THESIS

COMBATING POTATO MOP-TOP VIRUS: INFECTIOUS CLONE DEVELOPMENT AND SCREENING FOR RESISTANCE IN POTATO GERMPLASMS

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ABSTRACT

COMBATING POTATO MOP-TOP VIRUS: INFECTIOUS CLONE DEVELOPMENT AND SCREENING FOR RESISTANCE IN POTATO GERMPLASMS

Potato mop-top virus (PMTV) poses a significant threat to global potato production, resulting in yield losses and quality reductions. It causes spraing symptoms in tubers. PMTV is vectored by a soil-borne protist, *Spongospora subterranea* f. sp. *subterranean* (*Sss*). Currently, resistance to PMTV has not been identified in potato. To facilitate studies on PMTV biology and support the identification of resistant potato cultivars, an infectious clone of PMTV was constructed. This clone, generated by synthesizing full-length cDNA and producing in vitro RNA, enables the controlled inoculation of plants for resistance screening and pathogenesis studies. Infectivity and optimal doses of the PMTV infectious clone were tested using different quantities of PMTV RNAs on *Nicotiana benthamiana* and a susceptible potato cultivar, Yukon Gold. In both hosts, plants inoculated with 5 µg or higher of the infectious clone developed characteristic PMTV symptoms, including leaf yellowing, stunting and reduced root development. Control plants and those treated with a lower concentration (2 µg) remained asymptomatic. Molecular analyses using polymerase chain reaction (PCR) targeting the RNA-dependent RNA polymerase (RdRp), coat protein (CP), and Triple Gene Block (TGB) genes, as well as enzyme-linked immunosorbent assay (ELISA) targeting the CP protein, confirmed systemic PMTV infection. Following successful validation, the infectious clone was used to screen a diverse collection of wild-type potato germplasms from the *Solanum* Petota panel for

potential resistance to PMTV using quantitative PCR (qPCR). Eight species, including 14 potato lines, were identified as potentially resistant or tolerant to PMTV. This research provides a valuable tool for PMTV pathogenicity studies and resistance screening, thereby contributing to the development of effective PMTV management strategies.

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INTRODUCTION

Potato mop-top virus (PMTV)

Potato (*Solanum tuberosum*) holds the distinction of being the third most vital food crop globally. Potatoes are susceptible to various pathogens, resulting in significant reductions in quality and yield (Adolf et al., 2020). Among the issues in potato production, PMTV (genus *Pomovirus*, family *Virgaviridae*), is an emerging and destructive viral disease that threatens the potato industry (Adolf et al., 2020; Balendres et al., 2016; Falloon, 2008; Falloon et al., 2016; Gau et al., 2013). PMTV infection in potatoes causes necrotic arcs or flecks in tuber flesh, known as spraing symptoms (Sandgren et al., 2001).

PMTV is a positive-sense, single-stranded RNA virus with a tripartite genome. RNA1 encodes RNA-dependent RNA polymerase (RdRp), which is essential for viral replication (Savenkov et al., 1999). RNA2 contains genes for the coat protein (CP) and the CP-read through (CP-RT), which are crucial for vector acquisition and transmission, as deletions within CP-RT have been shown to abolish vector-mediated transmission (Reavy et al., 1998; Scott et al., 1994). RNA3 encodes the Triple Gene Block (TGB) protein for long-distance movement within a host plant (Kashiwazaki et al., 1995). The three RNAs of PMTV are highly conserved in isolates from around the world (Kalyandurg et al., 2017). There are generally two major strains of the virus recognized and coined as S (for "severe") and M (for "mild"). S strain tends to cause brighter yellow mosaic symptoms in the foliage. In contrast, the M strain could be symptomless, meaning the virus can go visually undetected, proving to be problematic regarding management strategies. In addition to the S and M strains, several other strains of PMTV are found in the Andean region

of Peru, where the virus is thought to originate (Kalyandurg et al., 2017). However, only the S strain has been reported from all the potato-growing regions of the world.

Powdery scab disease-PMTV disease complex

PMTV is transmitted by the soil-borne protist *Spongospora subterranea* f. sp. *subterranea* (*Sss*), which also causes powdery scab on potato tubers and root galls (Arif et al., 1995). The powdery scab disease is caused by the soil-borne obligate parasitic protist, *Sss,* in the order *Plasmodiophorida*. Powdery scab is particularly favored by cool, damp conditions and is an intractable disease (Balendres et al., 2016). The pathogen can survive in the soil for many years, and very low inoculum levels can cause relatively severe disease outbreaks, making control elusive. *Sss* can infect all underground organs of potatoes, stolons, tubers, and roots, where the pathogen stimulates the enlargement and division of host cells, leading to symptom development, mainly the formation of root galls. Depending on environmental conditions, initial tuber symptoms, which take 4–8 weeks to develop, are purplish-brown lesions (1–2 mm diameter) that subsequently enlarge into raised mature lesions that burst, exposing large masses of sporosori resulting in characteristic symptoms of the disease. There is limited genetic variation among *Sss* strains from around the world (Gau et al., 2013), with three clusters, two occurring in South America and the third group comprising samples from elsewhere. South American populations are generally more diverse than in other regions (Kalyandurg et al., 2017). This combined risk of both powdery scab and virus infection makes potato mop-top disease particularly challenging to manage. Estimates of loss caused by both pathogens are not available for the U.S. However, other countries, such as Australia, estimate an average of 4-5% annual loss of the gross production value (Wilson, 2016). In the Scottish seed industry, outbreaks of PMTV

have been reported to have caused up to 67% loss due to reduced yields and poor tuber quality (Davey et al., 2014).

Sss -PMTV interactions

The *Sss*-PMTV interaction is a complicated system to manipulate, and there has been little progress in understanding the molecular mechanisms of virus transmission (Adolf et al., 2020). PMTV is acquired by *Sss* during infestation of infected potato roots. When PMTV-infected primary zoospores, released from sporosori, come into contact with potato roots, this results in the formation of multinucleate plasmodium. Once PMTV infects potatoes, it replicates in the plant cells and disseminates into developing tubers. The multinucleate plasmodium can further develop into resting spores and secondary zoospores (Arif et al., 1995; Rochon et al., 2004). This continuous cycle of infection results in increased inoculum of both PMTV and *Sss*.

The virus is hypothesized to reside in zoospores (Arif et al., 2014; Rochon et al., 2004). In a previous study, freeze-fracture followed by transmission electron microscopy was used to determine whether PMTV can be detected in sporosori (Merz, 1995). It revealed the presence of rod-shaped particles, plausibly the PMTV located in cysts in a single sporosorus. The cyst-like structure encompassed the entire cell with no nucleus or membrane-bound compartments detected. Since virus replication requires living cells, the single sporosorus found may have been at the final stages of viral infection just before cell death or maybe an entirely undetermined structure. Since no tests were carried out to determine if the rod-shaped structures were indeed PMTV, the location of the virus within the vector is still unknown. Advances in microscopy coupled with availability of antibodies for PMTV CP will allow for a better characterization of virus presence within the vector. Yet to be addressed are virus location in the vector and its mechanism of transmission. Elucidation of these mechanisms will provide ideas for engineering

resistance or searching for mutated forms of host factors that could confer resistance to PMTV. Given that the importance of PMTV is rising in the U.S., a comprehensive understanding of the virus-vector system is vital to potatoes' future.

PMTV resistance has not been identified in potato

Currently, resistance to PMTV has not been identified in potato plants. Several studies in Europe and Asia have evaluated potato cultivars for PMTV resistance, but to date, no cultivars with resistance to PMTV have been identified (Kurppa, 1989; Sandgren et al., 2002; Tenorio et al., 2006). One challenge for screening for PMTV resistance is that virus incidence can vary widely from year to year in the same field because of the strong influence of the environment on disease incidence (Domfeh et al., 2015; Latvala-Kilby et al., 2009; Sandgren et al., 2002; Yellareddygari et al., 2018). Further complicating the issue is that PMTV can cause symptomless tuber infections, so tubers lacking necrotic arcs or spraing in the tuber can still harbor and proliferate the virus (Latvala-Kilby et al., 2009; Savenkov et al., 2003). Another drawback is that the current molecular assays only detect the PMTV coat protein. Field studies of PMTV are often hampered by the unpredictable abundance and spatial distribution of its vector, *Sss*, which varies yearly due to changes in soil type, weather, and cultivation practices. Moreover, *Sss* is notoriously difficult to culture *in vitro*, making controlled laboratory studies extremely challenging. In contrast, the infectious clone system provides a stable, reproducible platform for virus inoculation, independent of the natural vector. This system is ideal for dissecting virus biology, conducting mutational analyses, and screening germplasms for resistance.

Previously, a PMTV infectious clone was developed to study viral gene functions in tobacco, *Nicotiana benthamiana* (Savenkov et al., 2003). The study revealed that the absence of RNA 2 had a minimal impact on the accumulation or systemic movement of RNA 1 and RNA 3,

suggesting that the CP-encoding RNA is not essential for virus movement within the *N. benthamiana*. However, plants inoculated with only RNA 1 and RNA 3 did not develop the characteristic yellow mosaic symptoms typically seen when all three RNAs are present, suggesting that RNA 2 is necessary for symptom induction. Another study also reported differences in the pathogenesis between PMTV isolates in *N. benthamiana* and *N. tabacum* using infectious clones, further confirming the critical role of CP-RT in viral accumulation and symptom severity (Kalyandurg et al., 2017). Although the PMTV infectious clone has been successfully developed for tobacco plants, the absence of an infectious clone for potatoes has hindered the discovery of resistant potato cultivars.

In this study, I developed and functionally validated an infectious clone of PMTV in both a model host, tobacco (*N. benthamiana*), and a susceptible potato cultivar (Yukon Gold). Additionally, a diverse set of wild potato species and landraces from the Solanum section Petota (Hardigan et al., 2015) were screened for PMTV resistance. This work provides a powerful, foundational tool for future studies of PMTV-host interactions, as well as for identifying resistance and understanding its mechanisms.

Goals and objectives

The overarching goal of this research is to identify potato varieties that are resistant to PMTV in order to support sustainable disease management. The study has two main objectives: 1) to develop a PMTV infectious clone for potato; and 2) to screen diverse potato germplasms for PMTV resistance.

METHODS

PMTV infectious clone preparation

Full-length genomic cDNA clones of the PMTV isolate were synthesized by Twist Bioscience company and cloned into the pGWB402 plasmid (Supplementary Figure 1). The plasmids, each containing PMTV RNA1, RNA2, and RNA3 (encoding RdRp, CP, and TGB, respectively), were used as templates for high-fidelity polymerase chain reaction (PCR) using KAPA HiFi polymerase (Roche). Each 50 µL PCR reaction contained 1X KAPA buffer, 1.5 mM dNTPs, 1.5 µL of the corresponding forward primer (T7-RdRp, T7-CP, or T7-TGB), 1.5 µL reverse primer, 1 µL polymerase, and 1 µL plasmid DNA. PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 34 cycles of 98°C for 30 s, 65°C for 20 s, and 72°C for 2–4 min (2 min for CP and TGB, 4 min for RdRp), with a final extension at 72°C for 4 min. PCR products were resolved via agarose gel electrophoresis and purified using the GeneJet Gel Extraction Kit (ThermoFisher) according to the manufacturer's protocol. Capped RNA transcripts were synthesized using the RiboMAX™ Large Scale RNA Production System (Promega) with T7 polymerase. Each 20 µL reaction contained 4 µL T7 transcription buffer, 1.5 µL each of ATP, CTP, and UTP, 0.2 µL GTP, 1.5 µL cap analog, 2 µL enzyme mix, and 2000 ng of DNA template. Transcription was conducted at 37°C for 3 hours with the lid heated to 42°C. DNA template digestion was performed using 1 µL RQ1 DNase for 30 min at 37°C. RNA was purified by isopropanol and sodium acetate precipitation, followed by ethanol washes and resuspension in nuclease-free water. RNA concentration and integrity were determined using Nanodrop spectrophotometry and gel electrophoresis (Supplementary Figure 2), respectively.

Inoculation of tobacco and potato using the PMTV infectious clone

Equal volumes of RNA1, RNA2, and RNA3 were mixed with autoclaved 3X GKP buffer (50 mM glycine, 30 mM K_2HPO_4, 3% Celite, 3% bentonite), and rub-inoculated onto host plants. For each tobacco plant, two leaves were inoculated. To determine the infectivity and optimal doses of PMTV infectious clone for infection, different RNA mixtures (final RNA doses: 2 µg, 5 µg, 10 µg, 20 µg, and 30 µg) were prepared and mechanically inoculated onto four-week-old (four-five leaf stage) tobacco plants (*Nicotiana benthamiana*). Negative control plants were inoculated with buffer only. The plants were kept in a growth chamber at temperatures below 18°C for 4 to 6 weeks. This experiment was repeated independently five times to confirm reproducibility. Additionally, to further confirm that individual PMTV RNA segments are not independently infectious, tobacco plants were inoculated as described above using 10 µg of RNA. Treatments included a buffer-only control (C), individual RNA segments (RNA1, RNA2, or RNA3), and a positive control containing all three segments (RNA1+2+3). This experiment was independently repeated five times.

To confirm the infectivity of the PMTV infectious clone in potato, Yukon Gold potato plants (*Solanum tuberosum*) were inoculated as described above using three different RNA doses (5 µg, 10 µg, and 20 µg). For each potato plant, three leaves were inoculated. Plants inoculated with buffer only were used as negative controls. Inoculated plants were kept in a growth chamber for four to six weeks under the same environmental conditions as described above. This experiment was repeated independently five times.

Detection of PMTV RNAs in inoculated plants

After five weeks of inoculation with PMTV infectious clone, newly growth leaves were collected from symptomatic and asymptomatic plants for RNA extraction. The samples were

placed in 1.5 mL tubes, frozen in liquid nitrogen, and ground with a pestle. RNA was extracted using Quick-RNA Plant Miniprep Kit (Zymo, Irvine, CA), following the manufacturer's protocol. cDNA was synthesized with 1 µg of total RNA using the Verso cDNA Synthesis kit (ThermoFisher) and diluted 10-fold. The presence of all three PMTV RNA genome fragments was confirmed by PCR using gene-specific primers targeting the RdRp, CP, and TGB genes. Primers used in the analysis are listed in Table 1. PCR conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 39 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR products were resolved by gel electrophoresis.

To further confirm the presence of viral proteins after inoculation, newly developed leaf tissues (thumbnail size) from PCR-confirmed PMTV-infected tobacco and potato plants were tested using the enzyme-linked immunosorbent assay (ELISA). The ELISA test was performed using the PMTV Complete Kit 960 (BIOREBA, Reinach, Switzerland), following the manufacturer's instructions. Three tobacco plants were tested for each dose of infectious clone (2 µg, 5 µg, 10 µg, 20 µg, and 30 µg), and four potato plants were tested for each of the 5 µg, 10 µg, and 20 µg dose treatments. Each test included one positive and one negative control provided by the kit, along with four additional healthy leaf controls. Samples were considered positive if their absorbance values exceeded the threshold, defined as the mean absorbance of four healthy leaf controls plus three times their standard deviation.

Screening of wild-type potato germplasms for potential PMTV resistance or tolerance

The potato germplasms being screened in this study include a diverse set of wild species and landraces from the Solanum section Petota. The geographic sources of these germplasms are Peru, Bolivia, and Argentina (all from South America), which are also considered to be the center

of origin of PMTV, and *Sss* (Hardigan et al., 2015), making them promising candidates for identifying resistant cultivars. These germplasms were obtained from the National Plant Germplasm System (NPGS). A total of 27 lines, representing 12 tuber-bearing *Solanum* species, were selected for the preliminary screening, with each line having one control and three PMTV-inoculated plants.

All screening experiments were conducted using 5 µg of the PMTV infectious clone. After five weeks of inoculation with the PMTV infectious clone, newly developed leaves were collected and used for RNA extraction and cDNA synthesis as described previously. Quantitative PCR (qPCR) was conducted using specific primers designed for the PMTV CP gene (Table 1) (Zhou et al. 2019). qPCR was performed using iTaq Universal SYBR® Green Supermix (Bio-Rad). The cycling conditions were as follows: initial denaturation at 95°C for 30 s, followed by 39 cycles of denaturation at 95 °C for 10 s, 55 °C for 30 s, with an extension at 72 °C for 30 s. A final step of the melt curve was included to verify the specificity of the amplification.

Data analysis

Statistical differences in plant height, root length, root dry weight, and tuber weight were analyzed using one-way ANOVA followed by Fisher's LSD test for pairwise comparisons against negative controls ($p<0.05$). Normality of data was confirmed using Shapiro-Wilk test ($p>0.05$). To test the infectious clone dose responses in plant height, root length, root dry weight, and tuber weight, multiple comparison tests were performed on doses equal or higher than 5 µg using Kruskal-Wallis test followed by Dunn's test ($p<0.05$) or one-way ANOVA followed by Tukey's test ($p<0.05$) according to normality of datasets. Statistical differences in virus copy number in potato lines were analyzed using Kruskal-Wallis test followed by uncorrected Dunn's

test for pairwise comparisons against the susceptible Yukon Gold controls ($p<0.05$). All analyses were conducted in GraphPad Prism v10.2.3.

RESULTS

Symptomology of tobacco plants after inoculation by the PMTV infectious clone

Tobacco plants were inoculated with five different doses (2 µg, 5 µg, 10 µg, 20 µg, and 30 µg) of a PMTV infectious clone (mixture of RNA1, 2, and 3). All plants inoculated with varying doses of the infectious clone, except the 2 µg, developed apparent symptoms, including leaf yellowing and dwarfing and plant stunting (Figure 1A). Root sizes of these symptomatic plants were also smaller than controls (Figure 1B). Increased doses of the infectious clone from 5 µg to 30 µg did not result in more severe above- or below-ground plant symptoms. In contrast, control plants and plants treated with 2 µg of infectious clone showed no visible symptoms. Plant heights and root lengths were significantly reduced in plants treated with 5 µg or more of infectious clone compared to the control group ($p<0.05$ for plant height and $p=0.013$ for root lengths) (Figure 2A-B). Increasing the dose of the infectious clone from 5 µg to 30 µg did not further reduce plant height ($p= 0.598$) and root length ($p= 0.560$).

Molecular confirmation of PMTV infection in tobacco plants

To verify the successful infection of tobacco plants by the PMTV infectious clone, molecular analyses were performed using both PCR and ELISA. PCR detection with primers targeting the RdRp, CP, and TGB genes confirmed the presence of all three PMTV genome segments in plants that exhibited symptoms (Figure 3). Symptomatic plants inoculated with more than 5 µg of infectious clone consistently tested positive for all three viral genes. In contrast, no amplicons were detected in plants inoculated with 2 µg of infectious clone or in control plants (Figure 3). A plant housekeeping gene, 18S, was included in all tests to confirm the quality of samples. All plants inoculated with more than 5 µg of infectious clone were confirmed to be

positive, except one plant from 30 µg dose (symptomless). Overall, the infection rates for 2 µg, 5 µg, 10 µg, 20 µg, and 30 µg doses were 0%, 100%, 100%, 100%, and 80%, respectively.

To further confirm viral protein expression, ELISA was performed on new growth of symptomatic and asymptomatic plants targeting PMTV CP. Tobacco plants inoculated with 10 µg or higher doses of infectious clone showed positives, with 30 µg of infectious clone showing the higher positive rate (67%) than 10 µg (33%) and 20 µg (33%) (Table 2). Plants inoculated with lower doses, 2 µg and 5 µg, did not show positives.

Lack of infectivity of individual PMTV RNA1, 2, and 3 segments in tobacco plants

Tobacco plants inoculated with individual PMTV RNA segments (RNA1, RNA2, or RNA3) did not develop visible symptoms. In contrast, plants inoculated with the complete set of RNA segments (RNA1+2+3) exhibited clear and consistent symptoms, including leaf yellowing, dwarfing, and stunting (Figure 4A). Plant height and root development in plants treated with individual RNA segments was comparable to that of the negative controls, whereas they were significantly reduced in symptomatic plants inoculated with RNA1+2+3 (Figure 4; Figure 5A-B).

PCR analysis confirmed the systemic infection of the plants inoculated with RNA1+2+3 for all three virus genes (Figure 6). In contrast, no amplification was detected in plants inoculated with individual RNA segments and in control plants.

Infectivity of PMTV infectious clone in potato plants

Yukon Gold potato plants were inoculated with three different doses (5 µg, 10 µg, and 20 µg) of a PMTV infectious clone (mixture of RNA1, 2, and 3). Plants inoculated with 5 µg or more of RNA developed obvious symptoms, including leaf yellowing and curling and plant stunting. In some plants, the lower leaves abscised due to severe necrosis, while the upper leaves

clustered together but remained small and curled. (Figure 7A). Root sizes of these symptomatic plants were also smaller than control plants (Figure 7B). Overall, increased doses of the infectious clone from 5 µg to 20 µg did not result in more severe above- or under-ground plant symptoms. In contrast, control plants showed no visible symptoms.

Quantitative analysis of plant growth showed that plant heights were overall reduced in all three doses compared to the control, but only significantly different in the 20 µg treatment (p= 0.006). Root lengths were also significantly reduced in all dose treatments (p= 0.036 for 5 µg and p =0.025 for 20 µg), except the 10 µg treatment. For dry root weights, all three dose treatments showed significant reductions compared to the control (p<0.05). Tuber weights were reduced across the treatments, with a significant reduction found in the 5 µg treatment (p=0.029) (Figure 8A-D). Overall, increasing the dose of the infectious clone from 5 µg to 20 µg did not further reduce plant height (p=0.310), root length (p=0.921), dry root weight (p=0.665), and tube weight (p=0.242).

Molecular confirmation of PMTV infection in potato plants

To verify the successful infection of potato plants by the PMTV infectious clone, molecular analyses were performed using both PCR and ELISA. PCR detection with primers targeting the RdRp, CP, and TGB genes confirmed the presence of all three PMTV genome segments in plants that exhibited symptoms (Figure 9). Symptomatic plants inoculated with more than 5 µg of infectious clone consistently tested positive for all three viral genes. In contrast, no amplicons were detected in control plants (Figure 9). A potato housekeeping gene, actin, was included in all tests to confirm the quality of samples. All plants inoculated with PMTV infectious clone were tested positive, except one plant from 10 µg dose (symptomless). Overall, the infection rates for 5 µg, 10 µg, and 20 µg were 100%, 80% and 100%, respectively.

ELISA test on new growth of symptomatic and asymptomatic potato plants confirmed the presence of PMTV CP protein. Potato plants inoculated with 5 µg or higher doses of infectious clone showed positives, with 5 µg, 10 µg and 20 µg showing the positive rates of 50%, 75%, and 75%, respectively (Table 2).

Screening wild potato germplasms for PMTV resistance

Among 27 wild potato lines screened, there were 14 line exhibited significant reduction in PMTV accumulation compared to the susceptible control Yukon Gold potatoes ($p<0.05$) (Figure 10). These 14 lines represent eight *Solanum* species, including *Solanum berthaultii*, *Solanum boliviense*, *Solanum brevicaule, Solanum candolleanum, Solanum microdontum, Solanum raphanifolium, Solanum okadae,* and *Solanum tuberosum* subsp.*andigenum*. The lowest PMTV copy numbers were observed in lines, PI500053, PI458365, and PI265579, where in average, the $\log_2$-transformed virus copy numbers were reduced in 10.1, 7.8, and 5.2-fold compared to Yukon Gold potato controls, respectively. Additionally, all four lines from the *S. brevicaule* species were found to have significantly lower virus copy numbers than controls. The species, accession numbers, and plant identifiers for all screened lines are provided in Table 3.

DISCUSSION

The development and successful application of the PMTV infectious clone in this study represents an important advancement in PMTV research. It offers a reliable, vector-free platform for studying viral infection, replication, and host responses under controlled conditions. One of the major challenges in field-based PMTV studies is the unpredictable nature of its natural vector, *Sss*, which complicates consistent infection and resistance screening. By bypassing the need for vector transmission, the infectious clone system overcomes this limitation, enabling reproducible inoculation and facilitating systematic evaluation of plant responses. This is particularly valuable for screening potato germplasm and wild *Solanum* species for resistance, which is essential for breeding PMTV-resistant cultivars.

My study successfully developed a PMTV infectious clone to infect both tobacco and potato plants. Plants inoculated with PMTV infectious clone showed leaf yellowing, dwarfing, and stunting symptoms, which are consistent with symptoms observed in other studies (Calvert & Harrison, 1966; Harrison & Jones, 1970; Tenorio et al., 2006). Root development of these symptomatic plants was also hindered with reduced root length and weight compared to control plants. These symptoms were observed in both tobacco and potato at doses of 5 μg or higher.

PMTV infection caused overlapping symptoms in both tobacco and potato, but the symptom expression in potato displayed greater variability. Two distinct phenotypes were observed: one in which plants were severely stunted with yellow foliar blotching, and another showing a "mop-top" appearance with lower leaf drop and clusters of small, narrow leaves at the upper canopy. In contrast, tobacco plants showed a more uniform symptomatic profile, with consistent dwarfing and leaf chlorosis.

Infection of new growth by PMTV was molecularly confirmed using both PCR and ELISA through the detection of all three viral RNA segments and the CP protein, respectively. Although ELISA assays targeting the PMTV CP protein provided additional confirmation of infection, they exhibited lower sensitivity compared to PCR. In both tobacco and potato, some symptomatic plants tested negative by ELISA but were PCR-positive. This discrepancy is likely due to the lower sensitivity of ELISA, particularly when viral titers are low (Boonham et al., 2014). Interestingly, a higher percentage of ELISA-positive samples was observed in potato than in tobacco, suggesting that virus accumulation is greater in potato – potentially due to host-specific factors that promote viral replication and intercellular movement (Shemyakina et al., 2011). Together, these findings confirm that the developed PMTV infectious clone can effectively reproduce natural PMTV infection. While previous studies have demonstrated infection of tobacco by a PMTV infectious clone (Kalyandurg et al., 2017; Savenkov et al., 2003), this study is the first to demonstrate the successful infection of potato plants.

While several studies reported successful infection of tobacco using a PMTV infectious clone (Kalyandurg et al., 2017; Savenkov et al., 2003), the exact amount required to infect a host plant and associated infection efficiency is lacking. In this study, a clear dose-dependent threshold was observed, with consistent symptoms and detectable viral RNA only appearing when plants were inoculated with 5 µg or more of the PMTV infectious clone. Notably, tobacco plants inoculated with 2 µg dose did not show any symptoms of infection. Viral RNA was also not detected by PCR, indicating that 2 µg may be below the minimum threshold required for systemic infection. Furthermore, increasing the dose from 5 µg to 30 µg did not intensify symptom severity, suggesting a potential threshold effect rather than a dose-dependent gradient.

Inoculation with individual PMTV segments, RNA1, RNA2, or RNA3 did not cause any visible symptoms, and viral RNA was not detected in new growth tissues. This indicates that no single segment alone can infect the plant or move from the inoculation site to the new growth of the plant. Previously, Savenkov, 2003 found that a mixture of PMTV RNA1 and RNA3 was infectious and both RNAs were detected systemically in tobacco plants in the absence of RNA2. However, the typical PMTV infection symptoms were not developed without RNA2 (Savenkov et al., 2003). These results confirm that all three RNAs are required to establish a systemic and symptomatic infection.

To date, no sources of resistance or tolerance to PMTV have been deliberately incorporated into breeding programs and a reliable method for screening resistance or tolerance remains unavailable (Barker & Dale, 2006). Despite these limitations, several studies have made efforts to identify potential resistant cultivars and reduced sensitivity under field conditions. For example, Sandgren, et al. (2002) evaluated five potato lines in field trials in Sweden and identified the NY99 line as having a low incidence of PMTV infected tubers, which also showed low levels of virus accumulation (Sandgren et al., 2002). Other studies also tested the sensitivity of various market-type potato cultivars, where they showed the varying susceptibilities among cultivars (Domfeh et al., 2015; Yellareddygari et al., 2018).

The PMTV infectious clone provides an effective approach for screening wild-type potato species and cultivated potato germplasm for resistance to PMTV. The potato germplasms screened includes diverse wild species and landraces from *Solanum* section *Petota*, originating from Peru, Bolivia and Argentina. These regions are also considered the centers of origin for PMTV and its vector *Sss* (Hardigan et al., 2015), making them promising candidates for resistance screening. Based on initial rough screening, 14 lines, representing eight species, were

identified as potential resistance against PMTV. Of these lines, PI500053, PI458365, and PI265579 showed the lowest viral copy numbers, with fold differences ranging from 5.2 to 10.1 compared to the susceptible Yukon Gold potatoes. Potato lines showing significantly lower virus copy numbers will be further screened in both greenhouse and field conditions in the future.

In summary, this study reports the successful development and application of a PMTV infectious clone that enables vector-free inoculation of both tobacco and potato plants. Inoculated plants developed characteristic PMTV symptoms. Infection was confirmed in newly emerged leaves by PCR and ELISA, with 5 µg identified as the minimum effective dose. All three viral RNA segments were required for systemic and symptomatic infection. Importantly, this is the first study to demonstrate that an infectious clone can systemically infect potato, a key advancement for resistance screening. Screening of wild *Solanum* lines identified several potential resistant lines, showing significantly lower virus titers. These findings provide a strong foundation for identifying PMTV-resistant germplasm and support future breeding and genetic studies to develop sustainable resistance in potato.

FIGURES AND TABLES

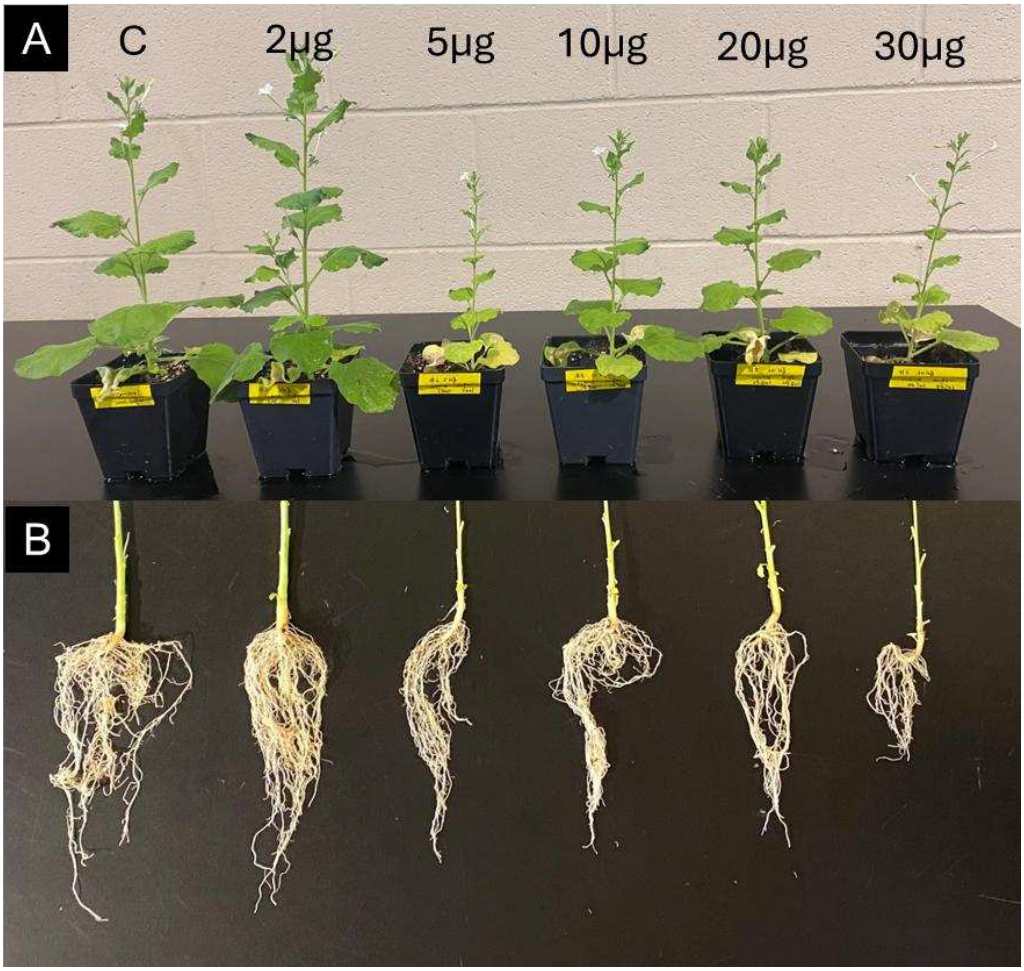

Figure 1. Tobacco plant and root symptoms after inoculation with different doses of PMTV infectious clone. Plant (A) and root (B) symptoms shown are after five weeks of inoculation. Plants were inoculated at the four-five leaf stage (about four weeks old) by rub-inoculating two leaves per plant. Treatments include buffer-only control and five different doses of infectious clone (2 µg, 5 µg, 10 µg, 20 µg, and 30 µg).

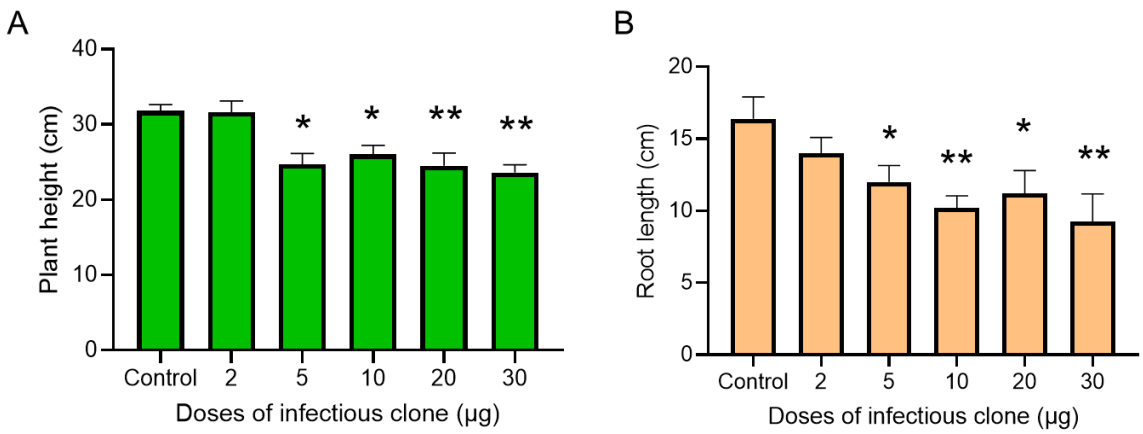

Figure 2. Tobacco plant heights and root lengths after inoculation with different doses of PMTV infectious clone. Plant heights (A) and root lengths (B) were measured after five weeks of inoculation. Treatments include buffer-only control and five different doses of infectious clone (2 µg, 5 µg, 10 µg, 20 µg, and 30 µg). Statistical analysis was performed using one-way ANOVA followed by Fisher's LSD test for pairwise comparisons against the negative control. Asterisks indicate significant difference between PMTV-inoculated treatment and control (*p<0.05, **p<0.01).

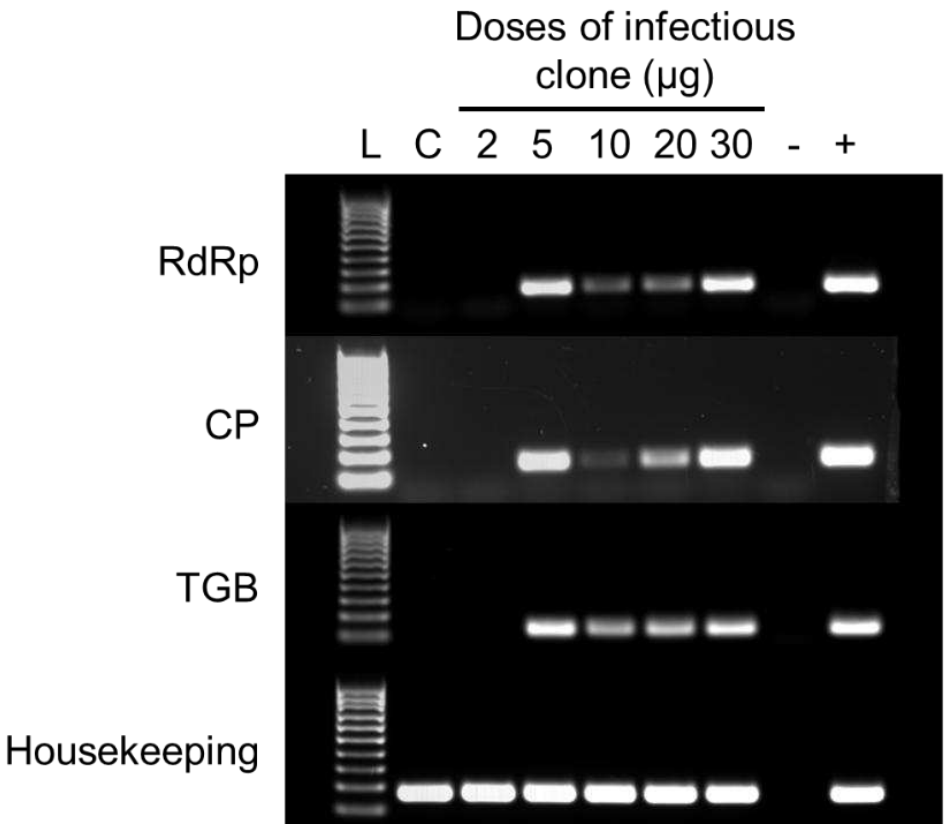

Figure 3. PCR confirmation of the presence of PMTV RNA1, 2, and 3 in the new growth of tobacco plants inoculated by different doses of PMTV infectious clone. Treatments include buffer-only control (C) and five different doses of infectious clone (2 µg, 5 µg, 10 µg, 20 µg, and 30 µg). The presence of PMTV RNA1, 2, and 3 genome fragments was determined by detecting their respective encoding genes, RdRp, CP, and TGB. Tobacco housekeeping gene, Nb18S-RT, was included in the PCR to ensure the success of RNA extraction and cDNA synthesis.

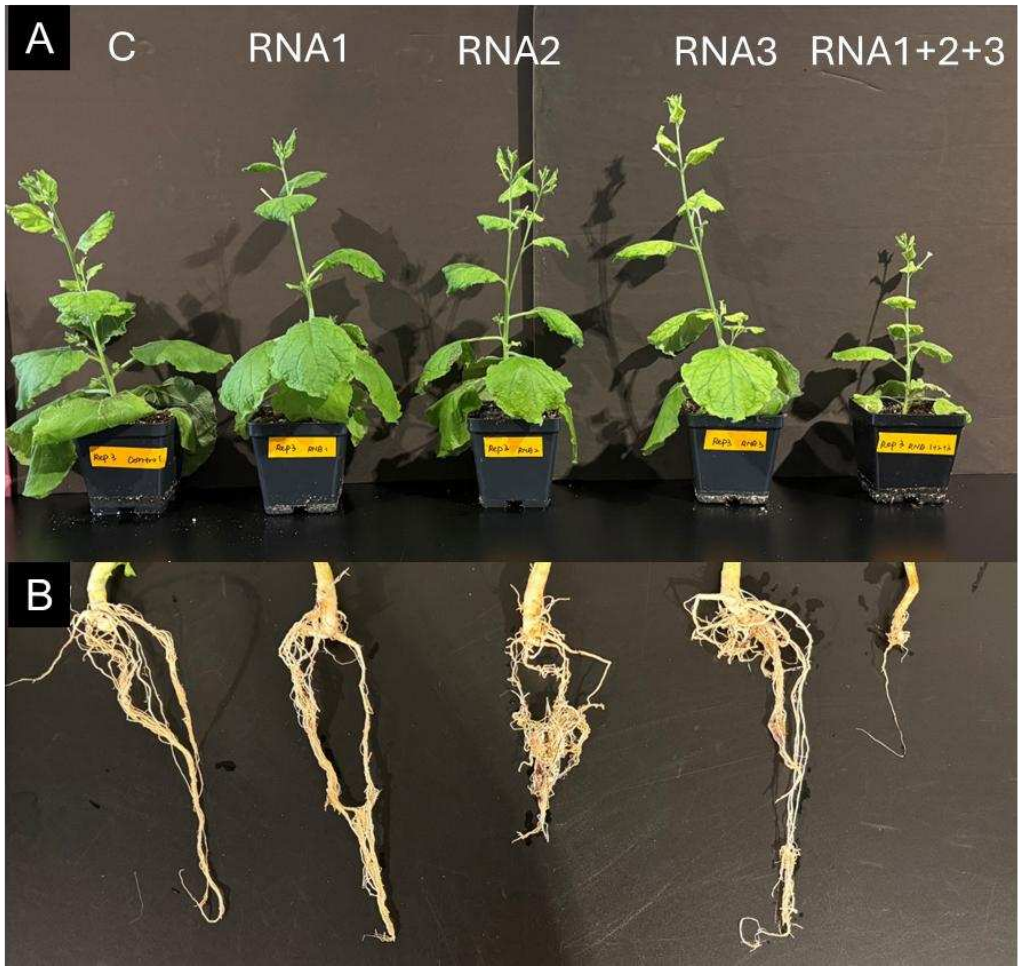

Figure 4. Tobacco plant and root symptoms after inoculation with individual PMTV RNA segments. Plant (A) and root (B) symptoms shown are after five weeks of inoculation. Plants were inoculated at the four-five leaf stage (about four weeks old) by rub-inoculating two leaves using 10 µg of RNA. Treatments include buffer-only control (C), RNA1, RNA2, and RNA3, and positive control (RNA1+2+3).

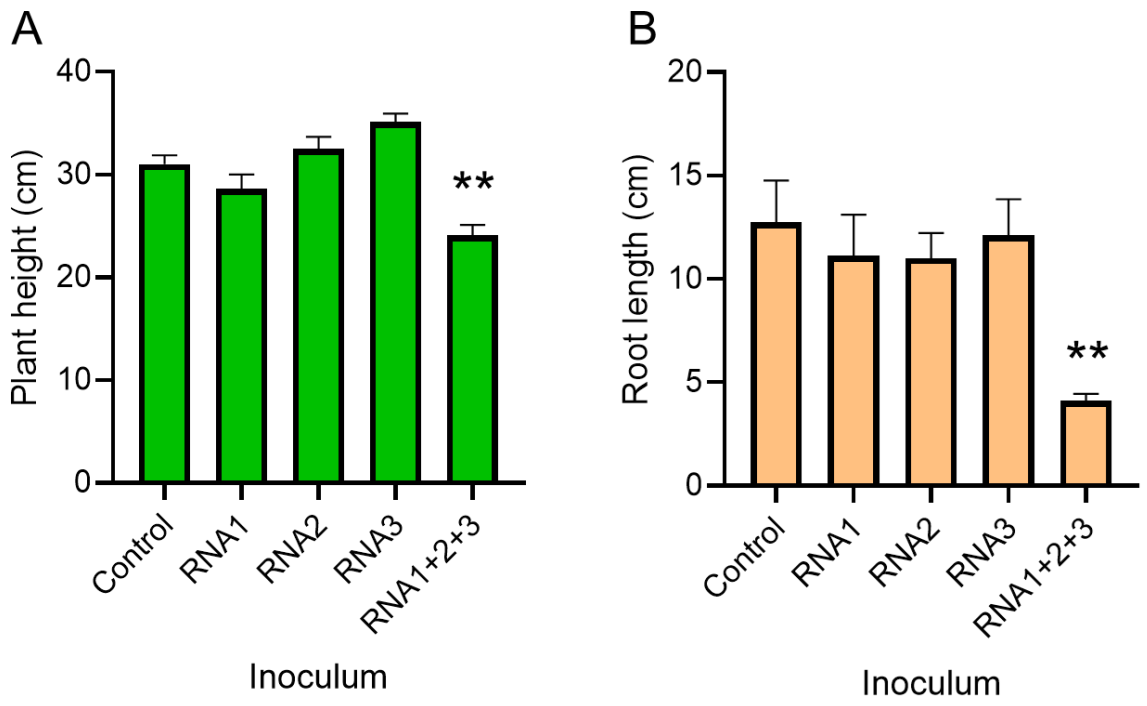

Figure 5. Infectivity of individual PMTV RNA segments on tobacco plant. Tobacco plants were rub-inoculated with individual PMTV RNA segments (RNA1, RNA2, RNA3), a combination of all three segments (RNA1+2+3, positive control), or buffer only (negative control). (A) Plant height and (B) root length were measured five weeks post-inoculation. Bars represent mean ± standard error (n = 4). Statistical analysis was performed using one-way ANOVA followed by Fisher's LSD test for pairwise comparisons against the negative control. Asterisks indicate significant differences from the control (**p<0.01).

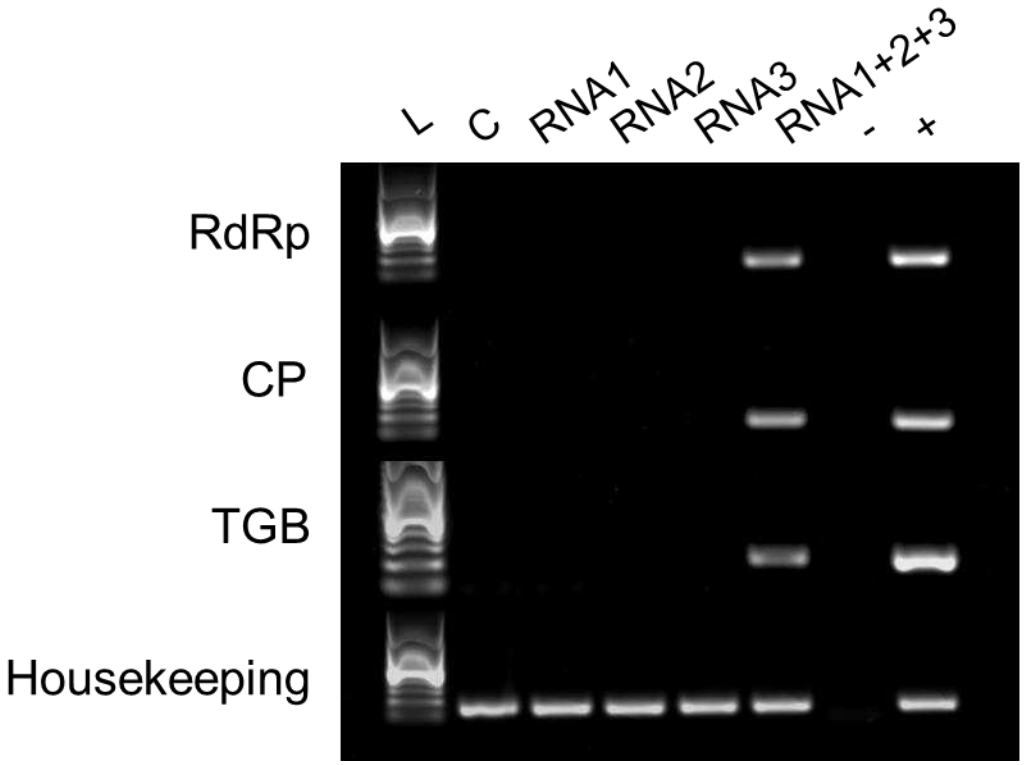

Figure 6. PCR detection of the presence of PMTV RNA1, RNA2, and RNA3 in the new growth of tobacco plants inoculated with individual RNA1, 2, or 3 segments. Treatments include buffer-only control (C), individual RNA segments (RNA1, RNA2, and RNA3), and a complete set of the infectious clone (RNA1+2+3). The presence of PMTV RNA1, 2, and 3 genome fragments was determined by detecting their respective encoding genes, RdRp, CP, and TGB. Tobacco housekeeping gene, Nb18S-RT, was included in the PCR to ensure the success of RNA extraction and cDNA synthesis.

Figure 7. Potato plant and root symptoms after inoculation with different doses of PMTV infectious clone. Plant (A) and root and tuber (B) symptoms shown are after seven weeks of inoculation. Plants were inoculated at the three-four leaf stage (about two weeks after germination) by rub-inoculating three leaves per plant. Treatments include buffer-only control (C) and three different doses of infectious clone (5 µg, 10 µg, and 20 µg).

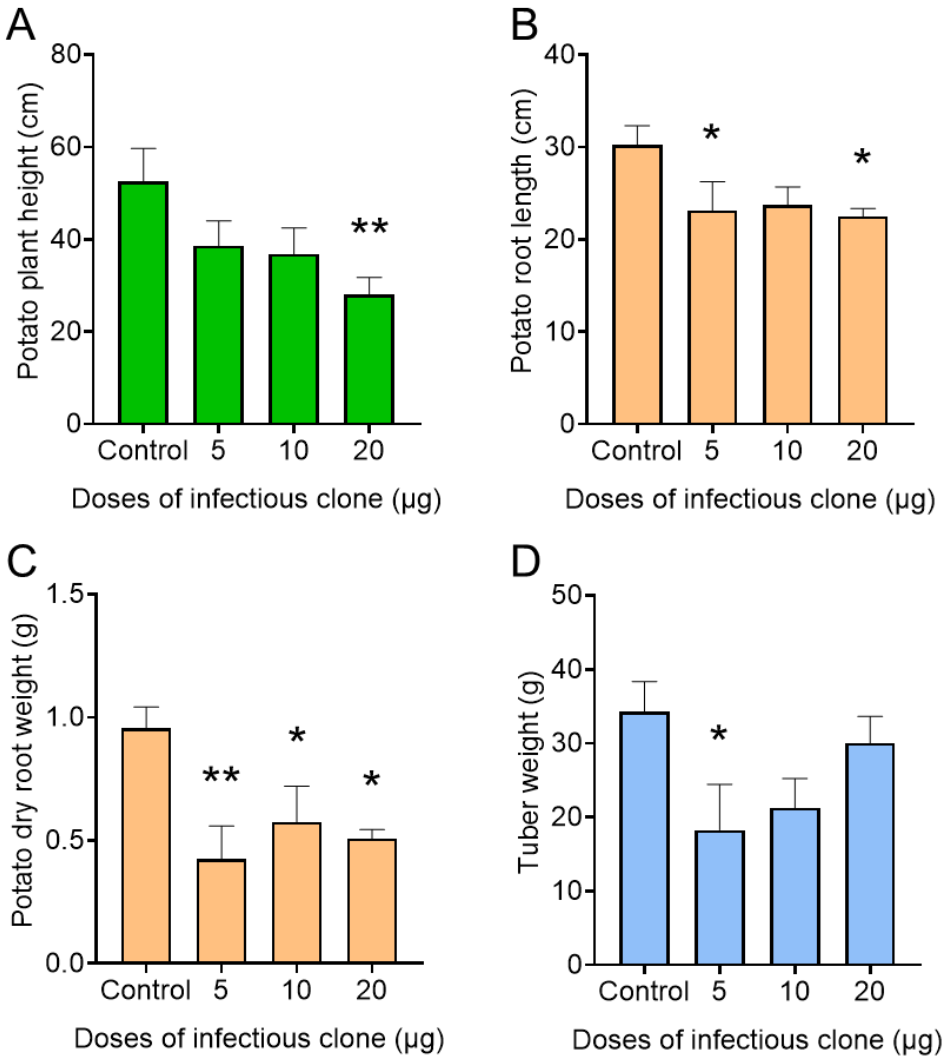

Figure 8. Potato plant heights, root lengths, dry root weight and tuber weight after inoculation with different doses of PMTV infectious clone. Potato heights (A), root lengths (B), dry root weight(C) and tuber weight (D) were measured after seven weeks of inoculation. Treatments include buffer-only control and three different doses of infectious clone (5 µg, 10 µg and 20 µg). Statistical analysis was performed using one-way ANOVA followed by Fisher's LSD test for pairwise comparisons against the negative control. Asterisks indicate significant difference between PMTV-inoculated treatment and control (*p<0.05, **p<0.01).

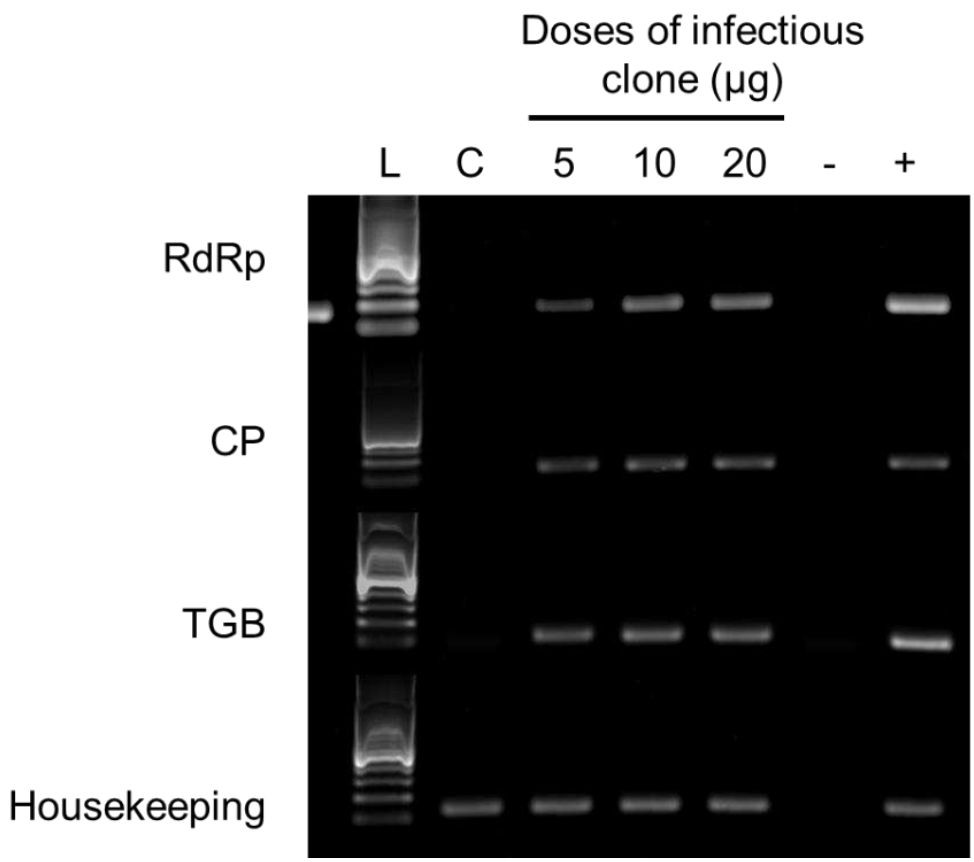

Figure 9. PCR confirmation of the presence of PMTV RNA1, 2, and 3 in the new growth of potato plants inoculated by different doses of PMTV infectious clone. Treatments include buffer-only control (C) and three different doses of infectious clone (5 µg, 10 µg, and 20 µg). The presence of PMTV RNA1, 2, and 3 genome fragments was determined by detecting their respective encoding genes, RdRp, CP, and TGB. Potato housekeeping gene, stAct, was included in the PCR to ensure the success of RNA extraction and cDNA synthesis.

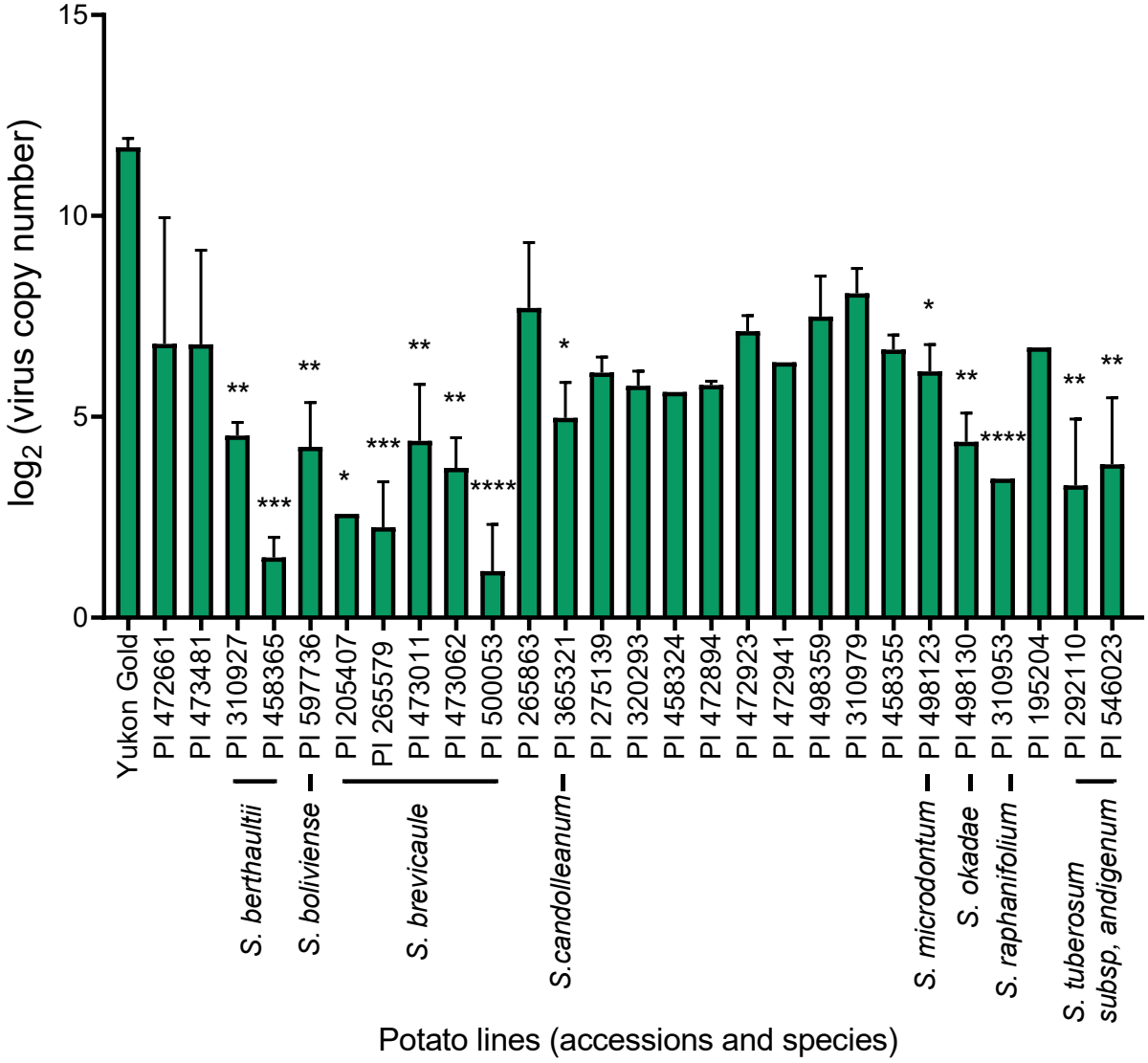

Figure 10. Screening of potato lines for potential resistance or tolerance to PMTV. Absolute quantification of PMTV coat protein (CP) RNA levels in new growth of inoculated potato cultivars five weeks post-inoculation. Virus accumulation is shown as log_2-transformed virus copy numbers. Bars represent means ± standard error. Statistical analysis was performed using Kruskal Wallis test followed by uncorrected Dunn's test for pairwise comparisons against the susceptible Yukon Gold control. Asterisks indicate statistically significant differences (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Species names are indicated below groups of accessions.

Table 1. Primer list used for PCR and qPCR analysis

Name	Strand	Sequence (5' to 3')	PCR size (bp)
RNA1-T7F	Forward	GGATCCTAATACGACTCACTATAGGGTATTTTATCAACTCTAACTAGCC	2,964
RNA2-T7F	Forward	GGATCCTAATACGACTCACTATAGGGTATTTTTAAGTCTAAACAGTTTG	3,134
RNA3-T7F	Forward	GGATCCTAATACGACTCACTATAGGGTATTTCAACTCTACCTAGCCGAGT	6,042
RNA1/2/3-R	Reverse	TGGTCTTGGATACCCTCCA	-
RdRp-F	Forward	GTTGATCCTAAGCTGTTACCGATGATT	132
RdRp-R	Reverse	CTTTGATACAGGCTTTGACTCCCA	
CP-F	Forward	ACCGAACGCTTTGGTGGAAGT	157
CP-R	Reverse	CGGAAGCTTCTCTCGGACCT	
TGB-F	Forward	ACTGGTGAGTTCGTAAAGCA	173
TGB-R	Reverse	AGTTCGCTGACCCTAAACCA	
CP-qF	Forward	GGGCGGGCAAGACGATTAG	96
CP-qR	Reverse	GTCCACCTCTGCGAGTTGATG	
Nb18S-F	Forward	ATGATAACTCGACGGATCGC	169
Nb18S-R	Reverse	CTTGGATGTGGTAGCCGTTT	
StActin-F	Forward	TGAACACGGAATTGTCAGCA	124
StActin-R	Reverse	GGGGTTAAGGGGGGCTTCAG	

Table 2. ELISA confirmation of the presence of PMTV coat protein in new growth of tobacco and potato plants after inoculation with PMTV infectious clone

Plant	Infectious clone dose (µg)	Total number of plants	Number of positive plants	Percentage of positive plants (%)
Tobacco	2	3	0	0
	5	3	0	0
	10	3	1	33
	20	3	1	33
	30	3	2	67
Potato	5	4	2	50
	10	4	3	75
	20	4	3	75

Table 3. Potato germplasms included in PMTV resistance screening

#	Species	Accession	Plant Name
1	*Solanum acaule*	PI 472661	OKA 3890A
2		PI 473481	HJT 5401
3	*S. berthaultii*	PI 310927	OCH s.n
4		PI 458365	HOF 1902
5	*S. boliviense*	PI 597736	SFVU 6531
6	*S. brevicaule*	PI 205407	BRU 1953. 19
7		PI 265579	COR 678A
8		PI 473011	HOF1800
9		PI 473062	OKA 4831
10		PI 500053	OKA 7512
11	*S. candolleanum*	PI 265863	EBS 1825
12		PI 365321	OCH S-24
13	*S. chacoense*	PI 275139	HJT 297
14		PI 320293	HHR 3706
15	*S. infundibuliforme*	PI 458324	OKA 4348
16		PI 472894	OKA 5381
17	*S. kurtzianum*	PI 472923	HOF1757
18		PI 472941	OKA4972
19		PI 498359	HOF 1756X1755
20	*S. microdontum*	PI 310979	ALN 64-11
21		PI 458355	OKA 4398
22		PI 498123	HHA 6653
23	*S. okadae*	PI 498130	1983 SD
24	*S. raphanifolium*	PI 310953	UGN 4229
25	*S. tuberosum subsp.andigenum*	PI 195204	CPC 712
26		PI 292110	UGN 5543
27		PI 546023	HOHL 253

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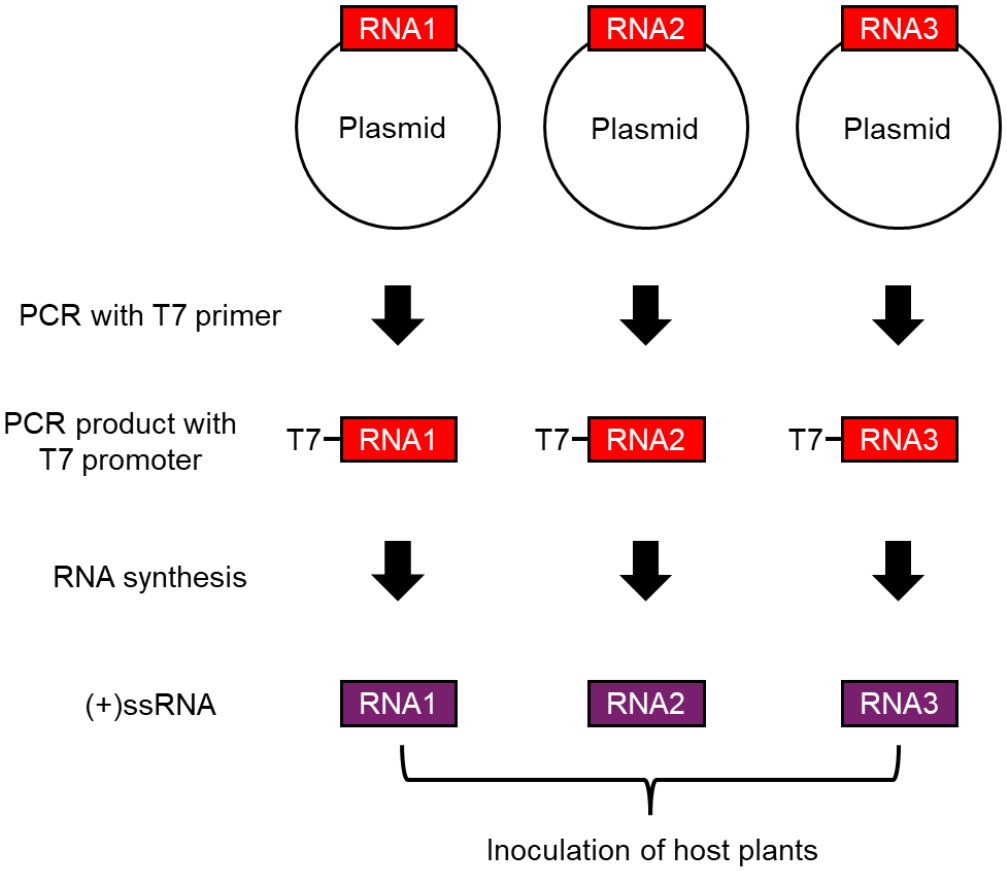
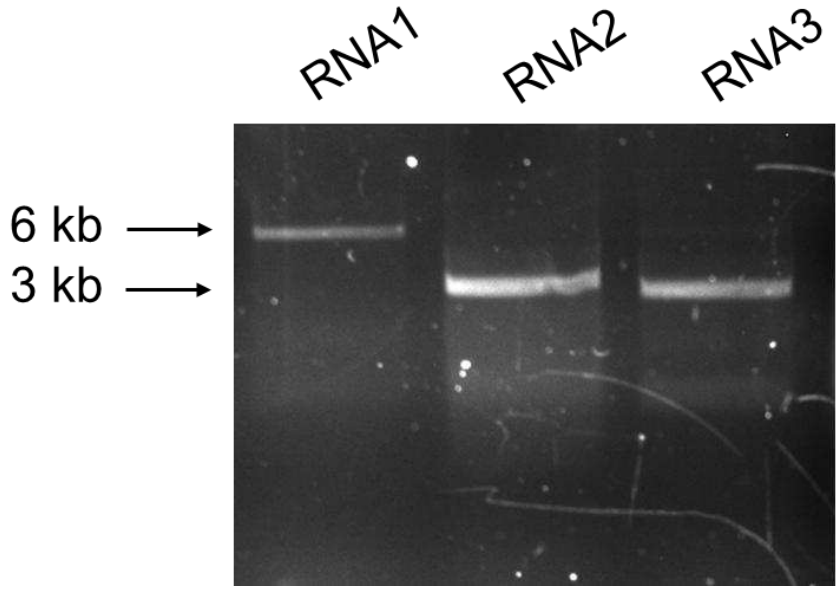


Supplementary Figure 1. Experimental workflow. Reverse transcription of PMTV RNA1, 2, and 3 using T7 promoter. Single stranded PMTV RNA1, 2, and 3 were mixed in 3X GKP buffer for inoculation of plants.



Supplemental Figure 2. Gel image showing successful synthesis of single-stranded PMTV RNA1, 2, and 3. Reverse transcription was performed using RiboMax™ large scale RNA production systems (Promega).