogens which may become perpetuated in new growth cycles. Disease may reduce yield and quality of the infected plant, and progeny tubers/ subsequent plants may be infected as well. Removal of diseased plants and continuous use of clean seed stocks is vital to the production of a vegetatively propagated seed crop, such as potato (Frost et al., 2013). A seed certification program, limiting the number of generations the crop could be field-propagated, and ensuring high quality with minimal disease of harvested seed potatoes is necessary for the success of the potato crop.

Enacted in 1912, the U.S. Quarantine Act gave the Animal and Plant Health Inspection Service (APHIS) jurisdiction to oversee the movement of nursery and plant stocks that may host harmful pathogens (Womach, 1997). Between 1913 and 1922, 22 seed certification agencies were established in multiple states. The seed certification program is considered to be a voluntary program. Regulation and tolerance differ by each state, which are independent agencies. The purpose of certification is "… to maintain and make available to the public high quality seeds of superior crop plant varieties so grown and distributed as to preserve genetic identity and purity" (Idaho Crop Improvement Association Rules of Certification). Certification is never a guarantee of a disease-free crop. It only indicates that the crop has met the certification agency standards; nonetheless, there are strict guidelines and routine inspections to ensure those tolerances are not exceeded.

Potato seed is initiated as lab-derived disease-free plant stock which is multiplied in vitro. Nodal cuttings are taken from explant stock, grown on media, and tested for pathogens. If pathogens are found, such as viruses, potato cuttings undergo one or more cycles of “clean-up” where these cuttings are subjected to heat or chemical treatments (Love et. al, 2003; Griffiths et. al, 1990). The clean-up is considered complete when no pathogens can be detected in the cuttings. Cuttings are then transplanted in greenhouses and plants are grown out. Pre-nuclear tubers (pre-nuclear generation) or minitubers are harvested from these cuttings and planted in potato seed producing fields for seed certification. The first year pre-nuclear tubers are planted in the ground is considered

the nuclear generation (G0). Subsequent generations grown in the field are referred to as G1 (second year planted in the field), G2, G3, and so on. Disease pressure increases with every consecutive generation (Sieczka and Thornton, 2003), so every state has a limited generation program in place in which potato seed originates from a pathogen-tested source and plants are grown for a maximum number of (7) years in the field (Idaho Crop Improvement Association Policies and Procedures; Love et al., 2003). The last generation allowed in Idaho is G6; however, most states allow between G5 and G6, with Alaska allowing G8. Certified seed is then sold to potato farmers who produce crop grown for fresh or processed use. In addition, Idaho growers must plant certified seed in Idaho unless planting their own year-out seed (Idaho Crop Improvement Association Policies and Procedures).

Inspection occurs five times (Idaho Crop Improvement Association Policies and Procedures): 1. Early summer inspection for presence of viruses and viroid pathogens. Viral symptoms (rugosity, leaf drop, mosaic, and necrosis) are easiest to see one to two months after planting. Viruses that affect seed quality include *Potato leafroll virus* (PLRV), *Potato virus Y* (PVY), *Potato virus X* (PVX), *Potato virus M* (PVM), and *Potato spindle tuber viroid* (PSTVd); 2. Late summer inspection for viruses and bacterial ring rot; 3. Harvest/storage inspection for a wide range of diseases including necrotic viruses, bacterial decay, and other tuber-borne diseases; 4. Postharvest inspection (winter grow-out) to look for variety mixture, viral infections, herbicide damage, and laboratory testing for bacterial ring rot. It is also common to find damaging soil-borne diseases, such as internal disorder or discoloration symptoms caused by *Tobacco rattle virus* (TRV) or *Potato mop-top virus* (PMTV). Winter-grow-out is conducted when 400-tuber sample from each seed lot are planted during the winter season in a warm-climate location such as Florida, Hawaii, or California, and visually inspected. Samples are collected for laboratory testing to determine disease incidence (Frost et al., 2013); and 5. Shipping point inspection which is performed by a federal-state inspector. The inspector also looks for the presence of zero-tolerance diseases, tolerances factors met by the ICIA, defects such as hollow heart and growth cracks, and verify seed lot identity. Once the seed lot passasses the shipping point inspection, the inspector tags the seed lot and seals the transportation vehicle (Bohl et al., 1992). If a seed crop fails inspection at any of these five events, the seed lot may be rejected (Lisinska and Leszczynski, 1989).

Tolerance limit is the maximum amount of certification factor which is allowed. For example, the seed certification allows a certain amount of tolerance for PVY, which causes potato tuber necrotic ringspot disease (PTNRD), and PLRV which causes net necrosis. Tolerance limit is set by the state disease and tolerances for certification change with each generation.

Zero tolerance factor is when none of the factor is allowed in a seed lot. Zero tolerance factors include bacterial ring rot, root-knot nematodes, and corky ringspot (CRS; symptom of infection by *Tobacco rattle virus*). Rejection of a seed lot results in disqualification, downgrading, recertifying, or roguing (Lisinska and Leszczynski, 1989).

In the past 10 years, PVY infection is the most common reason for rejection of seed potatoes from certification in the United States and Canada and is considered to be the most economically and scientifically important virus affecting potato production (Frost et al., 2013). PVY is a Potyvirus that is vectored by aphids. PVY can cause mild to severe leaf mosaic, leaf drop, and stunting symptoms on potato foliage and PTNRD on tubers. The most crucial primary inoculum sources for PVY aphid-transmission are infected potatoes within or near a potato field. Thus, disease-free planting material is a critical first step in limiting the spread of PVY (Frost et al., 2013).

In addition, two soil borne viruses that affect potato production are TRV and PMTV. TRV causes CRS in tubers which has a zero-tolerance factor in seed certification. TRV has tremendous host range with over 350 laboratory hosts and 100 natural hosts. On potato, TRV foliar symptoms may include mild mottling, stunting, or could be lacking. Tuber symptoms can be mild or severe necrotic arcing and spotting through tuber flesh, resulting in 100% tuber quality loss (Lisinska and Leszczynski, 1989). TRV is vectored by nematodes in the family Trichodoridae, also known as stubby root nematodes. Similarly, another soil-borne virus, PMTV, can cause necrotic arcs or lines in tubers. Depending on the cultivar, foliar symptoms include yellow blotching or mottling on leaves, chlorotic v-shaped markings, and extreme shoot stunting, but often are lacking in the foliage. Tuber symptoms include internal cracking, blotchy surface, and external necrotic rings and arcs (Lisinska and Leszczynski, 1989). PMTV is vectored by soil-borne plasmodiophoromycete *Spongospora subterranean*, the causal agent of powdery scab (Lisinska and Leszczynski, 1989). TRV and PMTV cause very similar symptoms in tubers, and visual diagnosis is not reliable since the two are virtually visually indistinguishable and require lab diagnostics as confirmation.

Seed certification is predicted to face new challenges which include the increased movement of seed potatoes, resulting in increased mobility of difficult-to-control soil-borne pathogens such as TRV and PMTV. Many potato varieties do not present clear symptoms when infected with potato viruses. Reliance on visual inspection alone will miss a proportion of infected tubers because many potato varieties do not display obvious symptoms. These asymptomatic tubers may act as inoculum source and risk spreading PMTV and TRV to new fields in seed tubers (Latvala-Kilby et al., 2009). The application of post-harvest tuber testing of seed nominees all will subsequently eliminate

contaminated seed tubers and help control of spread and the establishment of virus at new sites (Sokmen et al., 1998). While some states still rely upon visual inspection only, in Idaho, every seed lot sample must be assessed by enzyme-linked immunosorbent assay (ELISA) for postharvest testing to evaluate virus incidence (Frost et al., 2013). Such post-harvest tuber testing will require large scale laboratory diagnostics. Over the years, the potato industry has consistently exhibited a high demand for reliable diagnostic tools suitable for large-scale testing. ELISA testing has shown to be a cost-effective tool amenable to large-scale testing of field material compared to PCR (Bolotova et al., 2009), and that is why we have chosen to focus on developing an immuno-detection based methodology for the detection of PMTV and TRV. Our lab sensed a great need for detection tools for PMTV and TRV, and in this work, the objective of this study is to develop laboratory detection protocols for two soil-borne potato viruses, PMTV and TRV, and assess their performance against existing commercial diagnostic kits.

CHAPTER 2

DEVELOPMENT OF A TRIPPLE ANTIBODY SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY AND IMMUNOCAPTURE RT-PCR METHODOLOGY FOR THE DETECTION OF POTATO MOP-TOP VIRUS IN TUBER AND LEAF TISSUE

Introduction

Soil-borne *Potato mop-top virus* (PMTV) was first described by Calvert and Harrison (1966). Upon PMTV infection, tubers of susceptible potato cultivars exhibited brown concentric rings on tuber surface, cracking, blotchy discoloration, and internal necrotic arcs/flecks. These tuber symptoms, commonly called 'spraing disease' in Europe, resembled symptoms of *Tobacco rattle virus* (TRV). On potato foliage, PMTV could cause dwarfing of shoots (hence the name mop-top), yellow blotching or mottling, and chlorotic V-shaped markings on leaflets, depending on potato cultivar (Calvert and Harrison, 1966; Harrison and Jones, 1970). In potato plants, PMTV infection is not systemic, and not all progeny tubers are infected. Calvert and Harrison concluded that PMTV may eventually be self-eliminating in a potato plant. It was later discovered by Jones and Harrison (1969) that PMTV is vectored by plasmodiophoromycete *Spongospora subterranea* f. sp. *subterranea* (Sss). Sss itself is a potato pathogen, the causal agent of powdery scab (Jones and Harrison, 1969; Merz, 2008).

PMTV is in the family Virgaviridae, formerly classified in the genus furovirus, and is now re-classified in the genus Pomovirus as its type member (Torrance and Mayo, 1997; Adams et al., 2017). It is a rod-shaped single-stranded, positive-sense RNA virus with a genome comprised of three genomic RNAs. RNA-1 encodes replication-related proteins expressed from a single open reading frame (ORF) using a translational read-through mechanism. RNA-2 has four ORFs encoding triple gene block (TGB) proteins, involved in cell to cell movement as well as a class of long-distance RNA movement factors and a cysteine-rich 8K protein, thought to enhance virulence or play a role as a weak RNA silencer (Beuch et al., 2015). RNA-3 encodes the capsid protein (CP) and capsid protein read-through protein (CP-RT, involved in vector transmission) expressed by a translational read-through of the CP stop codon (Reavy et al., 1998; Ramesh et al., 2014). PMTV is an example of a virus where systemic movement in a plant is not dependent on CP (RNA-3). The TGB (RNA-2) proteins can facilitate systemic movement of capsidless RNA, producing symptomless infection. However, CP is required for symptom expression (McGeachy and Barker, 2000; Xu et al., 2004). Spontaneous

deletion of the TGB sequence from the virus has been found to occur naturally (Torrance et al., 1999), which may explain the erratic distribution of the virus in host plants. PMTV was shown to be low in genetic diversity, with all currently sequenced isolates of PMTV sharing at least 97% identity or more (Beuch et al., 2015).

The host range of PMTV includes plants from families of *Chenopodiaceae*, *Aizoaceae*, and *Solanaceae* as determined in laboratory host-range studies (Harrison and Jones, 1970). PMTV is now wide-spread throughout Europe, Asia, South America, and North America (Xu et al., 2004; Ramesh et al., 2014; Beuch et al., 2015). PMTV was first reported in the U.S. in 2003 when the presence of the virus was confirmed in symptomatic 'Shepody' tubers by ELISA and RT-PCR in Maine in 2002 and subsequently in symptomatic 'Russet Burbank' tubers from a commercial storage holding the 2001 Maine crop (Lambert et al., 2003). Since then, PMTV has been reported in many potato producing states including Washington, Idaho, Oregon, Colorado, North Dakota, and New Mexico (David et al., 2010; Crosslin, 2011; Whitworth and Crosslin, 2013; Mallik and Gudmestad, 2015; Kaur et al., 2016).

The development of 'spraing' caused by PMTV is influenced by multiple factors such as potato cultivar, post-harvest temperature treatments, storage period, and harvest date (Mølgaard, 1996). PMTV is hard to control because it can remain infectious in resting Sss cystosori for many years (Arif et al., 2014). Unfortunately, there are no known cultivars resistant to PMTV, although some varieties do not display obvious symptoms (Domfeh et al., 2015). Reliance on visual inspection of symptoms induced by PMTV will miss a large proportion of infected tubers and risk spreading PMTV to new fields in seed tubers (Latvala-Kilby et al., 2009). The application of post-harvest tuber testing will subsequently eliminate contaminated seed tubers and help to provide adequate control of spread and the establishment of PMTV at new sites (Sokmen et al., 1998).

Current methodologies to test for PMTV include using bait-plant bioassay, ELISA (Torrance et al., 1993; Gallo García et al., 2013), RT-PCR (Sokmen et al., 1998; Arif et al., 2014) and real-time fluorescent reverse-transcription polymerase chain reaction assay (Mumford et al., 2000). However, all detection systems for PMTV were developed outside of North America and could not be easily adapted to the cost-efficient potato testing in the U.S. Once PMTV was found to be widely distributed in the United States, methods of cost-efficient detection of the virus in tubers were in high demand, and our laboratory responded to the requests of the potato industry. In this study, we devised a triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) and immunocapture-reverse transcription (IC-RT)-PCR methodology suitable for detection of PMTV in tuber and leaf tissue from potato derived from the U.S. In the course of the development of these

assays, it became necessary to establish PMTV culture in a laboratory plant, *Nicotiana benthamiana*, to have a permanent source of PMTV positive control, and also to be able to purify virus antigen for specific antibody production. The objective of this study is to demonstrate the applicability of the developed methodologies for large-scale testing for PMTV in tuber samples and assess their performance to existing European commercial diagnostic kits.

Material and Methods

Virus source

Two PMTV isolates, one from Colorado and another from Idaho, were subjected to detailed studies and used as controls for the development of virus detection protocols. PMTV_CO was isolated from a sample initially submitted by Colorado State University for virus testing from Center, Colorado, in October 2015 (courtesy of Teresa Almeida). Two half-tuber samples exhibiting mild internal necrotic spots were tested for PMTV, TRV, *Alfalfa mosaic virus* (AlMV), PVY, PVM, *Potato virus A* (PVA), PVX, *Potato virus S* (PVS), and PLRV by RT-PCR (refer to Table 2.1 for primers used). Both samples tested positive for PMTV and PVS, and one of these samples was used for analysis and inoculation into *N. benthamiana*. The PMTV_ID isolate was provided by Katharine Carlise and Justin Ruhl (Simplot Food Group, Caldwell, Idaho); as part of the 19 tuber samples sent to the University of Idaho in October 2016, one sample “KF” tested positive for PMTV and PVS and subsequently was used to obtain nearly complete genome sequence for PMTV_ID.

Table 2.1. Primers used for initial potato virus testing.

Name of Primer	Direction	Primer Sequence 5' to 3'	Virus (standard abbreviation)	Reference
PMTV_1F	Forward	GTTTGT TGT TCT ATA ATT CTC GGA	*Potato mop top virus* (PMTV)	unpublished
PMTV_1R	Reverse	GCC TGA GGG GTT AAT TGC TA	*Potato mop top virus* (PMTV)	unpublished
TRV_1F	Forward	GAC GTG TGT ACT CAA GGG TT	*Tobacco rattle virus* (TRV)	Robinson (1992)
TRV_1R	Reverse	CAG TCT ATA CAC AGA AAC AGA	*Tobacco rattle virus* (TRV)	Robinson (1992)
AlMVcpF	Forward	CAT CAT GAG TTC TTC ACA AAA	*Alfalfa mosaic virus (AMV)*	unpublished
AlMVcpR	Reverse	TCA ATG ACG ATC AAG ATC GT	*Alfalfa mosaic virus (AMV)*	unpublished
PVM F	Forward	ATG GGW GAT TCA ACR AAG AAA	*Potato virus M* (PVM)	unpublished
PVM R	Reverse	GGC TAA AAA TAG TTA AAA ACC WA	*Potato virus M* (PVM)	unpublished
PVA2 F	Forward	CCA ACA CAG ACG CCC AAA AC	*Potato virus A* (PVA)	unpublished
PVA2 R	Reverse	TCC TCG CCA GAA AAC ATC CC	*Potato virus A* (PVA)	unpublished
PVX F	Forward	TAG CAC AAC ACA GGC CAC AG	*Potato virus X* (PVX)	Nie and Singh (2000)
PVX R	Reverse	GGC AGC ATT CAT TTC AGC TTC	*Potato virus X* (PVX)	Nie and Singh (2000)
PLRV F	Forward	CGC GCT AAC AGA GTT CAG CC	*Potato leaf roll virus* (PLRV)	Bostan and Peker (2009)
PLRV R	Reverse	GCA ATG GGG GTC CAA CTC AT	*Potato leaf roll virus* (PLRV)	Bostan and Peker (2009)
PVA1 F	Forward	GTT GGA GAA TTC AAG ATC CTG G	*Potato virus A* (PVA)	Singh (1998)
PVA1 R	Reverse	TTT CTC TGC CAC CTC ATC G	*Potato virus A* (PVA)	Singh (1998)
PVS F	Forward	TGG CGA ACA CCG AGC AAA TG	*Potato virus S* (PVS)	Bostan and Peker (2009)
PVS R	Reverse	ATG ATC GAG TCC AAG GGC ACT G	*Potato virus S* (PVS)	Bostan and Peker (2009)
S5585m	Forward	GGA TCT CAA GTT GAA GGG GAC	*Potato virus Y* (PVY)	Lorenzen et al. (2006)
o2172a	Forward	CAA CTA TGA TGG ATT TGC CGA CC	*Potato virus Y* (PVY)	Lorenzen et al. (2006)
n2258	Forward	GTC GAT CAC GAA ACG CAG ACA T	*Potato virus Y* (PVY)	Lorenzen et al. (2006)
n2650c	Reverse	TGA TCC ACA ACT TCA CCG CTA ACT	*Potato virus Y* (PVY)	Lorenzen et al. (2006)
n5707	Forward	GTG TCT CAC CAG GGC AAG AAC	*Potato virus Y* (PVY)	Lorenzen et al. (2006)
o6266c	Reverse	CTC CTG TGC TGG TAT GTC CT	*Potato virus Y* (PVY)	Lorenzen et al. (2006)
o2439c	Reverse	CCC AAG TTC AGG GCA TGC AT	*Potato virus Y* (PVY)	Lorenzen et al. (2006)
A6032m	Reverse	CTT GCG GAC ATC ACT AAA GCG	*Potato virus Y* (PVY)	Lorenzen et al. (2006)
n156	Forward	GGGCAAACTCTCGTAAATTGCAG	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
o514	Forward	GATCCTCCATCAAAGTCTGAGC	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
n787	Reverse	GTCCACTCTCTTTCGTAAACCTC	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
n2258	Forward	GTCGATCACGAAACGCAGACAT	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
o2172	Forward	CAACTATGATGGATTTGGCGACC	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
n2650c	Reverse	TGATCCACAACTTCACCGCTAACT	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
o2700	Reverse	CGTAGGGCTAAAGCTGATAGTAG	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
S5585m	Forward	GGATCTCAAGTTGAAGGGGAC	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
o6400	Reverse	GTAACTCCTAAACAAATGGTGGTTCG	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
n7577	Forward	ACTGCTGCACCTTTAGATACTCTA	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
YO3-8648	Reverse	CTTTTCCTTTGTTCGGGTTTGAC	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)
SeroN	Reverse	GTTTCTCCTATGTCGTATGCAAGTT	*Potato virus Y* (PVY)	Chikh-Ali et al. (2013)

Virus propagation in Nicotiana benthamiana *and maintenance in the laboratory*

The two Colorado half-tuber samples were kept in the dark at room temperature for 4 months and planted in 1-gallon pots under greenhouse conditions. Tuber-half “A” developed four shoots and tuber-half “B” developed one shoot. All shoots were individually labeled and periodically checked for PMTV infection through collection of young leaves subjected to RT-PCR and TAS-ELISA assays for PMTV. Tuber-half “A” foliage tested positive for PMTV in two out of four shoots; tuber-half “B” foliage tested negative for PMTV. The two PMTV-positive potato leaves from tuber-half “A” were

ground in inoculation buffer (50 mM sodium phosphate dibasic heptahydrate, 50 mM monopotassium phosphate, pH 7) 1:100 dilution (wt/vol) and used to mechanically inoculate the *N. benthamiana* plants at approximately 3 weeks old (three to four leaf stage). *N. benthamiana* is known to be a viable host for PMTV, but not for PVS, so *N. benthamiana* was used as a host to separate PMTV from PVS. Plants were tested three to four weeks after inoculation using ELISA and RT-PCR. PMTV_CO isolate was maintained in *N. benthamiana* using periodical re-inoculations of PMTV-infected *N. benthamiana* leaves homogenized in the inoculation buffer using 1:10 (wt/vol) dilution into young healthy *N. benthamiana* seedlings as a stable source of PMTV.

Virus purification

PMTV_CO was purified from *N. benthamiana* foliage using a modified procedure described by Torrance et al. (1993). Briefly, 100 g of systemically infected leaf tissue of *N. benthamiana* was harvested 16 to 25 days post-inoculation (dpi) and homogenized in 300 mL of cold extraction buffer (25 mM sodium acetate, 0.5 mL/L thioglycerol, pH 5.5). Leaf homogenate was squeezed through a double layer of cheesecloth and centrifuged at 10,000 rpm (JLA-16.25 rotor) at 4°C for 20 min. The pellet was resuspended in a Tris/urea buffer (50 mM Tris-Base, Urea, 0.15 g/100 mL, pH 8.7) at half the original volume and stirred at 4°C overnight. An equal amount of chloroform was added and stirred for 45 min at 4°C. The mixture was then centrifuged at 10,000 rpm (JLA-16.25 rotor) at 4°C for 15 min. An aqueous phase was collected and laid over a cushion of 55% (wt/vol) sucrose (in deionized water), followed by high-speed centrifugation at 32,000 rpm (Ti45 rotor) at 4°C for 180 min. The purified virus was resuspended in the storage buffer (0.1 M Tris solution, pH 8.7), and the virus was kept with 0.1% sodium azide at 4°C for long-term storage.

Protein analysis by polyacrylamide electrophoresis and Western blots

Purified virus preparations were mixed with the Laemmli's Tris-sodium dodecyl sulfate (SDS) sample buffer and denatured at 95°C for 10 min, and proteins were separated on 4-20% gradient, SDS-polyacrylamide gels (SDS-PAGE; Bio-Rad, Sunnyvale, CA). Protein markers from SDS-PAGE broad range standard (Bio-Rad cat. #161-0318) were used to estimate the size of the PMTV protein; the gel was stained using Coomassie Brilliant Blue G-250 (Bio-Rad). Immunoblotting (Western blot) was subsequently performed to confirm virus specificity. Proteins separated using 4-20% gradient, SDS-PAGE were transferred onto a nitrocellulose membrane (Bio-Rad) as described previously (Karasev et

al., 2010). The membranes were incubated for 16 to 20 h at 4°C with the respective PMTV-specific rabbit R34 polyclonal antiserum raised against bacterially expressed MBP-PMTV-CP at 1:20,000 dilution, followed by incubation with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma A-3687) at 1:30,000 dilution. The immune complexes were exposed by submerging the membranes with SIGMA *FAST*™ BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) substrate tablets (Sigma), and the color reaction was stopped by washing them in water.

Production of PMTV-specific antibodies

Polyclonal antiserum G1571, against the purified virus, PMTV_CO isolate, from *N. benthamiana* tissue was raised in goat following a series of six immunizations, with the first immunization in the presence of complete Freund's adjuvant and all subsequent immunizations with the presence of incomplete Freund's adjuvant (Cocalico Biologicals, Reamstown, PA). The general outline of the antibody production followed the previous descriptions (Karasev et al., 2010; Nikolaeva et al., 2012).

Polyclonal antiserum to the bacterially expressed capsid protein (CP) of PMTV was produced by B.D. Thompson, S. Eid, and O.V. Nikolaeva in 2012-2013 (unpublished), and used in this work for the development of immunodetection assays. Briefly, a synthetic nucleotide sequence coding for the PMTV CP was chemically synthesized by GenScript (Piscataway, NJ) and cloned in a pUC57 plasmid vector. This synthetic PMTV-CP gene was inserted into an expression vector pMAL-C2 (New England Biolabs, Ipswich, MA), and PMTV CP was expressed in *Escherichia coli* as a protein fused to a maltose-binding protein (MBP) tag according to a previously described methodology (Nikolaeva et al., 1995). The MBP-CP fusion protein was purified to near homogeneity by amylose resin affinity column chromatography, and polyclonal antiserum R34 was raised against MBP-PMTV-CP by Cocalico Biologicals in rabbit following a series of four immunizations, with the first one in the presence of complete Freund's adjuvant and all subsequent with the presence of incomplete Freund's adjuvant. The R34 rabbit polyclonal antibody raised against MBP-PMTV-CP was used as a detection antibody throughout all TAS-ELISA and immunoblot assays throughout this paper.

Development of TAS-ELISA assay for the detection of PMTV

A triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) for the detection of PMTV in tuber and leaf tissue utilized two PMTV-specific antisera, one (G1571) produced in goat against the purified virus, and one (R34) produced in rabbit against bacterially

expressed MBP-PMTV-CP (see the description above). TAS-ELISA tests followed the general protocol of Clark and Adams (1977), with some modifications also described in the protocol of Feng et al. (2014). Briefly, 96-well MaxiSorp microtiter plates (Nunc, Rochester, NY cat. No. 442404) were coated with 100 µL of the goat G1571 antiserum at 1:10,000 dilution in 20 mM sodium carbonate buffer (pH 9.6). Leaf or tuber tissue samples were ground in extraction buffer (1× phosphate buffered saline plus Tween® 20, 0.3% non-fat milk, and 2% polyvinylpyrrolidone) at a 1:10 (wt/vol) ratio. The Homex 6 grinding machine (Bioreba, Reinach, Switzerland) was used to homogenize leaf and tuber samples in meshed plastic bags. Sample extracts (100 µL each) were taken directly from the meshed bags used for homogenization and added to the pre-coated plates and incubated with plant extracts for 16 to 20 h at 4°C. Plates were washed six times using 1× phosphate buffered saline plus Tween® 20 (PBST) and then rinsed with deionized water. Detecting antiserum R34 from rabbit, at 1:10,000 dilution was applied to the wells in ELISA buffer (1× PBST and 0.3% non-fat milk). After incubation for 16 to 20 h at 4°C, plates were washed extensively with 1× PBST, and anti-rabbit (Sigma A-3687) immunoglobulin G (IgG) conjugates with alkaline phosphatase at 1:30,000 dilution in ELISA buffer were applied, and the plates were incubated 16 to 20 h at 4°C. After washing and adding p-nitrophenyl phosphate (Sigma_Aldrich CAS Number: 333338-18-4) substrate, the color reaction was monitored by measuring absorbance at 405 nm using an ELISA reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA). PMTV positive and negative samples were included in each ELISA experiment as controls. Samples were defined as positive if the absorbance value exceeded the healthy controls by three-fold.

Comparison of TAS-ELISA to three commercial kit

The newly developed TAS-ELISA methodology (coating goat G1571, detecting rabbit R34) for the detection of PMTV was compared to three commercial ELISA kits from three European diagnostic companies: SASA (Science and Advice for Scottish Agriculture, Edinburgh, Scotland), Bioreba (Reinach, Switzerland), and Neogen (Ayre, Scotland). Detecting assay using commercial ELISA kits was done according to manufacturers' instructions. For the SASA kit, coating with the mouse monoclonal antibody was done at 1:1,000 dilution, and the conjugated detecting antiserum was applied at 1:4,000 dilution. For the Bioreba kit, the coating was done at 1:1,000 dilution, and the conjugated detecting antiserum was administered at 1:1,000 dilution. For the Neogen kit, the coating was done at 1:500 dilution, the detecting antiserum was applied at 1:1,000 dilution, and the conjugate antiserum at 1:2,000 dilution. In addition to these four ELISA systems, the Plant Virology Lab

developed a fifth system in which the mouse antiserum from the SASA kit was utilized as coating at 1:1,000 dilution, followed by the rabbit R34 antiserum used as detection at 1:10,000 dilution. This comparison (summarized in Table 2.2) was conducted on various PMTV-infected samples including *N. benthamiana* leaf tissue, tuber skin/flesh tissue, and tuber sprout tissue. Samples were ground in extraction buffer at a 1:10 (wt/vol) ratio. Tubers were sampled by using the open end of a 1 mL pipette tip, to collect three punches 0.5 cm deep taken from three different areas of the tuber surface (top, middle, and bottom) and combined into one sample (Figure 2.1). All ELISA plates was loaded with the same plant extract and developed with the same p-nitrophenyl phosphate (Sigma_Aldrich CAS Number: 333338-18-4) substrate, and the color reaction was monitored by measuring absorbance at 405 nm using an ELISA reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA).

Table 2.2. Five ELISA systems for the detection of PMTV used in this study including three commercial kits: SASA, Bioreba, and Neogen. Commercial kits were compared to two homemade ELISA systems: coating G1571, detecting R34, and coating SASA, detecting R34.

System	Coating	Detection	Conjugate
Coating G1571, Detecting R34	G1571 1:10,000	R34 1:10,000	Anti-rabbit 1:30,000
Coating SASA, Detecting R34	SASA 1:1,000	R34 1:10,000	Anti-rabbit 1:30,000
SASA kit	SASA 1:1,000	SASA 1:4,000	
Bioreba kit	Bioreba 1:1,000	Bioreba 1:1,000	
Neogen kit	Neogen 1:500	Neogen 1:1,000	Conjugate Neogen 1:2,000

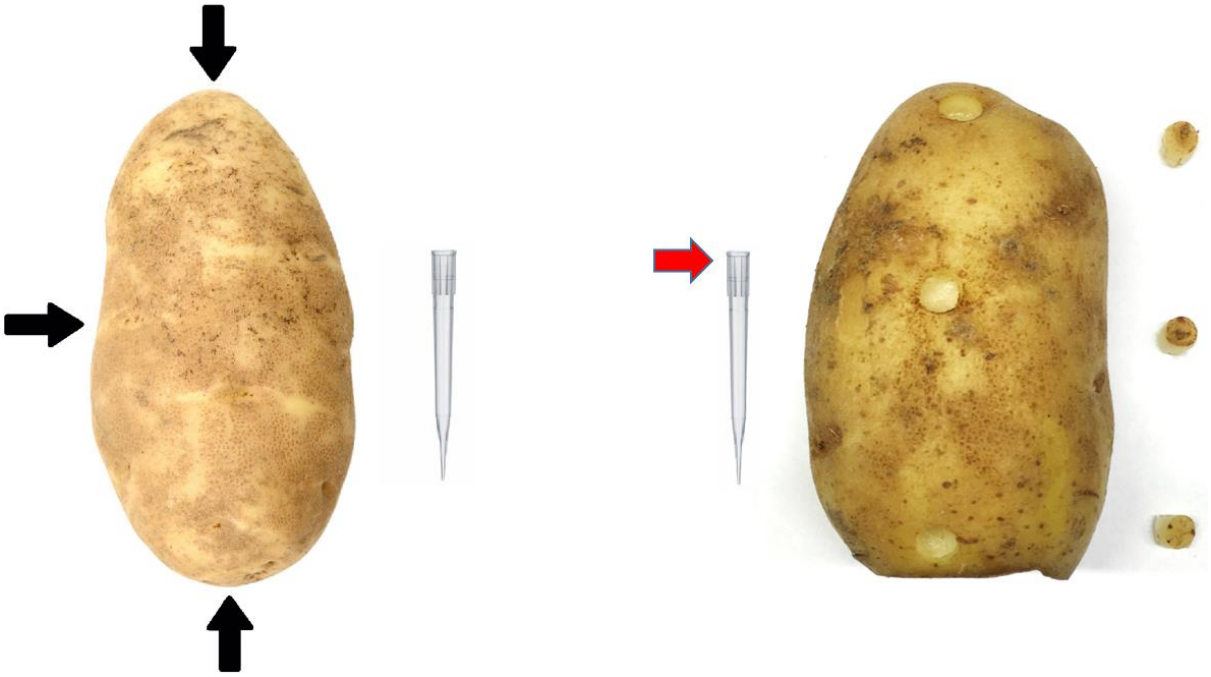

Figure 2.1. Method to extract tuber tissue. Tuber is typically left in intact. Using a 1 mL pipette tip, 3 punches 0.5 cm deep are taken from the surface (top, middle, and bottom) and combined into 1 sample. Tissue is ground in the ELISA grinding buffer and can be loaded onto ELISA and immuno-capture plates.

Immunocapture (IC)-RT-PCR for the detection of PMTV

An immunocapture-reverse transcription (IC-RT)-PCR methodology was performed as followed: 96-well skirted PCR plates (Eppendorf cat. No.951020460) were coated with 100 µL of the goat G1571 polyclonal antibodies at 1:10,000 dilution in 20 mM sodium carbonate buffer (pH 9.6) and incubated for 16 to 20 h or overnight at 4°C. Plates were washed thoroughly with deionized water. Most of the water was removed by hitting the plate upside down over a paper towel, and plates were stored at -20^{O}C until ready to be used. Leaf and tuber tissue samples were ground in extraction buffer (1× PBST, 0.3% non-fat milk, and 2% polyvinylpyrrolidone) at a 1:10 (wt/vol) ratio. Sample extracts (100 µL each) were added to the coated plates and incubated at 37°C for 2 to 4 h or 4°C overnight. Plates were washed six times using 1× PBST and then rinsed with deionized water.

Reverse transcription for first-strand cDNA synthesis, previously described by Chikh-Ali et al. (2013), was performed using virus particles captured by goat G1571 virus-specific antibody in wells of the pre-coated 96-well skirted PCR plates, in a 25-µL reaction mixture that contained M-MLV RT 5× Buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT) (Promega), 2.5 mM each deoxyribonucleotide triphosphate (dNTP), a mixture of oligo-dT and random hexamer primers 3 µM final concentration, 25 units of RNase Out Ribonuclease Inhibitor (Promega), and 200 units of

Moloney Murine Leukemia Virus Reverse Transcriptase (Promega). The reverse transcription reaction (RT) was performed in an Eppendorf Mastercycler Pro (Eppendorf North America) with an initial incubation at 25°C, increased to 42°C at 1°C per 30 s, held at 42°C for 45 min, and then increased to 60°C at 1°C per 2 min. The reverse transcriptase was inactivated by incubation at 70°C for 10 min.

PCR amplification was conducted in 20 µL of 10× Taq Buffer (500 mM KCl, 100 mM Tris HCl, 15 mM $MgCl_2$) containing 2.5 mM of each dNTP, 0.5 µM each forward primer and reverse primer, 2.5 units Taq DNA polymerase (Genscript), and 2 µL of cDNA template. Synthetic oligonucleotide primers PMTV_1F (5'-GTTTGTTGT TCTATAATTCTCGGA-3') and PMTV_1R (5'-GCCTGAGGGGTTAA TTGCTA-3') were used to amplify PMTV CP region from cDNA. Amplification was done with an initial incubation for 5 min at 95°C, followed by 11 cycles of 30 s at 95°C, 30 s at 62°C, and 2 mins at 72°C, another 22 cycles of 30 s at 95°C, 30 s at 56°C, and 2 mins at 72°C, and finally an extension at 72°C for 5 mins. A 10 µL aliquot of PCR reaction was separated onto 1.2% agarose gel containing ethidium bromide at 10 µg/mL and visualized under UV light. PMTV positive and negative samples were included in each immunocapture (IC)-RT-PCR experiment as controls. Samples were defined as positive if extract produced an amplicon 585 bp in size.

Nearly complete genome sequencing and sequence analysis

PMTV_CO genome was sequenced using a series of overlapping RT-PCR fragments (primers listed in Table 2.3) amplified on total PMTV_CO RNA extracted from infected *N. benthamiana* plants, and conventional Sanger sequencing. Total nucleic acids were extracted from infected leaves via Dellaporta methodology as described by Hicks et al. (1983) with some modifications. Briefly, plant tissue was homogenized in a Dellaporta solution (100 mM Tris, 50 mM EDTA, 500 mM NaCl, β-mercaptoethanol) at 1:10 (wt/vol) ratio, 10% SDS was added, homogenate vortexed to mix, and incubated at 65°C for 10 min. Potassium acetate (5M) was added, homogenate vortexed, and centrifuged at 12,000 rpm (Eppendorf 5430R rotor) for 10 min. The supernatant was transferred to a new 1.5 mL eppendorf tube, and after the addition of isopropanol, the tube was incubated on ice for 5 min, and centrifuged at 12,000 rpm (Eppendorf 5430R rotor) for 20 min. The pellet was washed with 70% ethanol, dehydrated, and resuspended in sterilized deionized (DI) water.

RT was performed using 4.5 µL of the total nucleic acid extract in a 25 µL reaction mixture that contained M-MLV RT 5× Buffer (50 mM Tris-HCl, 75 mM KCl, 3mM $MgCl_2$ and 10 mM DTT) (Promega Corp.), 2.5 mM each dNTP, a mixture of oligo-dT and random hexamer primers 3 µM final concentration, 20 units of RNase Out Ribonuclease Inhibitor (Promega), and 200 units of M-MLV

Reverse Transcriptase (Promega). RT was performed in an Eppendorf Mastercycler Pro (Eppendorf North America). Before the RT reaction, 4.5 µl of RNA template in addition to 8 µL of sterilized DI water was denatured at 70°C for 5 min; then, the reverse-transcription mix was added. The profile used here consisted of 25^{o}C for 2 mins; 17 Cycles of 30 sec at 25^{o}C; incubation at 42^{o}C for 45 mins; 18 Cycles of 2 min at 42^{o}C and RT deactivation at 70°C for 10 min.

PCR was performed using the parameters previously described for the immunocapture-RT-PCR methodology. 20 primer pairs were designed based on the sequences of reference isolates available in GenBank; these 20 primer pairs were used to amplify all three RNA components of PMTV (Table 2.3). After confirming the amplification of the expected PCR fragment visible on a 1.2% agarose gel, a 10 µL aliquot of each successful PCR reaction was treated with ExoSAP-IT™ PCR Product Cleanup Reagent (Thermofisher) and submitted for sequencing to Genewiz, Inc. (South Plainfield, NJ). Nearly complete viral genome segments were assembled using SeqMan Pro 13 (DNASTAR, Madison, WI). The sequences of PMTV isolates were aligned using Clustral X, and the analysis of the aligned sequences was completed using the program package RDP4.

Table 2.3. Primers used for amplification and Sanger sequencing *of Potato mop top virus* (PMTV).

Name of Primer	Direction	Primer Sequence 5' to 3'	Location
PMTV_RNA1_65F	Forward	GCGACCGCTTGCGATAAAACG	65
PMTV_RNA1_995R	Reverse	GCCACAATTAGGATCATCGTARAAGC	995
PMTV_RNA1_743F	Forward	CGATCACAGCACTCTTGGAGATGG	743
PMTV_RNA1_1850R	Reverse	CACATATCCTTCGCCAATCAAACC	1850
PMTV_RNA1_1542F	Forward	CTAGAGAGCGTGGTGCATAGAGTGTT	1542
PMTV_RNA1_2463R	Reverse	CATGAGCATGCTCGCCTTCG	2463
PMTV_RNA1_2248F	Forward	CTGACGTCGATCCTGATGACTCTGC	2248
PMTV_RNA1_3243R	Reverse	CAGCAGCTTCTCTACCGATTGTCAC	3243
PMTV_RNA1_2979F	Forward	GGTGAAATGTCATCGGACAACAGTG	2979
PMTV_RNA1_3992R	Reverse	CARYTTCCCTTCCTGCACAGATAACA	3992
PMTV_RNA1_3771F	Forward	GTGAACACTGTGCATGAAAGTCAGGG	3771
PMTV_RNA1_4767R	Reverse	GCTCGTCAGACGTCATCCTTGTGTTA	4767
PMTV_RNA1_4667F	Forward	ACCCYGATAAGATTGTGAATGCTTTG	4667
PMTV_RNA1_4454F	Forward	TAAGTTGGAAACATTTGAWGGTGCGG	4454
PMTV_RNA1_5606R	Reverse	GCGACAAGAATACTAACTGTGGGGCT	5606
PMTV_RNA1_5037F	Forward	TCTGCCGCATTGTCRTTGTTAGAGTC	5037
PMTV_RNA1_5994R	Reverse	ATCGTGTCTTGATCGCAGCAGC	5994
PMTV_RNA2_10F	Forward	CTCTACCTAGCCGAGTGCTTACTTGA	10
PMTV_RNA2_1134R	Reverse	ACAACTAGGCAGATTCGAGAACTCAG	1134
PMTV_RNA2_809F	Forward	GTGGATTCAAATCTACTGGTAAGCCC	809
PMTV_RNA2_1719R	Reverse	TGCCAATTCTCCGTTAATCAACTGC	1719
PMTV_RNA2_1551F	Forward	CGTGCAAGGAAAAGAGTACAACACTG	1551
PMTV_RNA2_2521R	Reverse	AACATAGCACCCACAAGCACAGTCAC	2521
PMTV_RNA2_1902F	Forward	GCCAGTACAGGGACGGTTCTAAGAG	1902
PMTV_RNA2_2907R	Reverse	TGATCGCAGCAGCAACTGGC	2907
PMTV_RNA3_114F	Forward	AGTGTTTTCTTGTGTCTTTTGCGGG	114
PMTV_RNA3_1124R	Reverse	CATCYTTGTTACGAATAGTGACACGC	1124
PMTV_RNA3_920F	Forward	GCTGCGTTRAGACTATTGCCTGGG	920
PMTV_RNA3_1929R	Reverse	GGACCTGTATAMGACGACAACACACC	1929
PMTV_RNA3_1718F	Forward	GCGTTTAATCCGTCAGACTGTGGGT	1718
PMTV_RNA3_2700R	Reverse	CCCTGTCCYTTAMCCGCACYACC	2700
PMTV_RNA3_2105F	Forward	ATGAAGAAYTATGCGGGTGGAGGT	2105
PMTV_RNA3_3107R	Reverse	GGAGTTGCACCCTTTTGAACGA	3107
PMTV_RNA1_4453F	Forward	CTAAGTTGGAAACATTTGAWGGTGCG	4453
PMTV_RNA1_5304R	Reverse	TGTCTTTCCTGCCCAGTTTCGTAAG	5304
PMTV_RNA1_5452R	Reverse	GCTAACAAACCAAACATGACATCACA	5424
PMTV_RNA3_1443F	Forward	ATCCTACTGGTAGTTATGACGATGCG	1443
PMTV_RNA3_2408R	Reverse	CAGCACTCATTATTCTGGTCAATCGG	2408

Results

Biological characterization

The University of Idaho Plant Virology Laboratory has tested tuber samples for the presence of PMTV since approximately 2007, primarily from industry personnel in the State of Idaho. However, since 2011 the number of samples submitted increased, and the geography of submitters expanded, including customers in Colorado. The two half-tuber samples from Center, CO were found positive for PMTV and PVS, using RT-PCR testing of the Dellaporta extracts from tuber skin and flesh. Although PMTV can cause internal necrotic arcs in tuber tissue (Figure 2.2), the submitted PMTV-positive Colorado tubers exhibited very mild internal necrotic spots (not shown). Later, during observations of other grown-out plants from other PMTV-positive tuber samples few PMTV-positive shoots displayed stunting (Figure 2.3 and Figure 2.4), while some PMTV-positive shoots were found entirely asymptomatic.

Figure 2.2. Example of tuber infected with PMTV. Internal necrotic arcs are visible on longitudinal cut.

Figure 2.3. Example of a single potato shoot found positive for PMTV, 7 weeks after planting. The shoot exhibited poor emergence and stunting.

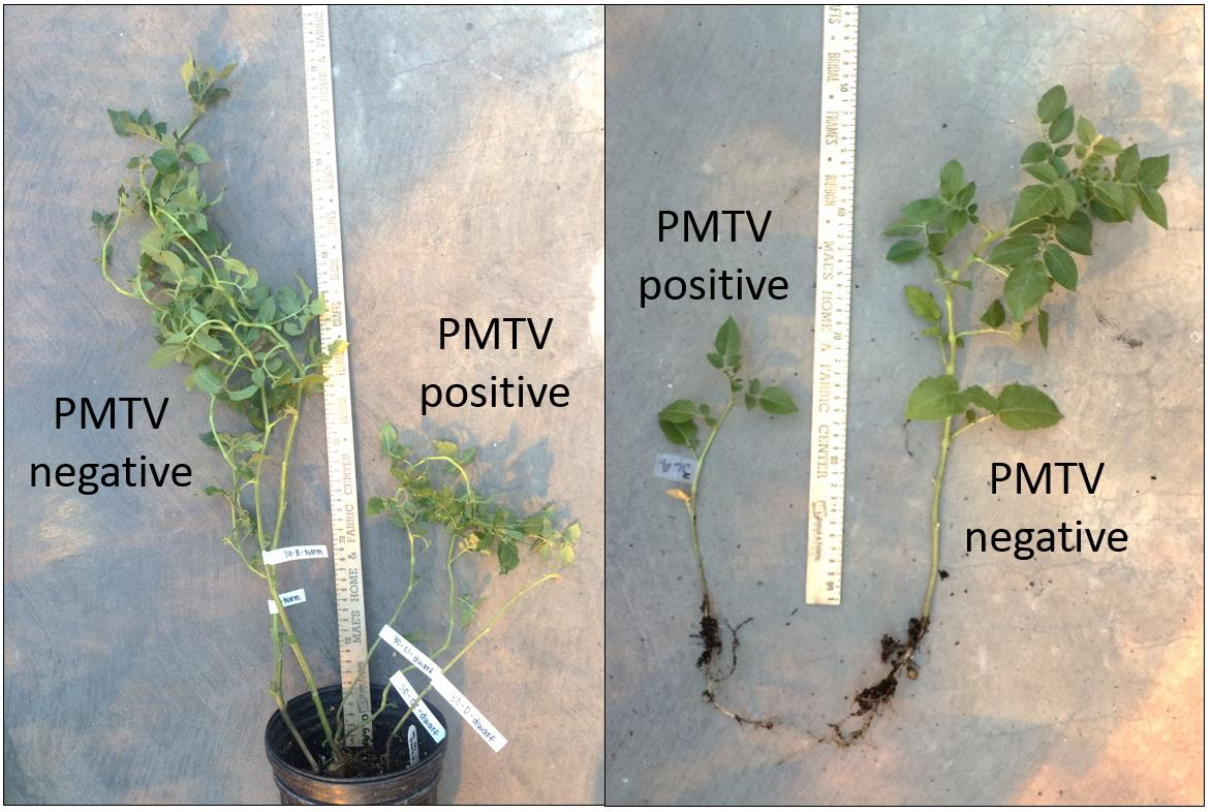

Figure 2.4. Example of potato plants grown from PMTV-positive tubers, with multiple shoots. Leaf tissue from all shoots was tested for PMTV individually. Note that virus apparently did not distribute evenly in the mother tuber, hence not all emerging shoots were positive. Shoots positive for PMTV tended to exhibit stunting.

PMTV-positive potato foliage systemically infected with PMTV_CO was used to mechanically inoculate the *N. benthamiana* plants. In this initial round of inoculations, from potato to *N. benthamiana*, inoculated and non-inoculated leaves of *N. benthamiana* were found PMTV-positive by ELISA and RT-PCR, but only about 30% of the inoculated *N. benthamiana* plants established systemic PMTV infection. Systemically PMTV-infected leaves of *N. benthamiana* was then used as inoculum to infect *N. benthamiana* seedling, producing systemic infection 100% rate. PMTV_CO isolate propagated was maintained in this convenient laboratory host in the greenhouse. The PMTV_CO isolate was established and maintained in *N. benthamiana* under greenhouse conditions, and provided a convenient supply of PMTV positive control. The infection in *N. benthamiana* produced mild mosaic symptoms visible two weeks post-inoculation. Symptoms were visible on younger leaves (Figure 2.5), and virus titer remained high in the younger leaves on infected *N. benthamiana,* easily detected by ELISA and RT-PCR. No stunting was observed and the virus did not seem to affect its host plant adversely.

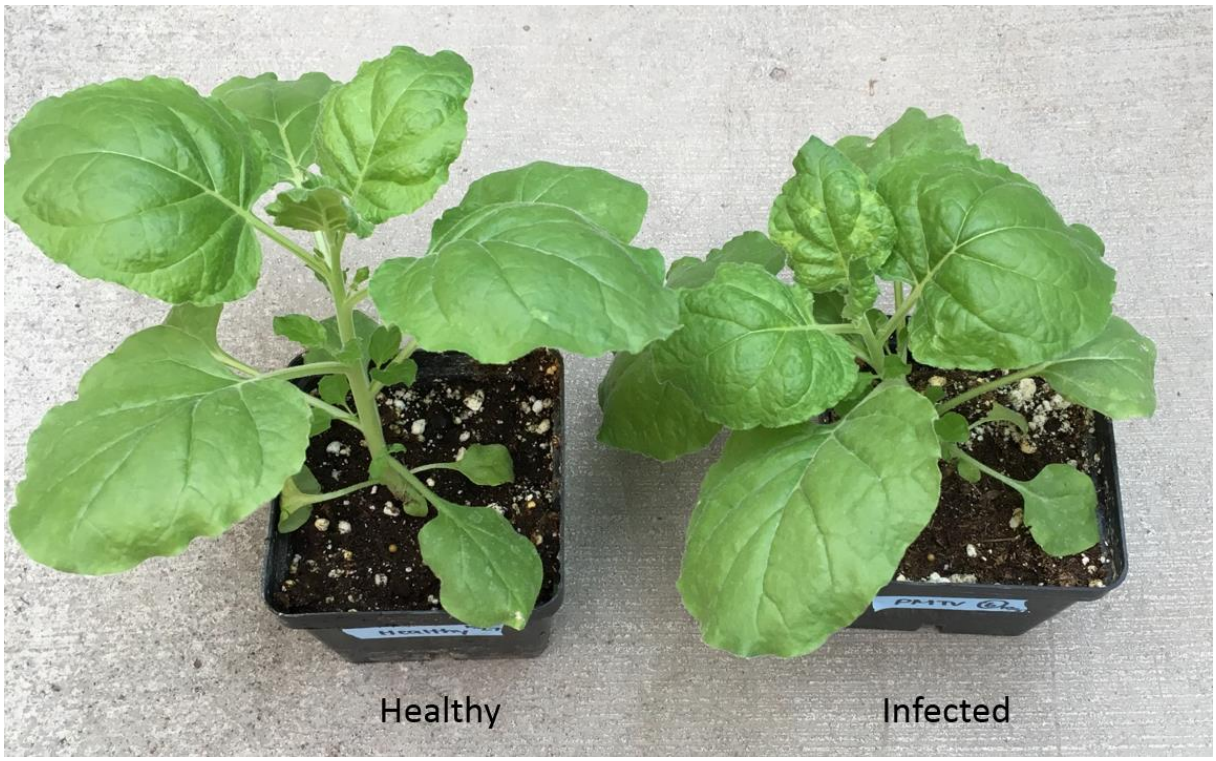

Figure 2.5. Image of a healthy *Nicotiana benthamiana* plant compared to a PMTV-infected *N. benthamiana* plant 11 days post inoculation (dpi). Symptoms are mild mosaic. PMTV is maintained in *N. benthamiana* to be used as control and reference isolate.

Virus purification and analysis

The modified procedure described by Torrance et al. (1993) resulted in a reasonably good yield reaching 1 mg of virus from 200 g of leaf tissue, and providing a sufficiently clean virus preparation of PMTV, when PMTV-infected *N. benthamiana* foliage was used for purification. A concentration of 1 µg per µL was obtained. When analyzed on a 4-20% gradient gel, PMTV preparation produced a major protein band of approximately 20 kDa and a slightly less prominent band of approximately 23 kDa stained with Coomassie (Figure 2.6A). Additional weak bands could be seen on a Coomassie-stained gel above and below the two most prominent bands in the purified PMTV preparation (Figure 2.6A). However, when analyzed with a Western blot, the detecting antibody R34 raised in rabbit against bacterially expressed MBP-PMTV-CP recognized only two bands, 23-kDa and 20-kDa (Figure 2.6B), which presumably represented the CP of PMTV. Previously, Torrance et al. (1993) noted that their purified PMTV virus preparation exhibited three protein bands, of 23.9, 21.5, and 20.5 kDa, suggesting PMTV virus particle is susceptible to partial degradation via plant proteases during virus purification.

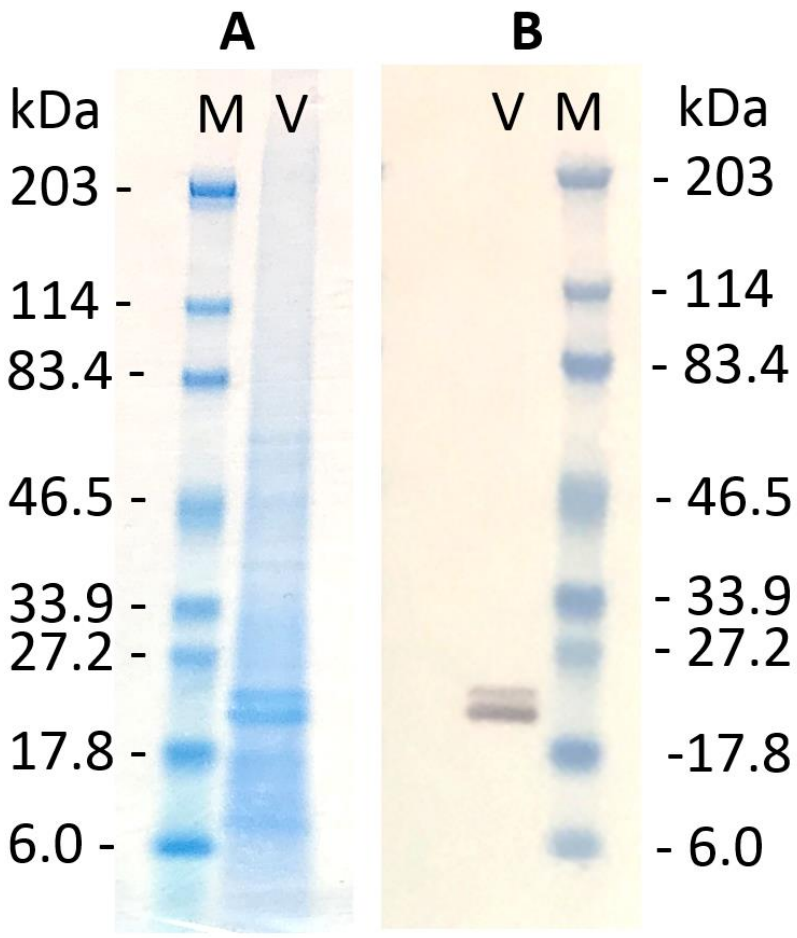

Figure 2.6. (A) Image of a coomassie stained SDS PAGE gel with PMTV_CO virus preparation. Preparation produced 1 major band around ~20 kDa, presumably coat protein (CP). (B) Image of Western blot with PMTV_CO virus preparation. Antisera raised against MBP-CP in rabbit was used as detecting antibody at 1:20,000 dilution. Preparations produced 1 major band around ~20 kDa, presumably CP.

Development of the TAS-ELISA tests for foliar and tuber samples

In preliminary experiments, the rabbit R34 antiserum produced against the bacterially expressed MBP-PMTV-CP antigen was found highly specific to PMTV CP when tested against purified virus, working well as a detecting antibody in Western blots (Figure 2.6B). However, the same rabbit R34 antiserum did not work as a coating antibody in ELISA (not shown), and hence another antibody was needed to develop one of the 'sandwich' ELISA formats. Once a purified PMTV preparation was obtained from *N. benthamiana*, it was immediately used as an antigen for the production of the G1571 antiserum in goat, to have a candidate for a coating antibody to be used in TAS-ELISA. Indeed, the G1571 antiserum was found suitable for coating in TAS-ELISA protocol when it was used at 1:10,000 dilution, in combination with the rabbit R34 antiserum used for detection at 1:10,000

dilution when tested against various dilutions of PMTV-infected and healthy *N. benthamiana* leaves (Figure 2.7).

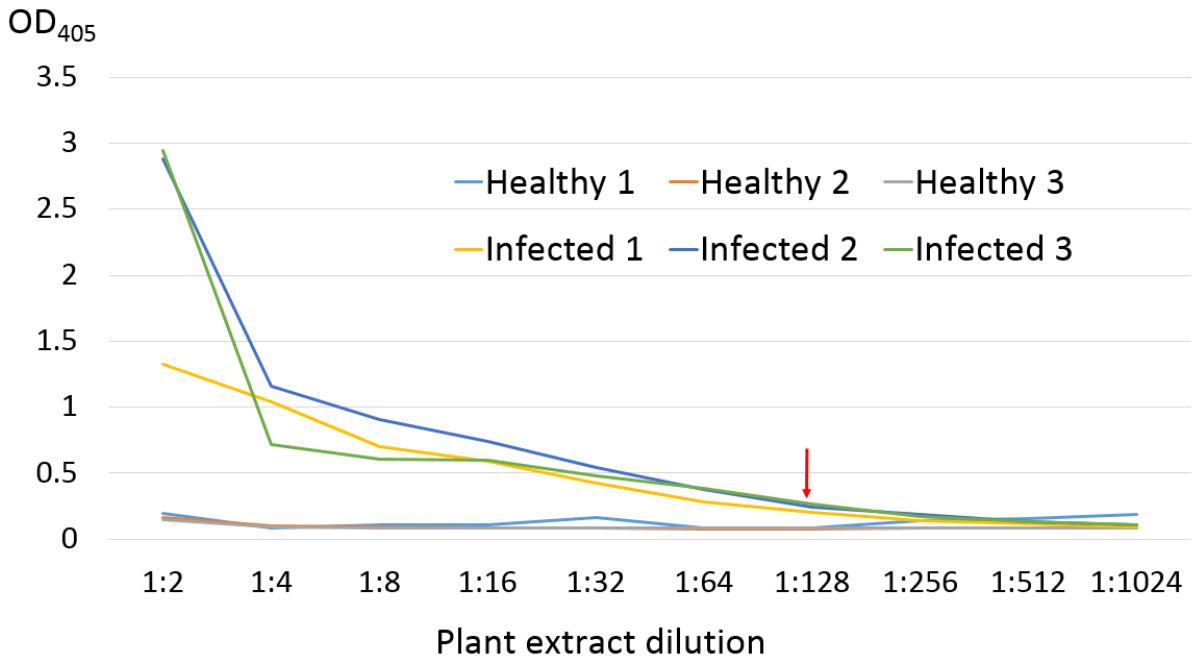

Figure 2.7. Titration curves for PMTV in *N. benthamiana* leaves. Detection by triple-antibody sandwich enzyme-linked immunosorbent assay (ELISA); virus antigen was captured by the goat G1571 polyclonal antibody (PAb) at 1:10,000 dilution. The rabbit R34 PAb was used as detecting antibody at 1:10,000 dilution. The plant extract was titrated along the X-axis: leaves of three individual infected were subjected to extraction in a sodium carbonate buffer at 1:10 (wt/vol) ratio; these extracts were diluted prior to loading onto the ELISA plate as indicated along the x-axis. OD_{405} = optical density at 405 nm. Red arrow indicated last dilution where PMTV presence exceeded the healthy controls three-fold was determined.

Assessment of TAS-ELISA and comparison to three commercial kit

This TAS-ELISA format (coating goat G1571, detecting rabbit R34) was compared to three commercial kits. This comparison was conducted on various PMTV-infected samples including *N. benthamiana* leaf tissue, tuber skin/flesh tissue, and tuber sprout tissue. The A_{405} signal-to-noise ratio (infected/healthy) was calculated and graphed for each ELISA system and type of tissue tested and presented at 24 hours after the addition of the substrate. The data obtained demonstrated that our home-made TAS-ELISA system utilizing the goat G1571 antiserum for coating and the rabbit R34 antiserum for detection produced excellent OD_{405} signals and signal-to-noise ratios (infected/healthy) when applied to foliar samples, however show equal sensitivity to commercial kits when tested with tuber tissue. To resolve the quality problem of the PMTV detection in tuber samples, various combinations of coating and detecting antibodies from all commercial ELISA kits were tested on the same PMTV-positive samples which lead to the development of a second home-made TAS-ELISA

system in which the mouse antiserum from the SASA kit was used for coating at 1:1,000 dilution, followed by the rabbit R34 antiserum used for detection at 1:10,000 dilution. Comparison of the five ELISA systems (summarized in Table 2.2) was done for all three different tissue sample types.

For PMTV testing in *N. benthamiana*, coating G1571 detection R34 as produced optical density (OD_{405}) signals 3.9× higher than the best commercial kit (SASA) (Figure 2.8A) and an A_{405} ratio (infected/healthy) 3.5× greater than the best commercial kit (SASA) (Figure 2.8B). Coating mouse SASA detecting R34 produced OD_{405} signals 10.1× greater than the best commercial kit (SASA) (Figure 2.8A) and an A_{405} ratio (infected/healthy) is 8.9× greater than the best commercial kit (SASA) (Figure 2.8B).

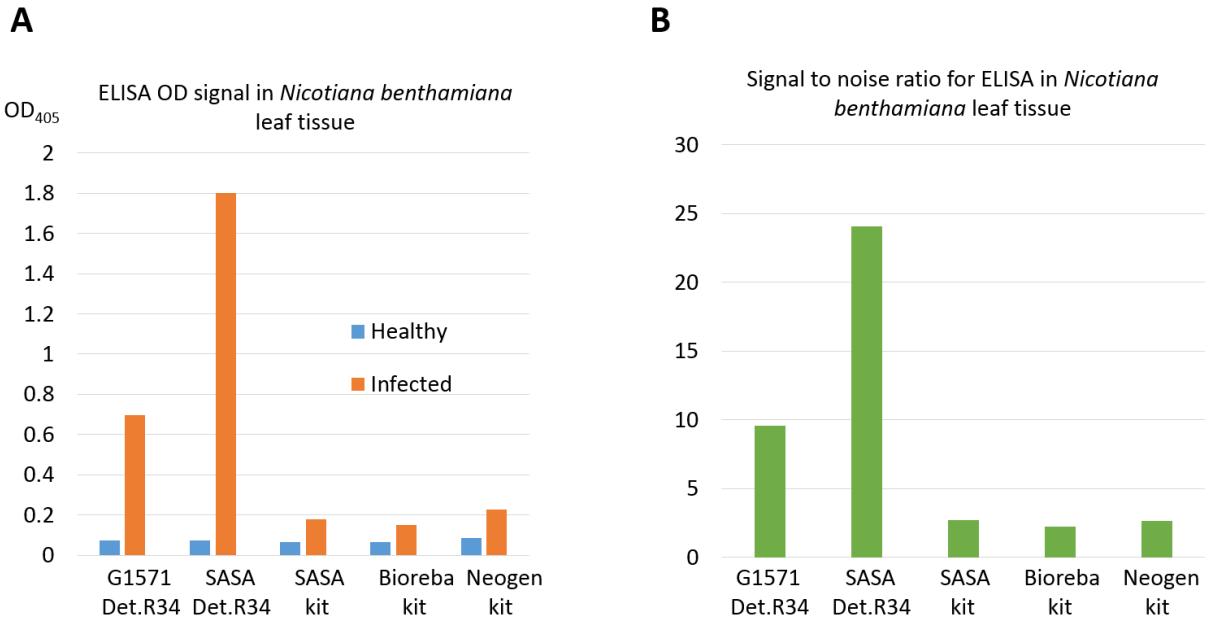

Figure 2.8. Comparing five ELISA systems for the detection of PMTV in *N. benthamiana* using three commercial kits: SASA, Bioreba, and Neogen. Commercial kits were compared to two homemade ELISA systems: coating G1571, detecting R34, and coating SASA, detecting R34. (A) Illustrates the infected and healthy OD_{405} signals. (B) Illustrates the infected to healthy ratio signal.

For PMTV testing in tuber tissue, coating G1571 detection R34 produced OD_{405} signals nearly the same as the best commercial kit (SASA), 0.18 and 0.17 respectively (Figure 2.9A) and an A_{405} ratio (infected/healthy) is also almost the same as the best commercial kit (SASA), 2.6 and 2.5 respectively (Figure 2.9B). Coating mouse SASA detecting R34 produced OD_{405} signals 4.7× more significant than the best commercial kit (SASA) (Figure 2.9A) and an A_{405} ratio (infected/healthy) is 3.9× greater than the best commercial kit (SASA) (Figure 2.9B).

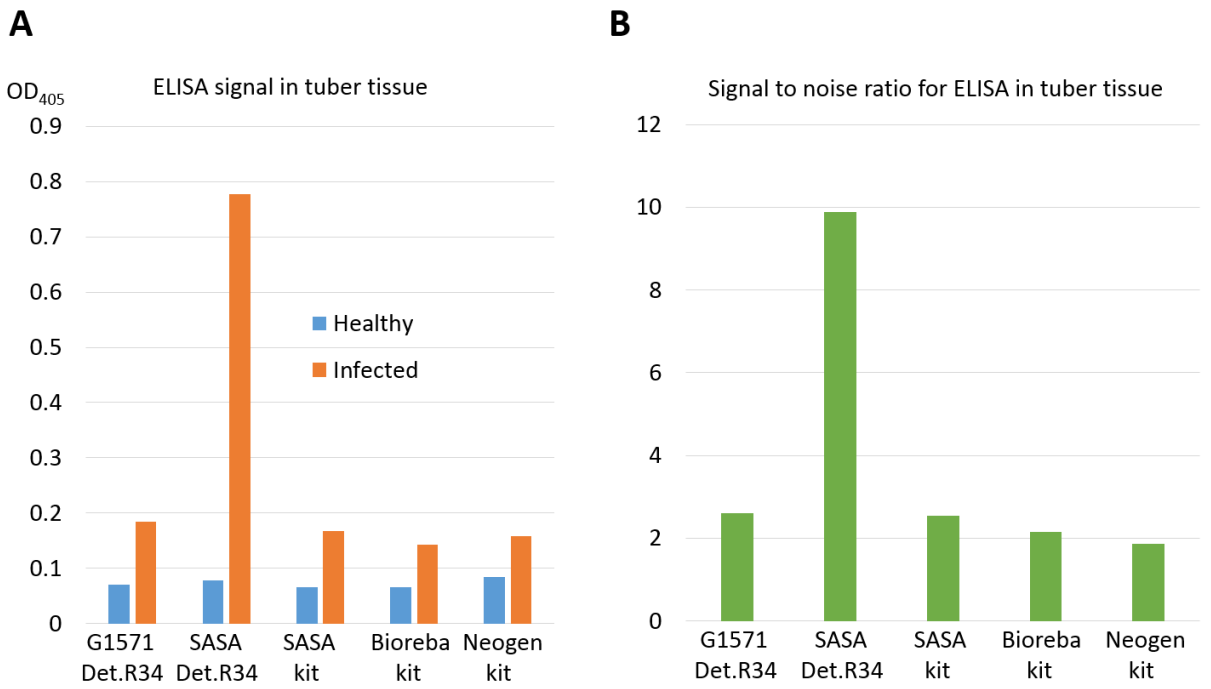

Figure 2.9. Comparing five ELISA systems for the detection of PMTV in tuber tissue using three commercial kits: SASA, Bioreba, and Neogen. Commercial kits were compared to two homemade ELISA systems: coating G1571, detecting R34, and coating SASA, detecting R34. (A) Illustrates the infected and healthy OD_{405} signals. (B) Illustrates the infected to healthy ratio signal.

For the PMTV sprout testing, coating G1571 detection R34 produced OD_{405} signals nearly the same as the best commercial kit (SASA), 0.18 and 0.17 respectively (Figure 2.10A) and an A_{405} ratio (infected/healthy) is also nearly the same as the best commercial kit (SASA), 2.3 and 2.6 respectively (Figure 2.10B). Coating mouse SASA detecting R34 produced OD_{405} signals 5.7× greater than the best commercial kit (SASA) (Figure 2.10A) and an A_{405} ratio (infected/healthy) is 4.3× greater than the best commercial kit (SASA) (Figure 2.10B).

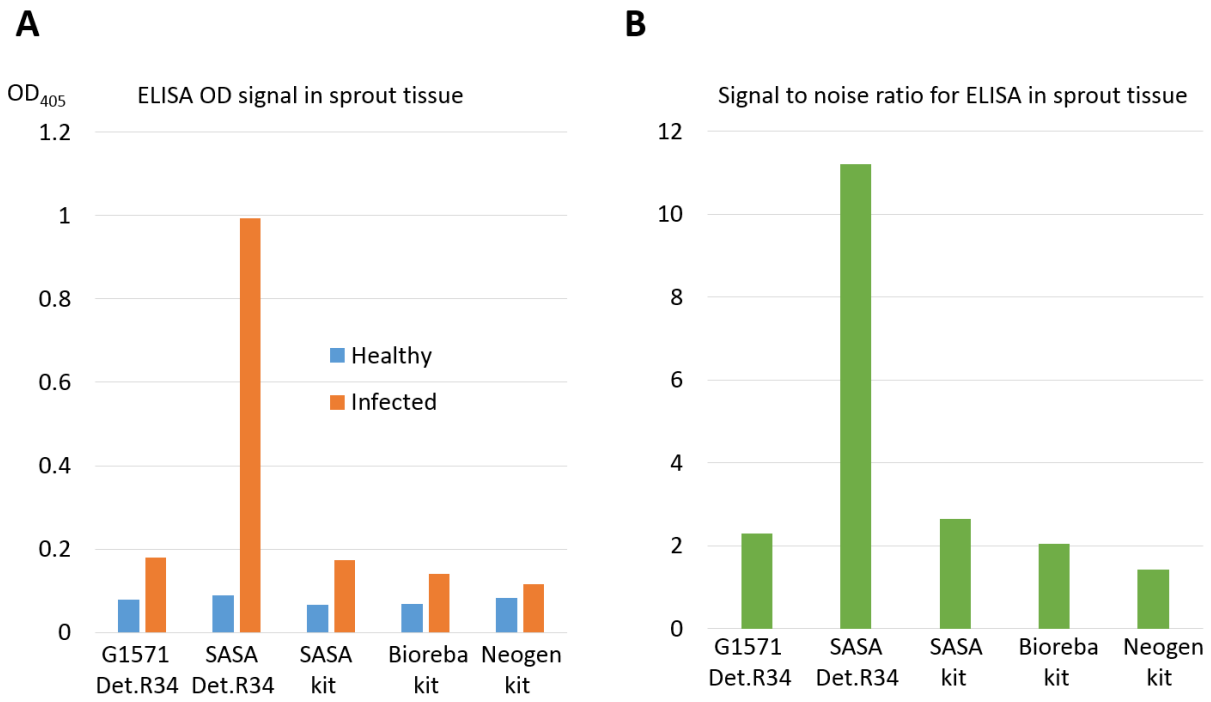

Figure 2.10. Comparing five ELISA systems for the detection of PMTV in sprout tissue using three commercial kits: SASA, Bioreba, and Neogen. Commercial kits were compared to two homemade ELISA systems: coating G1571, detecting R34, and coating SASA, detecting R34. (A) Illustrates the infected and healthy OD_{405} signals. (B) Illustrates the infected to healthy ratio signal.

Immunocapture-RT-PCR

Goat G1571 antisera was used as coating antibody at 1:10,000 dilution in IC-RT-PCR. Tuber and *N. benthamiana* tissue ground in extraction buffer at a 1:10 (wt/vol) was subjected to TAS-ELISA (coating goat G1571 1:10,000, detection rabbit R34 1:10,000, and anti-rabbit conjugate 1:30,000) concomitant with IC-RT-PCR testing (Figure 2.11). All PMTV-positive samples identified by TAS-ELISA produced PCR products of 585 bp in size in IC-RT-PCR. Apparently, the sensitivity and reliability of the PMTV detection using IC-RT-PCR were similar to the TAS-ELISA protocol.

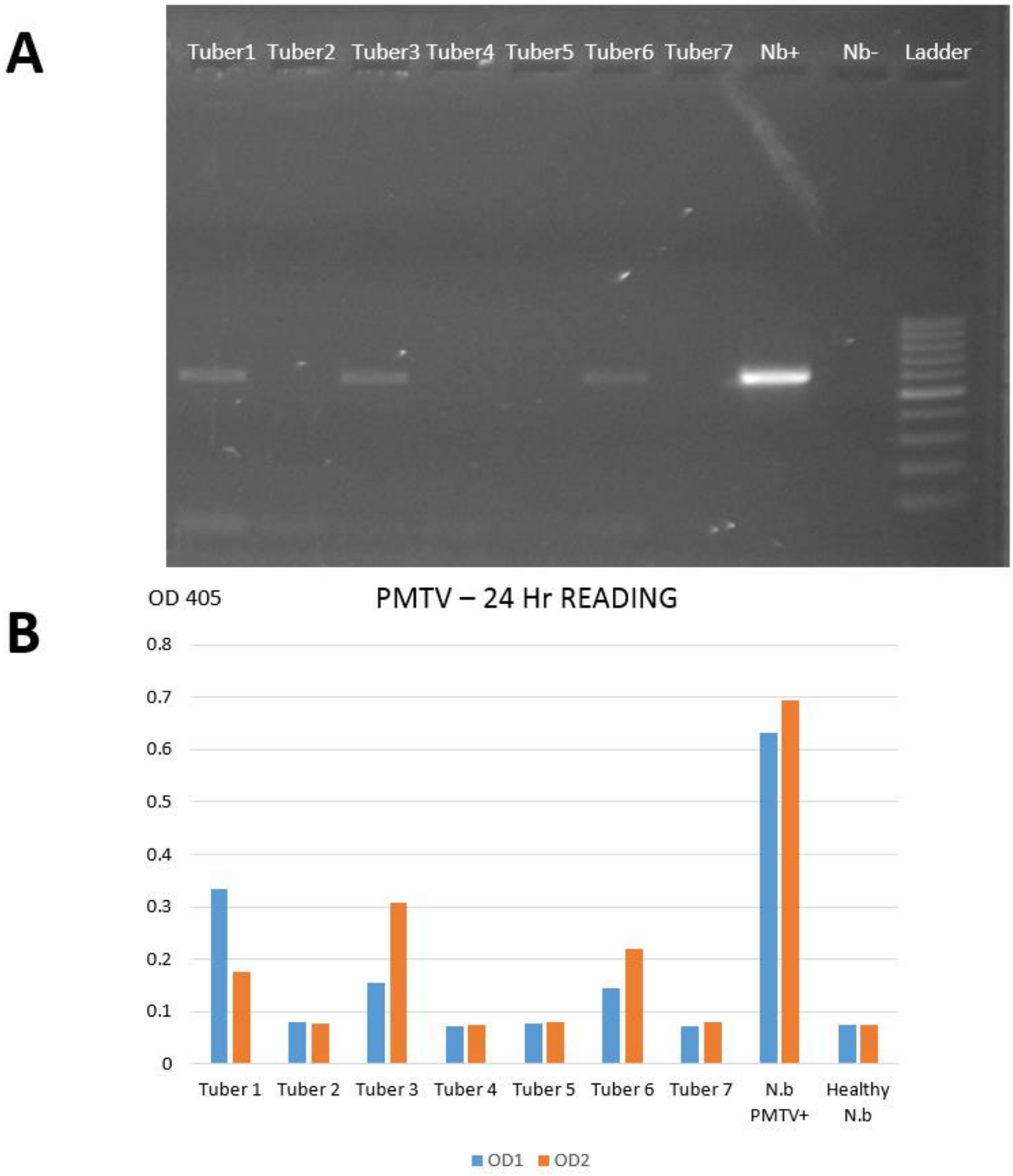

Figure 2.11. Comparison of immunocapture RT-PCR and TAS-ELISA for detection of PMTV in the same potato tubers and in *N. benthamiana* foliage. (A) Illustrates a gel image of immunocapture-RT-PCR (coating goat G1571 1:10,000). (B) Illustrates a TAS-ELISA graph (coating G1571 1:10,000, detecting rabbit R34 1:10,000, and anti-rabbit conjugate 1:30,000).

Sequencing and genome analysis

To confirm the sequence identity of PMTV_CO, 20 primers were designed for Sanger sequencing based on reference isolates from GenBank that shared 99% identity with the CP sequence obtained from the PMTV_CO isolate (Table 2.3). The near complete genome of PMTV covering most of RNA-1, RNA-2, and RNA-3 segments (Figure 2.12) was amplified from total nucleic acid Dellaporta extract using RT-PCR and sequenced with Sanger methodology as previously described (Green et al., 2017). RNA-1 of PMTV_CO contained a large single ORF coding for a 1,812-aa protein with easily identifiable motifs for methyltransferase (MT), helicase (HEL), and RNA-dependent RNA-polymerase (RdRp) associated with the role of RNA-1 in virus replication. This large ORF in RNA-1 was interrupted with a weak, opal stop-codon UGA, which could terminate the ORF to create a smaller, 1,303-aa protein containing only MET and HEL domains. RNA-2 of PMTV_CO had four ORFs coding for a 463-aa protein (HEL), a 119-aa protein (p13), a 190-aa protein (p21), and a 68-aa protein (p8). RNA-3 of PMTV_CO had a large single ORF encoding an 824-aa protein (p90). This ORF in RNA-3 was interrupted with an amber stop-codon UAG, which could terminate the ORF to create a smaller, 176-aa protein easily identifiable as the CP of the virus. Genetic organization of the PMTV_CO genome matched the known genome organization of PMTV (Harrison and Reavy, 2002). The nearly complete whole genome of the PMTV_CO isolate was compared to PMTV sequences available in GenBank database using BLASTn program. The PMTV_CO isolate shared over 99% nucleotide sequence identity with multiple strains reported from Europe and from North America. Nucleotide sequence of PMTV_CO RNA-1 (5,863 nt) was 99.9% identical to the Swedish PMTV isolate PF43 (GenBank accession KU955473), RNA-2 (2,788 nt) was 99.9% identical to the Canadian isolate Ch20 (KM822708), and RNA-3 (2,820 nt) was 99.9% identical to the to the Czech isolate Korneta-Nemilkov (DQ102381). An additional, nearly whole genome of an Idaho PMTV isolate PMTV_ID was also sequenced and compared to the PMTV_CO isolate. Both PMTV_ID and PMTV_CO isolates shared over 99% nucleotide sequence identity between each other in all three genome components, and both isolates were thus very close genetically to European and North American isolates of PMTV.

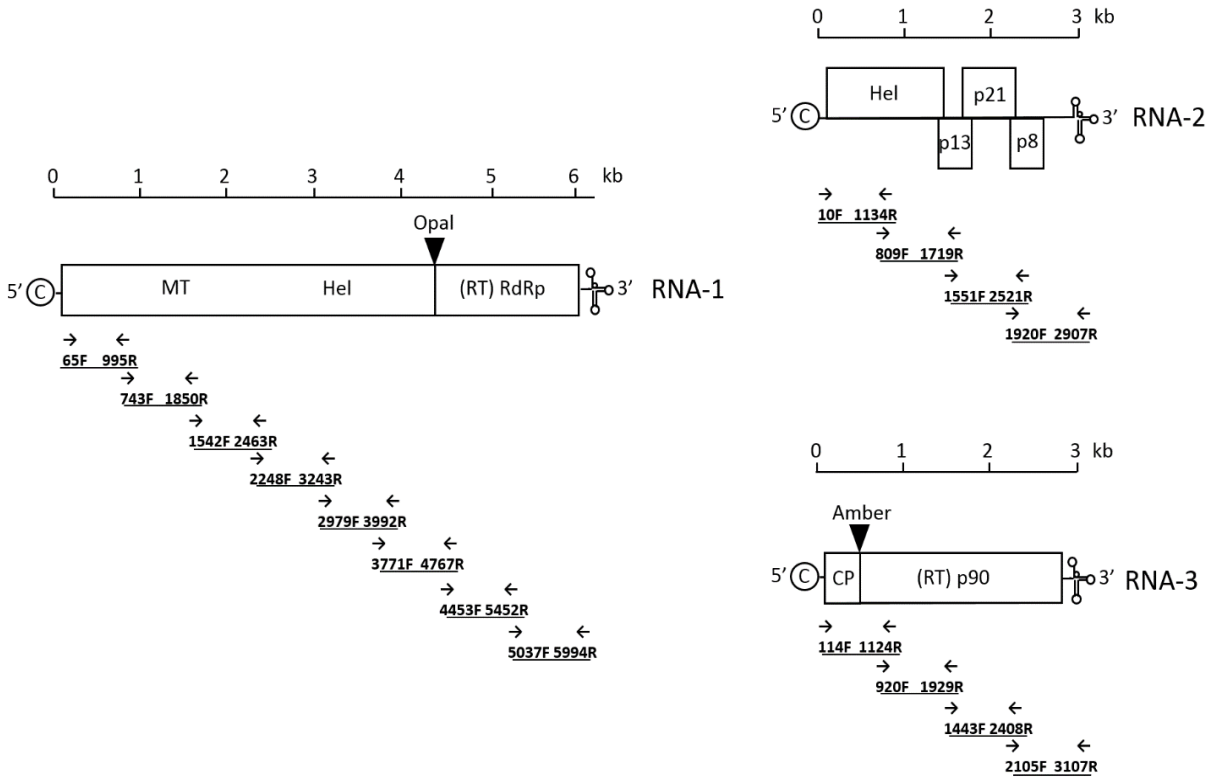

Figure 2.12. Image of sequencing coverage of PMTV RNA-1, RNA-2, and RNA-3 and genome organization of PMTV isolates PMTV_CO and PMTV_ID. Boxes indicate open reading frames in the three RNA species. All RNA species have a chemically modified ribonucleotide at the 5′-terminus, with so-called 'cap' structure and 3' termini have tRNA-like secondary structures.

Discussion

PMTV is an emerging potato pathogen in the United States. Since the first report of the virus in Maine in 2003 (Lambert et al., 2003), PMTV has been reported from nearly all major potato producing states including Idaho, Washington, Colorado and North Dakota (Crosslin, 2011; David et al., 2010; Kaur et al., 2016; Mallik and Gudmestad, 2015; Whitworth and Crosslin, 2013). However, until 2010, when PMTV was found in North Dakota (David et al., 2010), the virus was considered exotic and generally not widely prevalent in the U.S. From 2010 onward, a high demand for PMTV detection emerged across the U.S., and this work was conducted in order to create such tools, to satisfy the demand of the laboratories testing for the presence of PMTV. Several issues were addressed to develop diagnostic tools for PMTV testing. First, the genetic diversity of the virus present in different production areas had to be probed to determine if it may be difficult to create a universal PMTV diagnostic tool for all potato producing states. Second, a stable source of the PMTV positive control was needed to allow for sensitivity and specificity tests and for selection of the best

testing methodology. Third, initial protocols for sample collection and processing had to be developed. Since most of the prior research on PMTV testing was conducted outside of North America, mainly in Europe (Torrance et al., 1993; Sokmen et al., 1998; Mumford et al., 2000; Gallo García et al., 2013; Arif et al., 2014), applicability of the tools for PMTV diagnosis in the U.S. potato were tested and validated.

Testing for PMTV comes with a variety of challenges that make detection difficult. PMTV rarely infects potato systemically, and thus not all shoots will be PMTV-infected (Calvert and Harrison, 1966). Consequently, not all progeny tubers of an infected potato plant become infected and testing of select tubers may miss the PMTV infection. At the same time, visual diagnosis of PMTV is problematic since the virus often does not induce symptoms in the foliage, and tuber symptoms resemble symptoms caused by other viruses, such as TRV, AlMV, and PVY. Furthermore, PMTV is found to be unevenly distributed in a tuber and could be detected in some parts of a tuber sample but not in other parts of the same tuber (Sokmen et al., 1998). While PMTV appears to be self-eliminating in tubers in the absence of the plasmodiophorid vector (Calvert and Harrison, 1966), post-harvest tuber testing could be essential to prevent planting infected seed tubers and introducing virus to non-infected fields that may have powdery scab.

This paper describes a TAS-ELISA format, mouse antiserum from SASA used as a coating antibody at 1:1,000 dilution followed by rabbit R34 antiserum for detection at 1:10,000 dilution, which is four times more sensitive in the detection of PMTV than the current commercial kits available. An ELISA positive result was defined as the infected plant material producing a signal that exceeds the healthy plant signal three-fold. The mouse antiserum from SASA used for coating meets these expectations, whereas as goat antiserum as a coating and the three commercial kits did not. A clear threshold makes it easier to determine PMTV infection in tuber and foliar tissues. A possible explanation hypothesized to why the mouse antiserum from SASA used as a coating at 1:1,000 dilution followed by rabbit R34 antiserum for detection at 1:10,000 dilution is more sensitive than any other ELISA system. The reason may be due to competition for epitopes between coating and detecting antibodies in the 'sandwich' ELISA. The CP of PMTV harbors many epitopes, at least five according to Torrance et al. (1993). The goat G1571 antisera is a polyclonal antibody, which means this serum is composed of a population of multiple antibodies that recognize multiple epitopes; the same is true for the rabbit R34 antiserum. The mouse antiserum from SASA however is a monoclonal antibody, which means it is made up of only one antibody type that only recognizes one epitope of PMTV. Due to the nature of competitive epitope binding, a polyclonal antibody used for coating will

recognize and bind multiple or all epitopes available. When another polyclonal antibody is used for detection, those same, 'occupied', epitopes will not be available for detection because they are already bound to the primary (coating) polyclonal antibody, making fewer epitopes available for detection. Monoclonal antibodies used as coating are an effective strategy because they will only target one epitope, allowing all other epitopes to be accessible to the detection antibody. The implementation of this TAS-ELISA will be useful for both breeding and virus-free certification programs.

Immunocapture-RT-PCR is a combination of an immuno-detection specificity and molecular accuracy. This methodology allows the use of the same plant extract as for ELISA and Immunocapture-RT-PCR. Once the antigen (virus) is bound, all host compounds can be washed away. PMTV testing is mostly done on tubers. Theoretically, using the immunocapture-step should allow most of the carbohydrates to be washed away. However, testing with tuber tissue is often problematic because tubers have a high starch content, and the starch may interfere with regular RT-PCR tests. In this study, we observe results obtained from RT-PCR and ELISA do not correlate, and more tubers were scored as positive by ELISA. This observation has also been reported in other publications (Sokmen et al., 1998). Further investigation will be required to optimize this immunocapture-RT-PCR methodology for PMTV detection and achieve 100% correlation.

CHAPTER 3

POLYCLONAL ANTIBODIES SPECIFIC FOR TOBACCO RATTLE VIRUS AND THE DEVELOPMENT OF AN IMMUNOCAPTURE RT-PCR METHODOLOGY FOR THE DETECTION OF TOBACCO RATTLE VIRUS IN TUBER AND LEAF TISSUE

Introduction

The effects of *Tobacco rattle virus* (TRV) on tobacco was first described in Germany by Behrens (1899), who called it Mauche disease. Later, the virus was characterized by Quanjer (1943) (Robinson and Harrison, 1989). In the initial phases of characterization in the 1950s, TRV was categorized into two forms: M (multiplying) types that multiplied readily and were easily transmitted into tobacco by mechanical inoculation and NM (non-multiplying) types that transmitted mechanically only with difficulty. M and NM types cause similar symptoms and both contain characteristic rod-shaped particles. M types can give rise to M and NM types, but NM types never produced M types (Cadman, 1959). M types were found to contain two rod-shaped particle sizes, either long (175 nm, infective) or short (~75 nm, not infective but more abundant) in length and can be serologically detected. NM types only contained the longer rods and could not be serologically detected (Cadman, 1962). NM types became highly infective when prepared with aid of cold phenol (Sänger, 1968). It was later established that NM isolates of TRV are perpetuated by the larger genome nucleic acid (RNA-1) but do not produce nucleoprotein particles (CP) and M isolates of TRV contain both RNA-1 and RNA-2. RNA-1 is generally analogous among TRV isolates, but RNA-2 can be highly diverse thus TRV serology can vary on antigenic constitution depending on isolate. TRV serological relations has been studied with isolates from multiple different continents (Europe, North America, and South America). Isolates from different continents appear to only be serologically distantly related (Harrison and Woods, 1966).

TRV is in the family Virgaviridae, genus Tobravirus. It is a rod-shaped positive-sense bipartite RNA virus. RNA-1 is largely conserved among TRV isolates. RNA-1 contains four open reading frames (ORFs). The ORF nearest to the 5' terminus codes for a 134K protein and is terminated at an opal (UGA) stop codon. Read-through of this stop codon produces a 194K protein. Amino acid comparisons of the *Tobacco mosaic virus* (TMV) 194K protein to the TRV 134K protein showed strong homology, suggesting that both proteins are similarly involved in virus replication (Hamilton et al., 1987). The ORF that encodes for a 29K protein starts one base beyond the 194K protein stop codon

and, in turn, is followed by a 16K protein at the 3’ end of RNA-1. The 194K protein and 16K protein contributes in cell-to-cell movement (Hamilton et al., 1987). The 29K protein is homologous to TMV 30K protein which may be associated in virus transport. (Hamilton et al., 1987; Boccara et al., 1986). The 16K protein encodes a suppressor of RNA silencing that allows transient viral entry in meristems. This novel mechanism of meristem exclusion may be associated with the phenomenon of recovery in virus-infected plants in which upper leaves have little or no virus and are immune to secondary infection by the same virus (Martín-Hernández et al., 2008). RNA-2 can be highly diverse among different TRV isolates. RNA-2 encodes for CP (Bergh et al., 1985). There is sequence homology between RNA-1 and RNA-2 at both the 5’ and 3’ termini, but the extent of this homology varies between strains; in some strains the homologous region at the 3' end is large enough to include some or all of the open reading frame coding for the 16K and 29K protein of RNA-1 (Hamilton et al., 1987). RNA-2 of strain TCM contains an additional open reading frame, between the CP gene and the 3' terminal homologous region, coding for a 29K protein that is not homologous to that coded for by RNA-1 (Angenent et al., 1986). Serological properties correlate with transmissibility, indicating the factor determining vector transmissibility is located on the RNA-2 genome segment of TRV (Ploeg et al., 1993). Both RNA-1 and RNA-2 have a chemically modified ribonucleotide at the 5’-terminus, with so-called ‘cap’ structure (Pelham, 1979) and 3' termini have tRNA-like secondary structures (Van Belkum et al., 1987). TRV is unusual in that RNA-1 can replicate and establish systemic infection, as well as induce lesion type symptoms, independent of RNA-2 (Cadman and Harrison, 1959; Sänger, 1968).

TRV is vectored by *Trichodoridae* nematodes (Cadman, 1962), primarily genus *Trichodorus* and/or *Paratrichodorus*, commonly known as stubby root nematodes. TRV infestations can occur uneven in a field – have “hot spots” due to nematode populations manifesting in localized areas (Cooper, 1971). Adults and juvenile nematodes can transmit the virus, but it is probably not retained through the moult. The virus particles adhere to the oesophageal wall of the nematodes (Taylor and Robertson, 1970) and are thought to be ingested with saliva into root cells when the nematodes feed. TRV can be retained for many months by non-feeding nematodes (Van Hoof, 1970). There is no evidence for multiplication of the virus in the vector and it is probably not transmitted through nematode eggs (Ayala and Allen, 1968). Furthermore, virus transmission bait tests with single *Trichodoridae* nematodes have shown that a substantial degree of specificity occurs between vector species and Tobravirus serotypes. Tobravirus serotypes were associated with either *Paratrichodorus* or *Trichodorus* vector species but not both. This specificity was more apparent with associations

between *Paratrichodorus* vector species and tobravirus serotypes than with those between *Trichodorus* species. Different *Paratrichodorus* species were associated with different Tobravirus serotypes whereas *Trichodorus* species with the same species transmit similar serotypes by several *Trichodorus* species (Ploeg et al., 1992). *Paratrichodorus* species are most prevalent in the Pacific Northwest (Williams et al., 1996).

TRV has a wide host range, infecting over 100 natural hosts and more than 350 experimental hosts. One economically important crop that TRV can infect is potato, causing stem-mottle disease in foliage and corky ringspot (CRS) disease in tubers (Cadman, 1959). Stem-mottle disease is characterized by yellowish patterns on the leaves, leaf distortion, stunting, mottling, and occasionally accompanied by stem necrosis. TRV is inefficiently transmitted through tubers because often only one to two shoots are infected disease (Cadman, 1959). Typically, only a small fraction of progeny tubers become infected in the absence of viruliferous vectors (Harrison et al., 1983). In tubers, TRV can cause necrotic arcs, corky tissue throughout the flesh, and disfigure the tuber, decreasing the crop value (Van Regenmortel and Fraenkel-Conrat, 2013). In the Pacific Northwest, CRS tuber injury of 5-10% may result in potato crop rejection of an entire field (Williams et al., 1996). There is a plausible concern that TRV positive tubers (symptomatic and asymptomatic) can give rise to systemically infected plants, therefore can act as inoculum source to non-viruliferous nematodes if infected seed potatoes are planted (Crosslin et al., 1999). Potato cultivars are identified as tolerant, resistant, or susceptible/sensitive against TRV, although infection without expression of symptoms is common (Robinson and Dale, 1994). TRV has been detected in freshly harvested tubers (symptomless), as well as in tubers which have been stored, indicating that TRV is not eliminated during the storage period (Weidemann, 1995). TRV presents a unique dilemma in that NM types are predominately associated with potato plants with stem-mottle disease. NM types carry the larger genome nucleic acid (RNA-1) but lack CP; thus identification presents special problems (Harrison and Robinson, 1982). NM types of TRV are not detected by serological means such as ISEM and ELISA which is dependent on protein recognition of CP (Harrison et al., 1983). TRV is difficult to diagnose and differentiate from other tuber necrosis-causing viruses such as PMTV and PVY by visual symptoms. Effective control strategies rely on accurate diagnosis (Mumford et al., 2000). Hybridization methods using DNA complementary to nucleotide sequence of RNA-1 can detection NM and M forms (Harrison and Robinson, 1982). However, TRV is not uniformly distributed in CRS-affected tubers and appears to be in low concentration in tuber tissue, making accurate sampling and diagnosis a challenge (Crosslin and Thomas, 1995).

In the United States, TRV incidence has been reported in potato production areas in California, Colorado, Florida, Idaho, Michigan, Minnesota, North Dakota, Oregon, Washington, and Wisconsin (Yellareddygari et al., 2018). The threshold for seed certification for incidence of necrotic tuber is less than 0.5% (Halterman et al., 2012; United States Department of Agriculture 2004). Due to TRV similar symptomology and sometimes misidentification for PMTV or PVY, our laboratory attempted to fulfill demands to find methods of cost-efficient and reliable detection. In this study, our objective was to describe the development of a triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) and immunocapture-reverse transcription (IC-RT)-PCR methodology suitable for detection of TRV-M isolates in tuber and leaf tissue from potato. This was accomplished by establishing TRV (courtesy of James Crosslin; USDA-ARS) in a laboratory plant, *Nicotiana tabacum* cv. Samsun, to have a permanent source of TRV positive control, and also to be able to purify virus antigen for specific antibody production. We also studied a novel TRV isolate originally derived from potato, with properties intermediate between M and NM isolates of TRV.

Material and Methods

Virus sources

"BM 32123" isolate, along with two other isolates, were provided by James M. Crosslin, United States Department of Agriculture–Agricultural Research Service (USDA-ARS), Prosser, WA. TRV infected dried *N. tabacum* cv. Samsun leaf material was used as inoculum to mechanically transmit (grind in inoculation buffer at 1:10 dilution (wt/vol); described below) the virus to *N. tabacum* cv. Samsun in March of 2015, and maintained in a climate-controlled growth room. Inoculated plants were tested by RT-PCR for PMTV, TRV, AlMV, PVY, PVM, PVA, PVX, and PVS, to confirm other pathogens were not present (refer to Table 2.1 for primers used).

"TRV_ID" isolate was provided by the University of Idaho, Idaho Falls Research and Extension Center, courtesy of Melissa Bertram. Seven tuber samples were submitted to the University of Idaho for routine potato virus testing in September of 2016, and all seven were found to be TRV-positive and PVS-positive.

Virus propagation in Nicotiana tabacum *cv Samsun and maintenance in the laboratory*

To propagate TRV_ID, four out of the seven tuber-halves were kept in the dark at room temperature for 3 months until sprouting and planted in 1-gallon pots. After emergence, a total of 10 stems were produced among the 4 tuber-halves. Growing potato stems were individually labeled and tested for TRV infection through collection of young leaves subjected to RT-PCR and TAS-ELISA tests. Upon testing, only one out of 10 stems was found TRV-infected systemically. Foliage from that one TRV-positive potato shoot was used as inoculum source to inoculate *N. tabacum* cv. Samsun plants. Young tobacco transplants were mechanically inoculated approximately 3 weeks after planting (three to four leaf stage). Briefly, inoculum source was ground in inoculation buffer (50 mM sodium phosphate dibasic heptahydrate, 50 mM monopotassium phosphate, pH7) at 1:10 dilution (wt/vol). Three leaves were marked to be inoculated by puncturing a single hole per inoculated leaf with the back end of a cotton swab. Carborundum was sprinkled on the leaf surface, and a cotton swab submerged into the inoculum was gently rubbed against targeted leaves. Plants were observed daily starting at four days post-inoculation (dpi) for symptom development, and were assayed for TRV two weeks after inoculation by reverse transcription-polymerase chain reaction (RT-PCR). Isolates were maintained in *N. tabacum* cv. Samsun by periodical re-inoculation into young healthy *N. tabacum* cv. Samsun seedlings kept as a stable source of TRV.

Virus purification

Systemically infected leaf tissue of *N. tabacum* cv. Samsun was harvested at 7 to 14 dpi, and the virus was purified using a modified procedure described by Bergh et al. (1985). Briefly, 100 g of *N. tabacum* cv. Samsun leaf tissue was homogenized in 250 mL of cold extraction buffer (30 mM potassium phosphate buffer, pH 8, containing 10 mM sodium thioglycolate, 10 mM diethyldithiocarbonate). Leaf homogenate was squeezed through a double layer of cheesecloth and centrifuged at 13,000 rpm (JLA-16.25 rotor) at 4°C for 10 min. 4% PEG (MW 8,000 - 6.4 g) and 1.75% NaCl was added to the supernant and stirred at 4°C for 4 h. The mixture was then centrifuged at 19,500 rpm (JA25.5 rotor) at 4°C for 15 min. The pellet was resuspended in extraction buffer. Equal amount of chloroform was added and stirred for 45 min at 4°C. The mixture was then centrifuged at 10,000 rpm (JLA-16.25 rotor) at 4°C for 10 min. The aqueous phase was collected and added to 30 mM potassium phosphate buffer (PPB), pH 8. The mixture was laid over a cushion of 2.5% (wt/vol) sucrose (in deionized water), followed by a high-speed centrifugation at 38,000 rpm (70Ti rotor) at

4°C for 120 min. The final virus pellet was resuspended in the storage buffer (50 mM Tris, pH 8). The purified viruses were kept with 0.1% sodium azide preservative at 4°C for later use.

Protein analysis by polyacrylamide electrophoresis and Western blots

For Coomassie staining and western blots, purified virus preparation was mixed with the Laemmli Tris-sodium dodecyl sulfate (SDS) sample buffer and heated at 95°C for 10 min, and proteins were separated on 4-12% gradient SDS-polyacrylamide gels (Bio-Rad, Sunnyvale, CA). The SDS-PAGE gel was stained using Coomassie Brilliant Blue G-250 (Bio-Rad) with a SDS-PAGE broad range standard (Bio-Rad cat. #161-0318) protein marker. Immunoblotting was performed to confirm virus specificity. Separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad) as described previously (Karasev et al., 2010). The membranes were incubated for 16 to 20 h at 4°C with the respective rabbit R38 polyclonal antiserum at 1:20,000 dilution following incubation with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma A-3687). The immune complexes were revealed by incubating the membranes with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine (BCIP)/ nitro-blue tetrazolium (NBT) substrate (Sigma) and the color reaction was stopped by washing them in water

Production of TRV-specific antibodies and development of TAS-ELISA assay for the detection of TRV

Polyclonal antisera against purified TRV BM 32123 isolate was raised in rabbit R38 and guinea pig GP25 following a series of four to six immunizations, with the first one in the presence of complete Freund's adjuvant and all subsequent with the presence of incomplete Freund's adjuvant. Antibody production was performed by Cocalico Biologicals (Stevens, PA).

A triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) for the detection of TRV-M isolates in tuber and leaf tissue utilized two TRV-specific antisera, one in rabbit R38 and one in guinea pig GP25. Triple-antibody sandwich (TAS)-ELISA tests were performed following the general protocol of Clark and Adams (1997), with slight modifications. Briefly, 96-well MaxiSorp microtiter plates (Nunc, Rochester, NY cat. No. 442404) were coated with 100 µL of the rabbit R38 antiserum at 1:10,000 dilution in 20 mM sodium carbonate buffer (pH 9.6). Tissue samples were ground in extraction buffer (1× phosphate buffered saline (PBS) plus Tween® 20, 0.3% non-fat dry milk, and 2% polyvinylpyrrolidone) at a 1:10 (wt/vol) ratio. Tubers were sampled by using the open end of a 1 mL pipette tip to collect 3 punches 0.5 cm deep taken from different areas of the tuber surface (top, middle, and bottom) and combined into 1 sample (Figure 2.1). The Homex 6

grinding machine (Bioreba, Reinach, Switzerland) was used to homogenize leaf and tuber samples in meshed plastic bags. Sample extracts (100 µL each) were added to the coated plates and incubated with plant extracts for 16 to 20 h at 4°C. Plates were washed six times using 1× phosphate buffered saline plus Tween® 20 (PBST) and then rinsed with deionized water. Detecting guinea pig GP25 antiserum used at 1:30,000 dilution was applied to the wells in ELISA buffer. After incubation for 16 to 20 h at 4°C, plates were washed extensively with 1× PBST, and anti- guinea pig (Sigma A-5062) immunoglobulin G (IgG) conjugates with alkaline phosphatase at 1:30,000 dilution in ELISA buffer was applied, and the plates were incubated 16 to 20 h at 4°C. After washing and adding p-nitrophenyl phosphate (Sigma_Aldrich CAS Number: 333338-18-4) substrate, the color reaction was monitored by measuring absorbance at 405 nm using an ELISA reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA). TRV-positive and negative samples were included in each ELISA experiment as controls. Samples were defined as positive if the absorbance value exceeded the healthy controls by threefold.

Comparison of TAS-ELISA to commercial kit

A home-made enzyme-linked immunosorbent assay (ELISA) methodology for the detection of TRV was compared to the only commercial TRV ELISA kit available from Neogen (Ayre, Scotland). Testing with the commercial ELISA kit was done according to manufacturer's recommendations. Coating was done at 1:1,000 dilution; conjugated detecting antiserum was applied at 1:1,000 dilution. This comparison (ELISA systems summarized in Table 3.1) was conducted on TRV-infected samples including *N. tabacum* cv. Samsun leaf tissue and tuber skin/flesh tissue. All plates were loaded with the same plant extract grinded in extraction buffer at 1:10 (wt/vol) ratio and developed with p-nitrophenyl phosphate (Sigma_Aldrich CAS Number: 333338-18-4) substrate and the color reaction was monitored by measuring absorbance at 405 nm using an ELISA reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA).

Table 3.1. Two ELISA systems for the detection of TRV used in this study including one commercial kit from Neogen (Ayre, Scotland) and homemade ELISA system: coating R38, detecting GP25.

System	Coating	Detection	Conjugate
Coating R38, Detecting GP25	R38 1:10,000	GP25 1:30,000	Anti-guinea pig 1:30,000
Neogen kit	Neogen 1:1,000	Neogen 1:1,000	

Development of immunocapture(IC)-RT-PCR for the detection of TRV

An immunocapture-reverse transcription (IC-RT)-PCR methodology was devised for detection of TRV-M isolates in tuber and leaf tissue. 96-well skirted PCR plates (Eppendorf cat. No.951020460) was coated with 100 µL of the rabbit R38 polyclonal antibodies at 1:10,000 dilution in 20 mM sodium carbonate buffer (pH 9.6) and incubated at for 16 to 20 h or 4°C overnight. Plates were washed thoroughly with deionized water. Most of the water was removed by hitting the plate upside down over a paper towel and plates were stored at -20^{o}C until ready to be used. Tissue samples were ground in extraction buffer (1× PBST, 0.3% dry milk, and 2% polyvinylpyrrolidone) at a 1:10 (wt/vol) ratio. Sample extracts (100 µL each) were added to the coated plates and incubated 37°C for 2 to 4 h or 4°C overnight. Plates were washed six times using 1× PBST and then rinsed with deionized water.

Reverse transcription (RT) for first-strand cDNA synthesis, previously described by Chikh-Ali et al. (2013), was performed using virus particles captured by rabbit R38 virus-specific antibody in wells of the pre-coated 96-well skirted PCR plates, in a 25 µL reaction mixture that contained M-MLV RT 5× Buffer (50mM Tris-HCl, 75mM KCl, 3mM MgCl2 and 10mM DTT) (Promega), 10 mM each deoxyribonucleotide triphosphate (dNTP), a mixture of oligo-dT and random hexamer primers 3 µM final concentration, 25 units of RNase Out Ribonuclease Inhibitor (Promega), and 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega). The RT program was performed in an Eppendorf Mastercycler Pro (Eppendorf North America) with an initial incubation at 25°C, increased to 42°C at 1°C per 30 s, held at 42°C for 45 min, and then increased to 60°C at 1°C per 2 min. The transcriptase was inactivated by incubation at 70°C for 10 min.

Polymerase chain reaction protocol used throughout this paper was that of Robinson (1992). Primers were based off of sequences from TRV RNA-1. PCR was conducted in 20 µL of 10× Taq Buffer (500 mM KCl, 100 mM Tris HCl, 15 mM $MgCl_2$), containing 2.5 mM of each dNTP, 5 µM each forward primer and reverse primer, 2.5 units Taq DNA polymerase (Genscript), and 2 µL of cDNA template. Synthetic oligonucleotide primers TRV_1F (5'-GACGTGTGTACTCAAGGGTT-3') and TRV_1R (5'-CAGTCT ATACACAGAAACAGA-3') was used to amplify TRV RNA-1 region from cDNA (Robinson, 1992). Amplification was done with an initial incubation for 5 min at 95C, followed by 11 cycles of 30 s at 95C, 30 s at 62C, and 2 mins at 72C, another 22 cycles of 30 s at 95C, 30 s at 56C, and 2 mins at 72C, and finally an extension at 72C for 5 mins. A 10 uL aliquot of PCR reaction was fractioned onto 1.2% agarose gel containing ethidium bromide at 10 µg/mL and visualized under UV illumination. TRV positive and negative samples were included in each immunocapture(IC)-RT-PCR experiment as

controls. Samples were defined as positive if extract produced an amplicon size of 463 base pairs (bp).

Nearly complete genome sequencing and sequence analysis

BM 32123 and TRV_ID isolates were partially sequenced via Sanger sequencing using a series of overlapping RT-PCR fragments amplified on total RNA extraction from infected *N. tabacum* cv. Samsun. Total nucleic acids extracted using the Dellaporta methodology as described by Hicks et al. (1983) with some modifications, were used as templates for subsequent reverse transcription and PCR. Briefly, plant tissue was homogenized in Dellaporta solution (100 mM Tris, 50 mM EDTA, 500 mM NaCl, 0.001% β-ME) at 1:10 (wt/vol) ratio, 10% SDS was added, the solution vortexed to mix, and incubated at 65^{O}C for 10 min. After addition of 1/3 volume of 5 M potassium acetate, the mixture was vortexed, and centrifuged at 12,000 rpm (Eppendorf 5430R rotor) for 10 min. Supernatant was transferred to a new tube, isopropanol was added, and the mixture was incubated on ice for 5 min, then it was centrifuged at 12,000 rpm (Eppendorf 5430R rotor) for 20 min, supernatant was discarded and the pellet washed with 70% ethanol. Dry the residue. The resulting pellet was resuspended in sterilized DI water and used for reverse transcription immediately, or stored at -20^{o}C until use.

Reverse transcription (RT) was performed using 4.5 µL of the total nucleic acid extract in a 25 µL reaction mixture that contained M-MLV RT 5× Buffer (50mM Tris-HCl, 75mM KCl, 3mM MgCl2 and 10mM DTT) (Promega Corp.), 2.5 mM each dNTP, a mixture of oligo-dT and random hexamer primers 3 µM final concentration, 20 units of RNase Out Ribonuclease Inhibitor (Promega), and 200 units of M-MLV Reverse Transcriptase (Promega). RT was performed in an Eppendorf Mastercycler Pro (Eppendorf North America). Before the reverse-transcription reaction, 4.5 µL of RNA template was denatured at 70°C for 5 min; then, the reverse-transcription master mix was added. The thermocycler program here consisted of 25^{o}C for 2 mins; 17 Cycles of 30 sec at 25^{o}C; incubation at 42^{o}C for 45 mins; 18 Cycles of 2 min at 42^{o}C and RT deactivation at 70°C for 10 min.

PCR was preformed using the parameters previously described for the immunocapture-RT-PCR methodology. 13 primer pairs were designed from reference isolates from GenBank to amplify RNA-1 and RNA-2 of TRV (listed in Table 3.2). A 10 µL aliquot of a PCR reaction was treated using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermofisher) and submitted for sequencing to Genewiz, Inc. (South Plainfield, NJ). Nearly complete viral genome segments were assembled using SeqMan

Pro 13 (DNASTAR, Madison, WI). The sequences of PMTV isolates were aligned using Clustral X, and the analysis of the aligned sequences was performed using the program package RDP4.

Table 3.2. Primers used for amplification and Sanger sequencing of *Tobacco rattle virus* (TRV).

Name of Primer	Direction	Primer Sequence 5' to 3'	Location
TRV_RNA1_27F	Forward	GGTAGAACGTGCTAATTGGATTTTGG	27
TRV_RNA1_967R	Reverse	CTYTCAGGAGCAAAAAGCATAGCAGC	967
TRV_RNA1_721F	Forward	TGTCGGAGAAYGAGCTGTGGATGTA	721
TRV_RNA1_1797R	Reverse	ATACTATCACCTTGGTTTTGTGCGWG	1797
TRV_RNA1_1565F	Forward	TGARGCAATTCTGACTGARACGAARC	1565
TRV_RNA1_2646R	Reverse	CATCCCACACTCCCAGTCCGTG	2646
TRV_RNA1_2438F	Forward	CTTCCARGTGATGGGAGGTGARAGRT	2438
TRV_RNA1_3519R	Reverse	CYGCCACYTTCCTCAAYGCCAAC	3519
TRV_RNA1_3272F	Forward	RGTCGGAAARTTTGAYRTTGTTACWG	3272
TRV_RNA1_4197R	Reverse	GATCAGCCCGAATYTTYCCCACATC	4197
TRV_RNA1_4821F	Forward	GCAGTCATGGTTACRTATGGWGGAGA	4821
TRV_RNA1_5935R	Reverse	CYTCYTGATCGGTAAAATCGCCTTC	5935
TRV_RNA1_5756F	Forward	AYGTACTYGATGTCTGGAAGGTGGGT	5756
TRV_RNA1_6757R	Reverse	CCCCTTTTGCCTTTGTAACCATCA	6757
TRV_RNA1_4161F	Forward	CTGTTGTCTACGATGTGGGGAAGATT	4161
TRV_RNA1_5164R	Reverse	GCGCATGGACAGAATCACCTTTG	5164
TRV_RNA2_30F	Forward	GGCCGGGGTGTGTCTTTGA	30
TRV_RNA2_549R	Reverse	CTCCTCATCGTACATACCGTCTGTCA	549
TRV_RNA2_340F	Forward	GGCAATCGCATCCATTCGC	340
TRV_RNA2_1163R	Reverse	CACGTGACCCTCTGAAAGAGTATCTG	1163
TRV_RNA2_959F	Forward	CTTTTGGTAAATTCGATGACGCTTC	959
TRV_RNA2_1962R	Reverse	TTCGCAGGCAACACCACCG	1962
TRV_RNA2_1709F	Forward	CCGGACAAAGATATTACGCTGATGAT	1709
TRV_RNA2_2755R	Reverse	GCACAGCTTGCTCAGAACTGGAGTAG	2755
TRV_RNA2_2647F	Forward	AGTGCCGAGATGGATGCGAC	2647
TRV_RNA2_3644R	Reverse	TTGCCTTTGTAACCATCATCACTTTA	3644

Results

Biological characterization

The University of Idaho Plant Virology Laboratory frequently receive and test many tuber samples for various viruses affecting potato since approximately 2007. In September 2016, seven tuber samples were sent to the lab from Idaho Falls, Idaho for standard virus testing. All seven tuber samples were found positive for TRV and PVS, using RT-PCR testing of the Dellaporta extracts from

tuber skin and flesh. Although TRV can cause severe symptoms such as internal and external necrosis in tuber tissue (Figure 3.1), the submitted TRV-positive tubers from Idaho exhibited very mild internal necrotic spots (not shown).

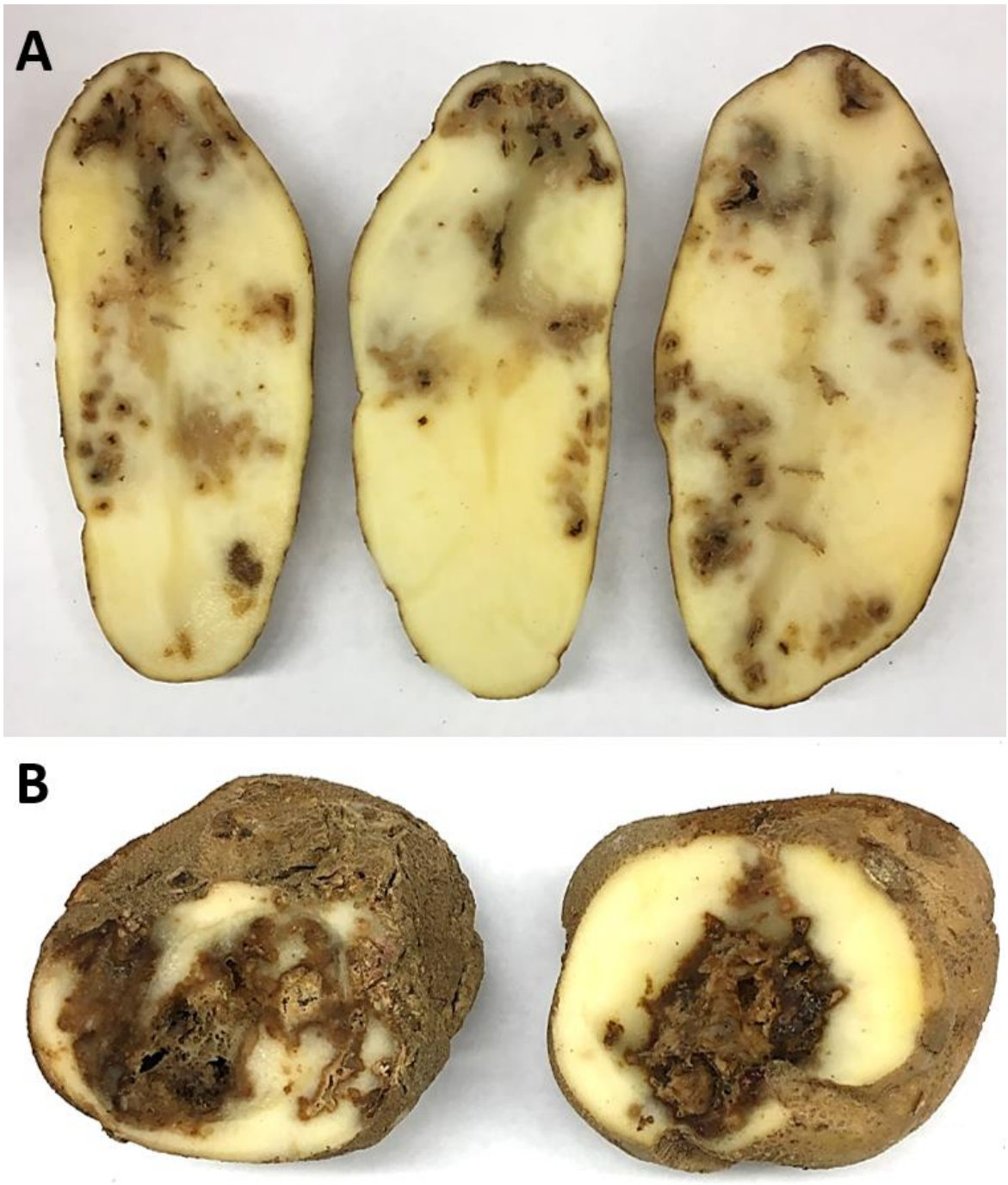

Figure 3.1. (A) Tubers infected with *Tobacco rattle virus* (TRV) exhibiting internal necrotic spots and necrosis (B) Tubers (cv. Spartan) infected with TRV exhibiting external cracking and internal necrotic arching and necrosis.

BM 32123 and TRV_ID isolates were established and maintained in *N. tabacum* cv. Samsun. For isolate BM 32123, the infection in *N. tabacum* cv. Samsun produced systemic severe mosaic symptoms and mottling visible 7 days post-inoculation and infection is confirmed by ELISA and RT-PCR. The virus does not kill its host plant (Figure 3.2).

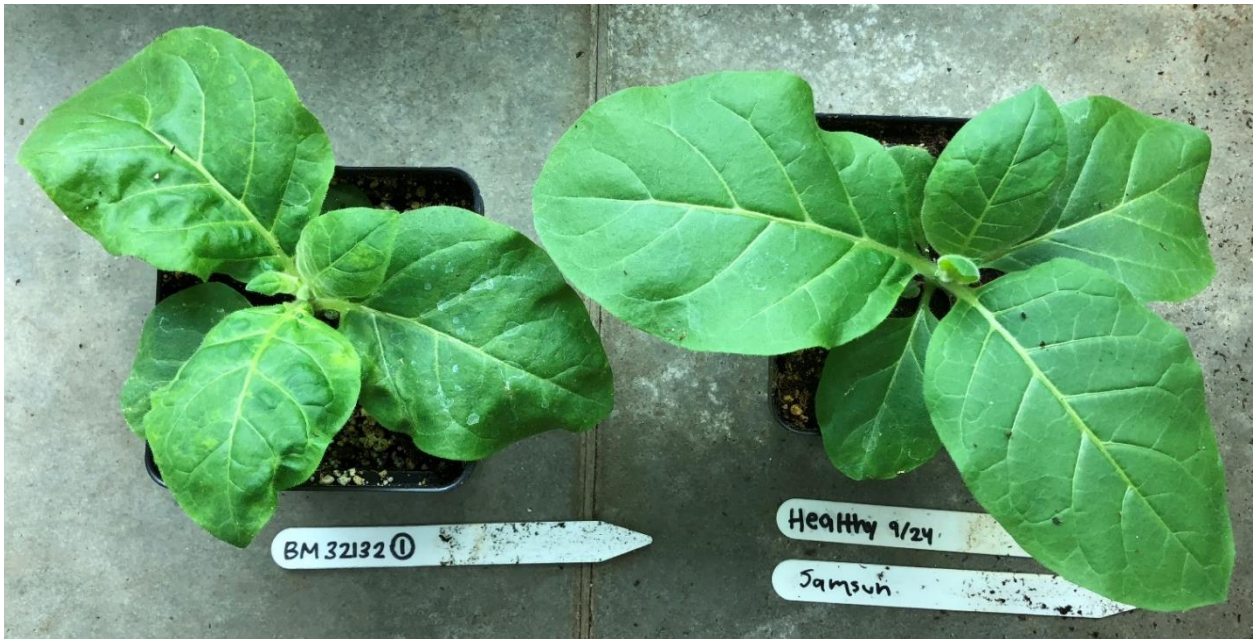

Figure 3.2. *Nicotiana tabaccum* cv. Samsun leaf, 1-week post-inoculation. Note systemic mottling and mosaic symptoms. Presence of TRV isolate BM 32123 in *N. tabaccum cv. Samsun* was confirmed by RT-PCR tests.

For isolate TRV_ID. Tuber halves were grown out and a single TRV-infected stem was detected. Under greenhouse conditions, single stem exhibited severely stunted growth (Figure 3.3) and TRV presence in young potato foliage was confirmed by both ELISA and RT-PCR. When isolate TRV_ID was transferred onto *N. tabacum* cv. Samsun, the initial systemic infection in *N. tabacum* cv. Samsun produced chlorotic/necrotic ringspots on non-inoculated leaves visible 2 weeks post-inoculation (Figure 3.4). However, when tested by TAS-ELISA and RT-PCR, TRV_ID isolate tested positive by RT-PCR (targeting RNA-1), but inconclusive or negative by ELISA. Subsequent re-inoculations from tobacco to tobacco produced similar test results (RT-PCR positive for RNA-1 and RNA-2; and inconclusive or negative ELISA signal).

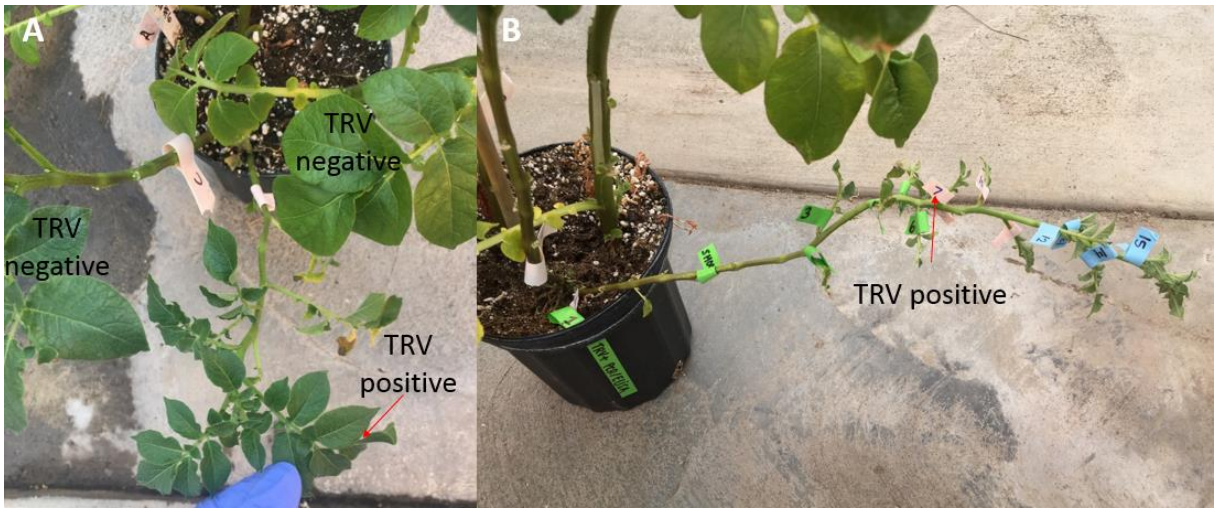

Figure 3.3. Potato plants grown from TRV-positive tuber from Idaho, with multiple shoots. Leaf tissue from all shoots was tested for TRV individually. Note that virus apparently did not distribute evenly in the mother tuber, hence not all emerging shoots were positive. TRV infected shoot exhibit severe stunting. (A) Single TRV-infected shoot 7 weeks post planting (B) Same shoot 10 weeks post planting.

Figure 3.4. Individual Nicotiana tabacum cv. Samsun leaf, 2 weeks post-inoculation. Note chlorotic/necrotic ringspots on non-inoculated leaf. Presence of TRV isolate TRV_ID in *N. tabacum cv. Samsun* was confirmed by RT-PCR tests.

Virus purification and immunodetection

The modified procedure described by Bergh et al. (1985) resulted in a high yield reaching 1.5 mg of the BM 32123 virus from 60 g of tobacco leaf tissue, and produced a very clean virus preparation. A single major protein band of approximately 23 kDa was visualized after Commassie staining in SDS-PAGE (Figure 3.5A) and Western blotting (Figure 3.5B). We concluded that this is the capsid protein (CP) of TRV.

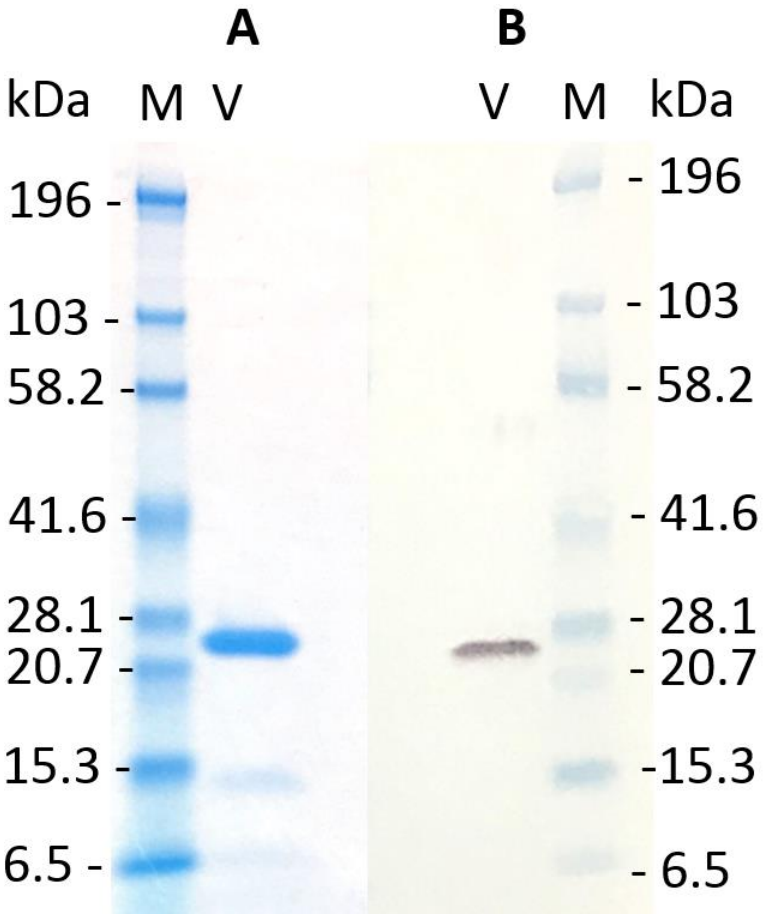

Figure 3.5. (A) Image of a Coomassie stained SDS PAGE gel with TRV isolate BM 32123 virus preparation. Preparation produced 1 major band around ~23 kDa, presumably coat protein (CP). (B) Image of western blot with TRV isolate BM 32123 virus preparation. Antisera raised against purified virus in guinea pig GP25 was used as detecting antibody at 1:30,000 dilution. Preparations produced 1 major band around ~23 kDa, presumably CP.

Development of the TAS-ELISA tests for foliar and tuber samples and comparison to commercial kit

BM 32123 purified virus preparation was used as an antigen for the production of antisera in rabbit R38 which was used as coating at 1:10,000 dilution, followed by guinea pig GP25 antiserum for detection at 1:30,000 dilution. Indeed, this combination was able to detect TRV when tested against various dilutions of TRV-infected and healthy *N. tabacum* cv. Samsun leaves (Figure 3.6). This TAS-

ELISA format was compared to a commercial ELISA kit from Neogen (Ayre, Scotland) where mouse antisera was used as coating at 1:1,000 dilution followed by conjugated detecting antiserum applied at 1:1,000 dilution (Table 3.2), using various TRV-infected tissue including tuber tissue and *N. tabacum* cv. Samsun foliage and tuber tissue. Results demonstrated the rabbit R38 antisera produced greater A_{405} ratios (infected/healthy) in *N. tabacum* cv. Samsun leaf tissue compared to commercial TRV ELISA kit from Neogen, whereas in tuber tissue, the rabbit R38 antisera produced A_{405} ratios (infected/healthy) nearly equal ratios as Neogen commercial kit. The A_{405} ratio (infected/healthy) was calculated and graphed for each ELISA system and type of tissue tested. Signals and ratios graphed are data obtained after substrate has been added for 5 hrs.

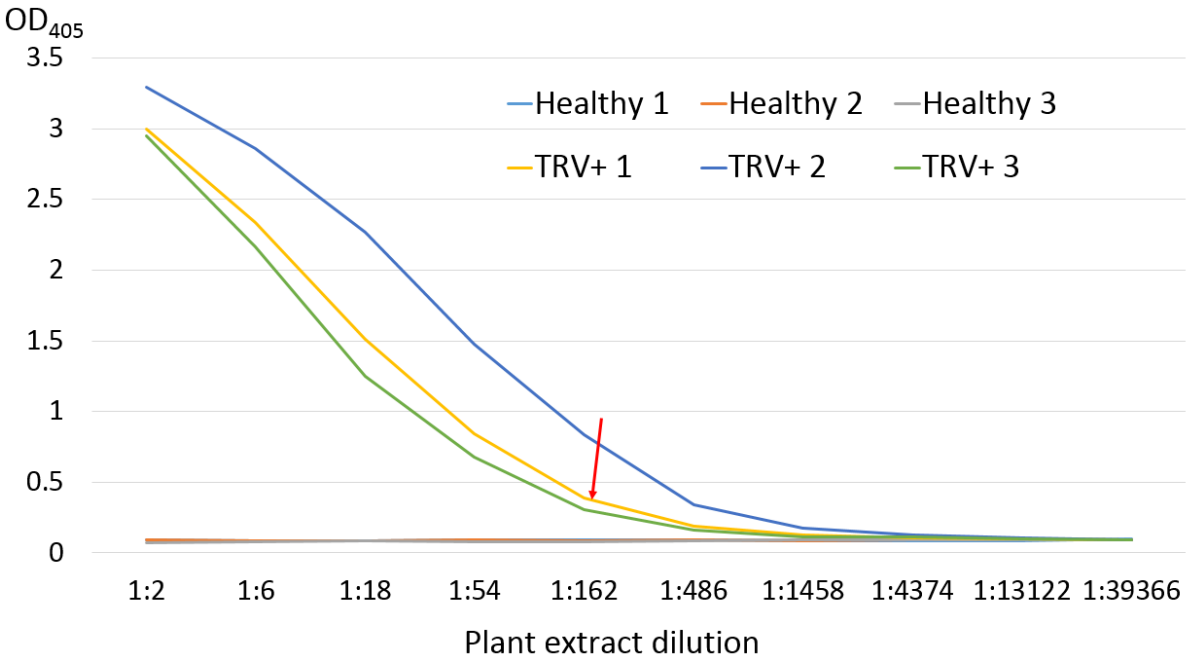

Figure 3.6. Titration curves for TRV in *N. tabacum* cv. Samsun leaves. Detection by triple-antibody sandwich enzyme-linked immunosorbent assay (ELISA); virus antigen was captured by the rabbit R38 polyclonal antibody (PAb) at 1:10,000 dilution. The guinea pig GP25 PAb was used as detecting antibody at 1:30,000 dilution. The plant extract was titrated along the X-axis: leaves of three individual infected were subjected to extraction in a sodium carbonate buffer at 1:10 (wt/vol) ratio; these extracts were diluted prior to loading onto the ELISA plate as indicated along the x-axis. OD_{405} = optical density at 405 nm. Red arrow indicated last dilution where TRV presence exceeded the healthy controls three-fold was determined.

For TRV testing in *N. tabacum* cv. Samsun, coating R38 detecting GP25 produced OD_{405} signals 3.3× greater than Neogen commercial kit (Figure 3.7A) and A_{405} ratio (infected/healthy) is 2.5× greater than Neogen commercial kit (Figure 3.7B).

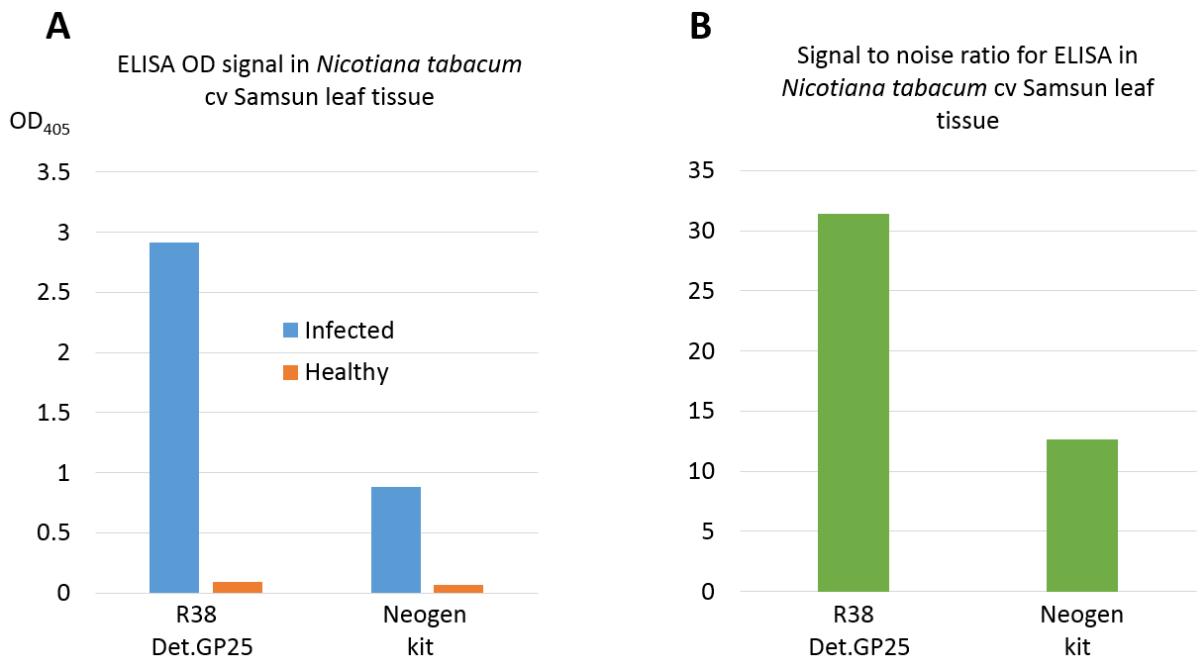

Figure 3.7. Comparing two ELISA systems for the detection of TRV in *N. tabacum* cv. Samsun using commercial kit from Neogen and homemade ELISA system: coating R38, detecting GP25 (A) Illustrates the infected and healthy OD_{405} signals. (B) Illustrates the infected to healthy ratio signal.

For TRV tuber testing, our home-made ELISA, coating R38 detecting GP25, produced OD_{405} signals 2.4× greater than Neogen commercial kit (Figure 3.8A) and A_{405} ratio (infected/healthy) is 1.5× greater than Neogen commercial kit (Figure 3.8B).

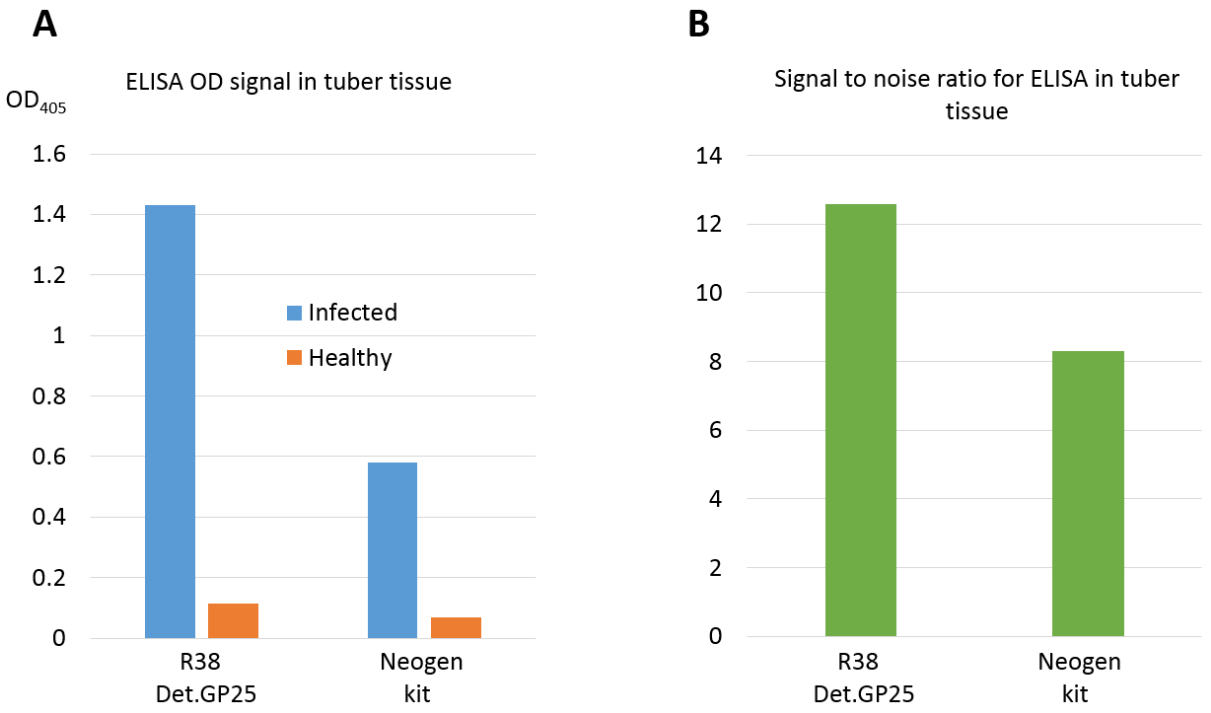

Figure 3.8. Comparing two ELISA systems for the detection of TRV in tuber tissue using commercial kit from Neogen and homemade ELISA system: coating R38, detecting GP25 (A) Illustrates the infected and healthy OD_{405} signals. (B) Illustrates the infected to healthy ratio signal.

Immunocapture-RT-PCR

Our test results demonstrated the IC-RT-PCR (coating R38 antibody at 1:10,000 dilution) methodology could detect TRV in infected tuber tissue with positive samples producing a 463-bp amplicon (Figure 3.9).

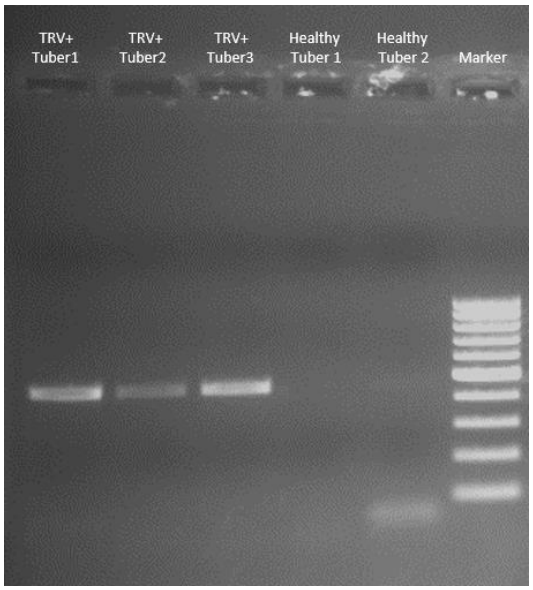

Figure 3.9. A gel image of immunocapture-RT-PCR (coating rabbit R38 1:10,000).

Sequencing and genome analysis

To characterize the genomes of TRV isolates studied in this work, a set of primers (Table 3.2) was designed to amplify the nearly whole genome of TRV (both RNA-1 and RNA-2) as overlapping RT-PCR fragments suitable for Sanger sequencing (Figure 3.10). For the isolate BM 32123, the sequence determined for the RNA-1 genome segment spanned 6,696 nt, (missing 13 nt at the 5' and 82 nt at the 3' termini), and for the RNA-2 segment 3,504 nt (missing approximately 32 nt at the 3'-end). RNA-1 had four open reading frames (ORFs) encoding, in the 5' to 3' direction, a 131-kDa protein, a 194-kDa protein, a 27-kDa protein, and a 15.4-kDa protein; the 194-kDa protein was apparently expressed as a read-through product containing the entire 131-kDa protein (Figure 3.10). RNA-2 of the isolate BM 32123 had three ORFs encoding, in the 5' to 3' direction, a 22-kDa protein (CP), a 35-kDa protein, and a 32-kDa protein (Figure 3.10). The BM 32123 sequences for RNA-1 and RNA-2 were compared to TRV sequences available in the GenBank database using BLASTn program, and were found 99.2% and 99.4% identical to the TRV isolates MI-1 (GQ903771) and BM (AY166663) collected in Michigan and Washington, respectively.

For the isolate TRV_ID, the sequence determined for the RNA-1 genome segment spanned 6,696 nt, (missing 34 nt at the 5' and 1 nt at the 3' termini), and for the RNA-2 segment 3,538 nt (complete RNA-2). RNA-1 had four ORFs encoding, in the 5' to 3' direction, a 131-kDa protein, a 194-kDa protein, a 27-kDa protein, and a 15.4-kDa protein; the expression of the 194-kDa protein occurred as a read-through product (Figure 3.10). RNA-2 of the isolate TRV_ID had three ORFs encoding, in the 5' to 3' direction, a 22-kDa protein (CP), a 35-kDa protein, and a 32-kDa protein (Figure 3.10). The TRV_ID sequences for RNA-1 and RNA-2 were compared to TRV sequences available in the GenBank database using BLASTn program, and were found 99.1% and 99.3% identical to the TRV isolates MI-1 (GQ903771) and Cot2 (AY166662) collected in Michigan and Colorado, respectively.

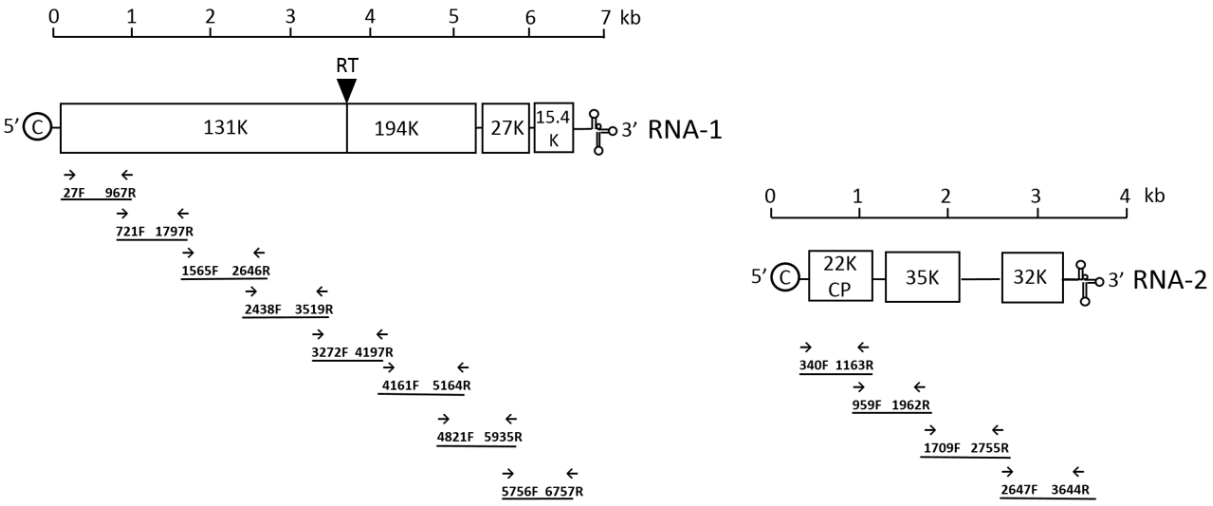

Figure 3.10. Image of sequencing coverage of TRV RNA-1 & RNA-2 and genome organization of TRV isolate BM 32123 and TRV_ID. Boxes indicate open reading frames in the two RNA species. Both RNA-1 and RNA-2 have a chemically modified ribonucleotide at the 5'-terminus, with so-called 'cap' structure and 3' termini have tRNA-like secondary structures.

Discussion

Movement of symptomatic and symptomless TRV infected seed tubers is a concern as a means of dissemination of the virus and introduction to previously unaffected sites (Xenophontos et al., 1998). However, TRV in many ways is an inefficient virus. TRV is inefficiently transmitted through tubers because often few shoots are infected on a potato plant (Cadman, 1959). Generally, only a small fraction of progeny tubers become infected, suggesting that the virus will rarely become established in fields as a result of planting infected seed tubers (Harrison et al., 1983). In addition, nematode transmissibility is dependent on the presence of RNA-2. Most potato plants with stem-mottle disease are predominately associated with NM types lacking RNA-2 and therefore lack the ability to transmit through the nematode vector. With the lack of viruliferous vectors, TRV is essentially self-eliminating. NM types is no threat as inoculum source and therefore should not be a concern. The mechanism in which M types switch to NM types is unknown but it is very common in potato, with past data reporting M types giving rise to up to 95% NM types and only 5% M types. This may explain the usual behavior we observe with the TRV_ID isolate, which is originally derived from a potato host. Over time and subsequent re-inoculations, RNA-2 appears to have dissipated in the plant host, hardly detectable by ELISA. TRV may exist solely as infectious RNA-1 (with no associated proteins) and, as a result, cannot be detected by ELISA. Detection of these NM isolates, which appear to be the most common type found in potato, has been overcome by the development of reverse-transcription-polymerase chain reaction (RT-PCR) tests for TRV detection (Mumford et al., 2018).

Effective and long term solutions to TRV infestations should include the use of systemic nematicides (such as aldicarb Timek 15G) and fumigants (such as 1,3-dichloropropene Telone II) to control incidence of virus, subsequently control spraing and on total numbers of trichodorid nematodes. D-D kills the trichodorid vectors, whereas aldicarb and oxamyl (oximecarbamates- inhibit the cholinesterase of nerve tissue) did not kill them but apparently affect their behavior (decrease nematode mobility, ability to locate host roots, or change feeding habits). None of the nematicides chemicals are phytotoxic (can be applied at planting) or decreased tuber yield, however the Fumigant (D-D) is phytotoxic- must be applied several weeks before planting, are costly to apply, and require special equipment (Alphey et al., 1975). Across 11 university and private research trials in the Pacific Northwest conducted from 1990-1994 have shown all treatments with Telone (trichloropropene + chloropicrin) soil fumigants not only protect potatoes against CRS but also increase tuber yields (Williams et al., 1996).

CHAPTER 4

CONCLUSIONS

As a general conclusion, we have developed efficient and sensitive methods to detect two soil-borne potato viruses, PMTV and TRV, in both foliar and tuber samples. The two Idaho isolates PMTV_ID and TRV_ID were found closely related to the known virus isolates found in other states, exhibiting low genetic diversity and suggesting a universal applicability of the developed detection methodologies to PMTV and TRV isolates across the U.S.

Comparisons of five ELISA systems for the detection of PMTV in *N. benthamiana,* tuber tissue, and sprout tissue were done using three commercial kits: SASA, Bioreba, and Neogen and two homemade ELISA systems: coating G1571, detecting R34, and coating SASA, detecting R34. All three commercial kits performed poorly when tested with the three different tissue types. None of the three commercial kits produced positive absorbance values exceeding the healthy controls three-fold. The rabbit R34 antiserum produced against the bacterially expressed MBP-PMTV-CP antigen was found to work well as a detecting antibody but did not work as a coating antibody in ELISA. Once PMTV was established in *N. benthamiana,* purified virus was obtained to produce the goat G1571 antiserum. The G1571 antibody was used coating in the home-made TAS-ELISA protocol where it was used at 1:10,000 dilution, in combination with the rabbit R34 antiserum used for detection at 1:10,000 dilution. This home-made TAS-ELISA protocol was 3.5X more sensitive when it came to *N. benthamiana* tissue, however, results were less impressive when it came to tuber and sprout tissue. G1571 as coating was found to have the same sensitivity as the three commercial kits. The other home-made ELISA where the mouse antiserum from the SASA kit was used for coating at 1:1,000 dilution but was followed by the rabbit R34 antiserum used for detection at 1:10,000 dilution was found to be more sensitive in all three tissue type testing. For PMTV testing in *N. benthamiana*, coating mouse SASA detecting R34 produced A_{405} ratio (infected/healthy) is 8.9× greater than the best commercial kit (SASA). In tuber tissue, 3.9× greater than the best commercial kit (SASA), and in sprout testing, 4.3× greater than the best commercial kit (SASA). The implementation of this TAS-ELISA will be useful for both breeding, virus-free certification programs, and research.

PMTV infected tubers can be detected by immunocapture-RT-PCR, however this methodology is shown to be less sensitive than ELISA. The starchy tissue in tubers may play a role in

interference with RT-PCR. Further investigation will be required to optimize this immunocapture-RT-PCR methodology for PMTV detection.

Comparison of two ELISA systems for the detection of TRV in *N. tabacum* cv. Samsun and tuber tissue using commercial kit from Neogen and our home-made ELISA system: coating R38, detecting GP25. While the Neogen commercial kit produced positive absorbance values exceeding the healthy controls three-fold in the two different tissue types testing, our home-made ELISA systems: coating R38, detecting GP25 was found to be superior in foliage tissue testing and more or less the same sensitivity for tuber testing as the commercial kit from Neogen. For TRV testing in *N. tabacum* cv. Samsun, coating R38 detecting GP25 produced A_{405} ratio (infected/healthy) is 2.5X greater than Neogen commercial kit. In TRV tuber testing, coating R38 detecting GP25 produced A_{405} ratio (infected/healthy) is 1.5X greater than Neogen commercial kit. TRV detection can be done by immunocapture-RT-PCR, but again, this methodology is shown to be less sensitive (and more expensive) than ELISA.

TRV detection is complicated though because RNA-1 can replicate and establish systemic infection in some hosts independent of RNA-2, which encodes for CP, resulting in a 'capsidless' virus infection and can present special problems for identification since isolates lacking RNA-2 are not detected by serological means such as ISEM and ELISA which is dependent on protein recognition of CP. Detection of these NM isolates, which appear to be the most common type found in potato, has been overcome by the development of reverse-transcription-polymerase chain reaction (RT-PCR) tests for TRV detection.

The use of high-quality seed is essential for the production of a profitable potato crop. The use of virus-free seed is the most effective strategy for managing PMTV and TRV. Planting clean seed is important because both viruses can survive in infected seed tubers and spread from one season to the next, as well as act as inoculum source to non-infected fields with viruliferous vectors. The impact of my thesis is the efficient and effective immunodectection methods that we have developed as a means a to analyze for TRV and PMTV will help the seed certification process by providing them with a more sensitive assay (4X more sensitivity) for the detection of PMTV than the current go-to standard SASA kit for large scale testing, allowing them to detect these diseased seeds more accurately before they escape seed certification.

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