Characterisation of a Virus from Tomato

by

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Abstract

Tomato (*Solanum lycopersicum*) material with mosaic symptoms was sent to the university by a local commercial glasshouse for examination. In order to determine the causative agent of any infection, a series of experiments was undertaken. From initial analysis viral nucleic acid sequences were obtained by inoculating indicator plants with sap obtained from the diseased leaves, and it was shown that a non-microbial infectious agent was transmissible. Following this, it was found possible to extract viral nucleic acids from the inoculated plants, and the results suggested the possibility of a mixed infection by two pepino mosaic virus strains, as well as the presence of a possible *Potyvirus*. The rapid amplification of cDNA ends was the method used to attempt to obtain a full-length sequence of pepino mosaic virus. A partial sequence recovered corresponded to the genes encoding the Triple Gene Block and the coat protein of the viral genome. A rapid detection method using the dot blot technique was also explored and the results showed promise for future research in this area.

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List of Abbreviations

Abbreviation	**Explanation**
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GSP	Gene specific primer
IPTG	Isopropyl β-D-1-thiogalactropyrandoside
MEGA7	Molecular evolutionary genetics analysis version 7
NaCl	Sodium Chloride
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PepMV	Pepino mosaic virus
POTX	Potexviruses
POTY	Potyviruses
PVX	Potato virus X
PVY	Potato virus Y
RACE	Rapid amplification of cDNA ends
RdRP	RNA dependent RNA polymerase
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SOC	Super optimal broth with catabolite repression
TGB	Triple Gene Block
TMV	Tobacco mosaic virus
Tris HCl	Tris(hydroxymethyl)aminomethane hydrochloride
X-Gal	5-bromo-4chloro-3-indolyl-β-D-galactopyranoside
UTR	Untranslated region

Chapter 1: Introduction

Introduction to plant pathogens

Plant pathogens are considered a real and omnipresent danger. Pathogens for plant life come in many forms, bacterial, viral and fungal, that can cause a multitude of different diseases which result in a variety of outcomes for the infected plant. However, perhaps the most dangerous are viral pathogens, as it is estimated there are more than 700 known plant viruses which cause diseases in a wide range of hosts (Strange & Scott, 2005). While many diseases caused by pathogenic bacteria, fungi, and parasites can be controlled using bactericides, fungicides, and pesticides, the control of viral infections in agricultural crops and wild plants is more difficult (Stevens, 1983). RNA viruses are particularly capable of genetic recombination, which allows for the adaptation against countermeasures at an extraordinary rate (Roossinck, 1997) . Furthermore, the transmission of viruses makes the risk of viral infection more potent. While some viruses may require a vector, such as an aphid, others are easily transmitted via mechanical means, and may be unwittingly introduced and spread around a commercial setting with great ease by the employees who work there (Broadbent & Fletcher, 1963). Viruses have a separate taxonomic system away from the conventional biological classification scheme, the Baltimore classification, where viruses are classified initially on the structure of their genome. To date there are seven classifications; Double stranded DNA viruses (I: dsDNA), single stranded DNA (II: ssDNA), double stranded RNA (III: dsRNA), positive sense single stranded RNA (IV: (+)ssRNA), negative sense single stranded RNA (V: (-)ssRNA), single stranded RNA reverse transcribing (VI: ssRNA-RT), and, double stranded DNA reverse transcribing (VII: dsDNA-RT) (Baltimore, 1971). The majority of plant viruses are RNA viruses, with an estimated 75% of those consisting

of (+)ssRNA genomes, and an estimated 10% of being (-)ssRNA (Raikhy & Tripathi, 2017).

Tomato industry and economic impact of viral infection

In 2016 an estimated 18 million tonnes of tomatoes were produced within the European Union, with an approximate two thirds of these crops originating in Spain and Italy (Eurostat, 2017), two countries which have been severely impacted by viruses infecting tomato (Pagán, *et al*., 2006; Tiberini, *et al*., 2011).

There are several viruses which infect tomatoes, the most prominent being Tomato Spotted Wilt virus (TSWV), Tomato Yellow Leaf Curl virus (TYLCV), Cucumber mosaic virus (CMV) (Scholthof, *et al*., 2011), and Pepino mosaic virus (PepMV) (Jones, *et al*., 1980).

Crop loss among the viruses vary, with some reports of crop losses exceeding one billion dollars worldwide, as is the case with TSMV (Adkins, 2001). For Pepino mosaic virus, infection of tomato crops was not shown to affect the overall yield of fruits, however, infection of the virus did significantly affect the quality of fruits rendering infected fruits unmarketable (Spence, *et al.*, 2006).

Description of Genus's of interest to the study

Potyvirus

The *Potyvirus* genus is the largest group of plant viruses and is estimated to account for 30% of known plant viruses with at least 180 defined members (Riechmann, *et al*., 1992). Viral species in the genus are positive sense single stranded RNA viruses. The genomic arrangement is linear and composed of a single segment of approximately 10K nucleotides (Dougherty & Carrington, 1988). The type species of the *Potyvirus* genus is potato virus Y (PVY; family *Potyviridae*, genus *Potyvirus*),

which is comprised of multiple strains, with recombinant and ordinary strains (Karasev, *et al*., 2011).

The genome of PVY as with other members of the *Potyvirus* genus, is a monopartite sequence, which encodes for a polyprotein gene, and has a 5' non-coding region and a 3' poly(A) tail (Turpen, 1989). There is one large open reading frame, which produce 10 proteins (Robaglia, *et al*., 1989). The first is the P1 protein (185-1036), which acts as a protease involved in the autoproteolytic cleavage of the C terminus and is also a component for genome amplification (Yang, *et al.*, 1998). HCPro (1037-2404) is thought to be a multifunctional protein, involved in the suppression of antiviral defences within plants, and the transmission by aphids (del Toro, *et al*., 2014). The P3 protein (2405-3499) is thought to be involved in the replication of viral RNA due to the association with cylindrical inclusions at early stages of infection (Rodríguez-Cerezo, *et al.,* 1993) and a determinant in the symptom severity expressed by turnip mosaic virus (Jenner, *et al*., 2003). There is a small 6K1 protein (3500-3655) which has been a subject of limited studies, however it has been suggested that the protein may have a replicative function (Waltermann & Maiss, 2006). CI protein (3656-5557) and 6K2 protein (5558-5713) are both involved in viral movement, working in tandem to move between cells, and as result are an integral part of viral propagation (Spetz & Valkonen, 2004). The NIa protein (bases 5714-7009) contains two domains, the first is the VPg, used in the synthesis of viral RNA, and the second is a proteinase and is thought to interact with the NIb protein (bases 7010-8572), which is believed to act as an RNA-dependent RNA polymerase (Li, *et al.*, 1997). Finally, the coat protein gene (bases 8573-9373) encodes for the capsid around the virus. Due to the differences in amino acid sequences for the coat protein, it has been found that it is a possible to differentiate

and identify potyviruses and strains based on the coat protein encoding sequences (Shukla & Ward, 1989).

Transmission of the virus has been recorded by two main methods: mechanical transmission through infected tools (Fageria, *et al*., 2015), and through aphid vectors (Boguel, *et al.*, 2011; Fox, *et al.,* 2017).

It has been reported that tomato plants infected with PVY present with chlorosis and yellowing of the leaves (Massumi, *et al.*, 2009). However, a second study found that symptoms are dependent on the strain of PVY, for example *Lycopersicon esculentum* three strains of PVY were asymptomatic, but detectable by enzyme-linked immunosorbent assay (ELISA), and one strain of PVY caused mosaic symptoms (Aramburu, *et al*., 2006).

Tobamovirus

Tobamovirus is a genus of positive sense single stranded RNA viruses which infect a variety of plants of the Solanaceae family and the type member of this genus is tobacco mosaic virus (TMV; family *Virgaviridae*, genus *Tobamovirus*). The virus is a rod-shaped virus (**Figure 1.**) containing a single RNA segment and was the first virus to be discovered in 1886 by Adolf Mayer, and named by Martinus Beijerinck in 1898, who at the same time coined the term "virus". Similar to the *Potexvirus* species the genome is roughly 6.4K nucleotides in length and is a non-segmented. Like with *potato virus X*, TMV contains a m^7G^5'ppp^5'Gp cap on the 5' end. Traditionally it is assumed that TMV, does not contain a poly(A) tail on the 3' end, however it has been found that in some mutations the polyadenylated tail is present (Li, *et al*., 2014), as has been found with mutants from other Tobamovirus species (Guo, *et al*., 2015).

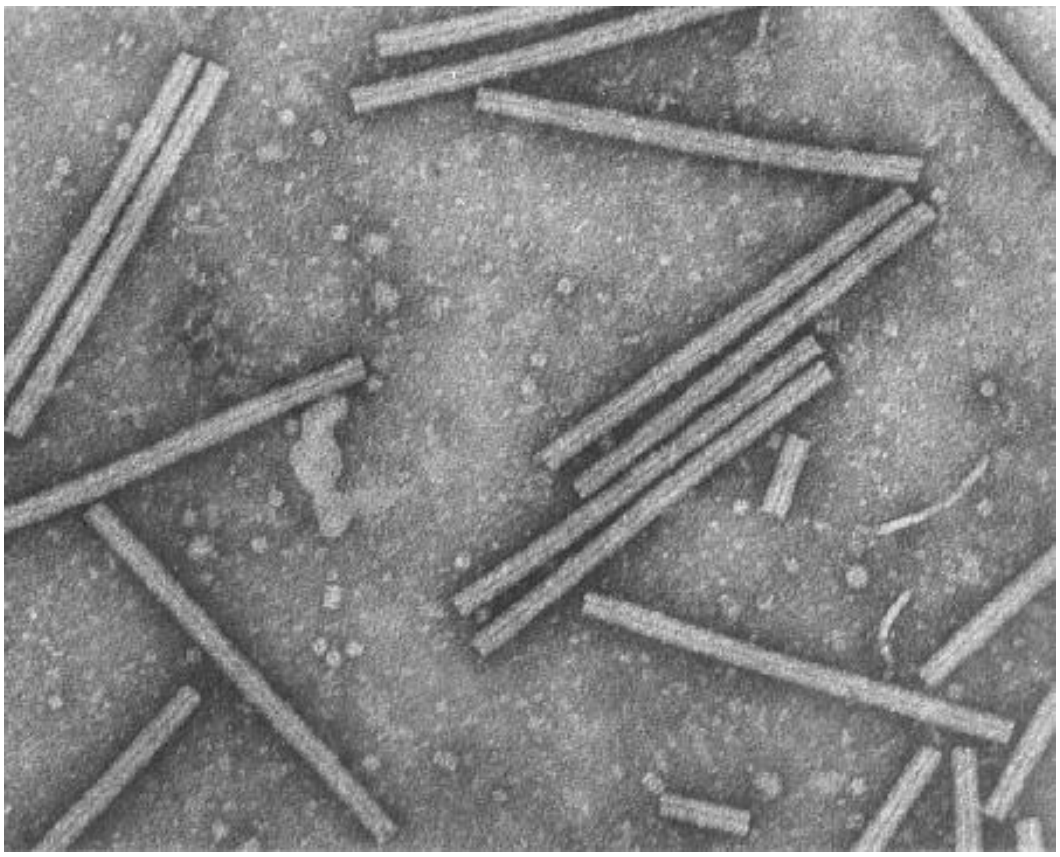

Figure 1.1. Electron micrograph displaying the morphology of Tobacco mosaic virus rod shaped particles (Scholthof, 2005).

The TMV genome contains four open reading frames; ORF1 (69-4919) and ORF2 (69-3419) encode a 183kDa protein and a 126kDa protein, respectively, involved in viral replication. Both the 183K and 126K proteins contain conserved methyltransferase and helicase domains, with the 183K protein also containing a conserved polymerase domain *(Yamaji, et al.*, 2006). ORF3 (4903-5709) encodes a 30Kd movement protein involved in the movement of virus particles during infection. The movement protein is thought to bind to both actin and tubulin to facilitate viral RNA through plasmodesmata (McLean, *et al*., 1995). Finally, ORF4 (5712-6191) encodes for the 17.5Kd coat protein (Saito, *et al*., 1987).

The virus is readily transmitted between plants in a variety of ways. TMV is easily transmitted by simple contact between infected and non-infected leaves. The virus

has also been found to be transmitted through seed, as well as through aphid vectors. There is evidence to suggest that agricultural workers can spread the virus, particularly if they are smokers with studies having found viable TMV RNA in the saliva of smokers as well as in human stool samples (Balique, *et al.*, 2012).

The symptoms caused by TMV vary between host plant, but in tomato can include mosaic, necrosis, mottling, and if fruits are present, discolouration, reduced fruit yield, and delayed fruit ripening (**Figure 1.2**). The symptoms displayed are also thought to be dependent on the environmental conditions, the age of the infected plant, as well as the strain of infecting virus (Fletcher & MacNeill, 1971).

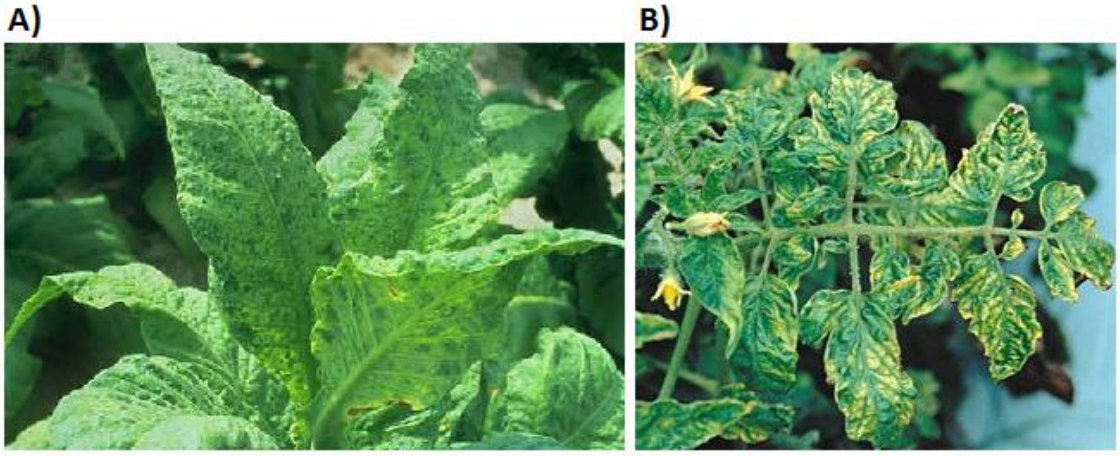

Figure 1.2. Symptoms of TMV displayed on two species of plant. **A)** Mosaic symptoms typically seen on tobacco leaves during infection (Scholthof, 2005). **B)** Chlorosis symptoms seen when TMV infects tomato (Scholthof, 2005).

Potexvirus

The potexviruses are a genus of positive sense single stranded RNA viruses which infect a wide variety of plants (Sonenberg, *et al.*, 1978). To date there are 34 species of virus within the genus with the type species being Potato virus X. These viruses infect plant species ranging from *Chenopodium*, potato, tomato, tobacco, sesame, and members of the *Cucumis* genus (Short & Davies, 1987). Viruses within the genera

are between 6 and 8kb in length. They contain a m^7G^5'ppp^5'Gp cap on the 5' end and the 3' end is polyadenylated. The genomes encode for five proteins; ORF1 which encodes an RNA-dependent RNA polymerase, which contains methyltransferase, helicase, and RNA polymerase domains (Park, *et al*., 2014). ORF2-4 encodes the triple gene block which is involved in the structure and movement of the virus through the host plant and present in all species of the *Potexvirus* genus (Verchot-Lubicz, *et al*., 2007). ORF5 encodes for the coat protein, which in addition to protecting the virus genome, is also required for movement between plant cells (Rakitina, *et al.*, 2005). The structure of the potexviruses are described as flexible filamentous (Kendall, *et al*., 2013).

Potexviruses are mainly transmitted by mechanical means, as is the case with viral species such as senna mosaic virus (Rezende, *et al*., 2017), pepino mosaic virus (Ling, 2008), and cymbidium mosaic virus (Hu, *et al.*, 1994). However, there are other means of transmission of potexviruses that have been recently discovered. The strawberry mild yellow edge virus has been found to be transmitted by aphids (Lavandero, *et al.*, 2012) and the possibility that bamboo mosaic virus is spread by dipteran insects has been reported (Chang, *et al*., 2017).

Potexviruses cause a variety of symptoms according to the virus and the infected plants, however the main symptoms presented tend to be mosaic and chlorosis on infected leaves (Short & Davies, 1987).

Potexviruses of interest to this project

Potato virus X

History

Potato virus X (PVX; family *Alphaflexiviridae*, genus *Potexvirus*) was originally identified in 1931 as a separate virus to what was at the time called "rugose mosaic" in potato, which was found to be a combination of PVX and PVY (Smith, 1931). However, this was not the first recorded case of PVX, in 1925 James Johnson observed that sap from potatoes which produced a faint mottle on leaves readily infected tobacco (Johnson, 1925), although at this time this was not identified as PVX.

Virus distribution

As with PepMV, PVX appears to be a New World virus, although this cannot be confirmed due to the lack of information and reports available, therefore it is not possible to say for certain where the first outbreak of PVX occurred. What is certain, is that the virus has spread throughout the world infecting crops of the *Solanaceae* family. In Asia, the virus has been reported to infect plants of different families in China; in *Orychophragmus violaceus,* reported for the first time in Beijing in 2008 (Li, *et al*., 2008); Squash crops in Chongqing province *(Ling, et al.*, 2010); potato cultivars, Shangdong province (Jing, *et al.*, 2003), and Sichuan province (ZhongKang, *et al*., 2005); two reports in India of PVX in potato cultivars in the Punjab (Sharma & Kang, 2003) and West Bengal (Santanu & Amitava, 2003) regions. There have also been reports of PVX infecting crops in Iran (Khakvar, *et al*., 2005), Japan *(Kagiwada, et al.*, 2002), Lebanon (Abou-Jawdah, *et al*., 2001), Pakistan (Khan & Saif-ur-Rehman, 2002), and Turkey (Bostan & Haliloglu, 2004). In Africa, the virus has been found

infecting tomato crops in Algeria (Nechadi, *et al.*, 2002), Tunisia (Ben Moussa, *et al*., 2000), potato crops in Egypt (El-Araby, *et al*., 2009), Tanzania (Chiunga & Valkonen, 2013), and weed species in Ethiopia (Alemu, *et al*., 2002). Considering the virus might be considered a New world virus there are relatively few reports from the Americas pertaining to PVX, with only reports from Argentina (Clausen, *et al*., 2005), Brazil (Silva, *et al*., 2005), Greenland *(Neergaard, et al.*, 2014), and the USA (Robertson, *et al*., 2011) issuing recent notices of the virus in crops. PVX has been found in several countries in Europe, infecting a hybrid dock (*Rumex patientia* L. *x Rumex tianschanicus* A. Los) in the Czech Republic (Petrzik, 2009), and *Petunia* cultivars in Hungary (Baracsi, *et al*., 2002). Finally, there has been incidence of PVX in commercial potato fields in Tasmania, Australia (Lambert, *et al*., 2007).

Transmission

As with other potexviruses, PVX is readily transmitted by mechanical methods, such as when a healthy plant encounters infected tools and machinery, as well as contact with infected clothing by workers (Manzer & Merriam, 1961). Several vectors have been described for PVX, the first is the fungus species *Synchytrium endobioticum* (Nienhaus & Stille, 1965), which itself is a pathogen of potatoes, but has also been found to infect other members of the *Solanum* genus (Hampson & Haard, 1980). The grasshopper, *Melanoplus differentialis*, was discovered to be a potential vector for PVX, despite infectivity of the virus decreasing as a result of the digestive tract of the grasshopper, the virus remained infective for six hours (Walters, 1952).

Viral properties

PVX is a single-stranded positive-sense RNA virus and is the type species of the *Potexvirus* genus, with the viral particles described as flexuous filaments (Atabekov,

et al., 2007). The full genomic sequence is approximately 6435 bases in length when excluding the 3' poly (A) tail (Skryabin, *et al*., 1988). The virus has a 5' m^7GpppG cap (Sonenberg, *et al*., 1978) which has been observed in various plant viruses and acts as a messenger for viral protein synthesis (Zimmern, 1975) (Klein, *et al*., 1976). The genome has five ORFs; ORF 1 (bases 85-4453), preceded by an 84 base 5' leader sequence; three overlapping ORFs forming the TGB, ORF 2 (4486-5164), ORF 3 (5147-5492), ORF 4 (5427-5637); and ORF 5 (5650-6361) followed by a 76 base UTR (Huisman, et al., 1988). ORF 1 encodes for a single protein (166Kd), a replicase, which contains viral methyltransferase, a viral helicase, and an RNA dependent RNA polymerase domain (Davenport & Baulcombe, 1997). ORF 2 (25Kd) that encodes for a viral helicase, as well as a P-loop NTPase, along with ORF 3 (12Kd), and ORF 4 (7Kd), which encode for a plant viral movement protein and a viral coat protein, respectively, make up the TGB (Samuels, *et al*., 2007). Finally, ORF 5 (25Kd) encodes for the viral coat protein (Huisman, *et al*., 1988).

Pepino mosaic virus

History

Pepino mosaic virus (PepMV; family *Alphafelixviridae*, genus *Potexvirus*) was first observed in 1974 in pepino crops (*Solanum muricatum*) in the Canete valley, Peru, where the leaves of the pepino crop presented with yellow mosaic and was later found to be an undescribed *Potexvirus* that was later named pepino mosaic virus (Jones, *et al*., 1980).

Virus distribution

While PepMV was originally located in Peru, it was later shown to be present in the Central, Southern and Andean regions (Soler, *et al*., 2002). In 1999 an unknown

virus had been identified in tomato crops (*Lycopersicon esculentum*) in the Netherlands. Analysis by transmission electron microscopy indicated that the virus was a *Potexvirus*, with inoculation of indicator plants and serological tests identifying the virus as PepMV (van der Vlugt, *et al*., 2000). Subsequently, PepMV has been isolated in glasshouses around the world. In Africa, a Moroccan report in 2016 indicated that PepMV was widespread among tomato cultivars with an average infection rate of 21%, with the virus also present in seven regions of the country (Imane, 2016). In 2011, the first report of PepMV infecting tomatoes from farms in the Limpopo Province, South Africa was issued, however to date this is the only report of PepMV in South Africa (Carmichael, *et al*., 2011).

Asia, like Africa, has limited reports of PepMV infection in tomato crops with isolates detected near Latakia, Syria in 2008 (Fakhro, *et al*., 2010), while in China, the first report of PepMV infecting tomato was in 2003 in Shanghai (YaoLiang, *et al.*, 2003).

South and Central America have widespread reports of PepMV infections with PepMV being endemic in Peru (Soler, *et al.*, 2002). Isolates have also been recorded in Chile, with the first reported incident in 2001 (Ramirez & Bustamante, 2001), with further reports of the virus in the Central region of Chile in 2005 (Yanten Carreno, *et al*., 2005). PepMV was detected in samples of currant tomato (*Solanum pimpinellifolium*) from three provinces (Manabi, Esmeraldas, and Guayas) in Ecuador in 2005 (Soler, *et al*., 2005), and at one glasshouse in Jocotitlán, Mexico in 2010 (Ling & Zhang, 2011).

There have been reports of PepMV in tomato cultivars from four provinces in Canada; Alberta in 2005 (French, *et al.*, 2005); in glasshouses in British Columbia in 2009 *(Ling, et al*., 2008); Ontario, with the first report in 2001 (French, et al., 2001),

and subsequent reports in 2007 (Saboruin, *et al*., 2007), and 2008 (Ling, *et al.*, 2008); and Quebec in 2010 (Gilbert, *et al.*, 2010). Several states in the United States have recorded outbreaks of PepMV in tomato glasshouse crops; Alabama (Ling, *et al*., 2008), Arizona (Ling, *et al*., 2008; French, *et al.*, 2001; Maroon-Lango Guaragna, *et al*., 2003), California (Ling, *et al*., 2008), Colorado (Ling, *et al*., 2008; French, *et al*., 2001; Maroon-Lango Guaragna, *et al*., 2003), Florida (Maroon-Lango Guaragna, *et al.*, 2003) , Maryland (Maroon-Lango, *et al*., 2005), Minnesota (Lockhart, 2007), Oklahoma (Maroon-Lango Guaragna, *et al*., 2003), and Texas (Ling, *et al*., 2008) (French, *et al*., 2001; Maroon-Lango Guaragna, *et al*., 2003).

In Europe PepMV is widespread, as of 2017, 19 countries reporting active infections by the virus. Two of these, Spain (mainland) (Pagán, *et al*., 2006), and Italy (Sicily) (Tiberini, *et al.*, 2011), both have endemic infections of the virus in glasshouses infecting tomato fruits. Austria, Belgium (Verhoeven, *et al*., 2003) (Hanssen, *et al.*, 2008), Bulgaria, Cyprus (Papayiannis, *et al*., 2012), Denmark, France (Verhoeven, *et al*., 2003; Cotillon, *et al.*, 2002), Germany (Verhoeven, *et al*., 2003; Schwarz, *et al*., 2010), Greece (Efthimiou, *et al*., 2010), Hungary (Forray, *et al*., 2004), Ireland, Lithuania (Šneideris, *et al*., 2013), Netherlands (van der Vlugt, *et al.*, 2000) (Verhoeven, *et al*., 2003), Poland (Pospieszny & Borodynko, 2002; Pospieszny & Borodynko, 2006; Hasiów-Jaroszewska, *et al.*, 2009), Switzerland (Stäubli, 2004; Ramel, *et al*., 2007), Turkey (Özdemir, 2010), Ukraine (Verhoeven, *et al*., 2003), and the United Kingdom (Verhoeven, *et al*., 2003; Mumford & Metcalfe, 2001) have all reported sporadic occurrences of PepMV infections of tomato in glasshouse settings.

Transmission

While no vector has been identified for PepMV, the virus is known to be transmitted between plants by mechanical means such as human contact or horticultural tools (Wright & Mumford, 1999). It has been found that when bees are used in crop pollination, they are able to spread the virus between tomato crops (Lacasa, *et al*., 2003). PepMV is also capable of being transmitted through hydroponic systems (Schwarz, *et al*., 2010), or irrigation systems, where the infected roots of a plant release virions into the water and have been shown to remain infectious for up to three weeks (Mehle, *et al*., 2013). A related study which examined the transmission of PepMV by the fungal vector *Olpidium virulentus*, noted that PepMV was only transmitted in drainage water, when the fungal vector was present on the roots of the tomato plant, but not when only the virus or fungus was present alone (Alfaro-Fernández, *et al.*, 2010). Research examining transmission routes of the virus between tomato crops has also revealed that the virus is not seedborne (Ling, 2008) except for cases where the seed exterior is contaminated by PepMV, in which case the virus can be transmitted, but the rate of incidence is considered low (del Carmen Códoba-Sellés, *et al*., 2007).

Viral properties

PepMV is a member of the *Potexvirus* genus and is a single-stranded positive-sense RNA virus with the viral particles described as flexuous filaments. The full genomic nucleotide sequence of the virus is on average 6410 bases in length when excluding the 3' poly (A) tail, measuring approximately 508 x 12.5 nm (Jones, *et al*., 1980). The genome consists of five open reading frames (ORFs); ORF 1 (bases 87-4406), and is preceded by a short untranslated region (UTR); the triple gene block (TGB) which is

comprised of three overlapping ORFs; ORF 2 (4432-5136), ORF 3 (5117-5488), ORF 4 (5340-5594); and ORF 5 (5633-6346), followed by a short UTR, which also contains the poly (A) tail (Aguilar, *et al*., 2002). ORF 1 codes for a single protein with a molecular weight of 164 Kd, which comprises three replicase domains that are found in other potexviruses: a methyltransferase, a viral helicase, and an RNA-dependant RNA polymerase. ORF 2 (26 Kd) also encodes for a viral helicase, with ORF 3 (14 Kd) and ORF 4 (9 Kd) forming the TGB which is believed to have a role in the movement of the virus between cells within the host. Finally, ORF 5 (25 Kd) encodes for the coat protein (Cotillon, *et al*., 2002). To date there are six recognised strains of PepMV; Peruvian (Moreno-Pérez, *et al*., 2014), Chilean 1 & 2 (Ling, 2007), US1, US2, and EU (Li, *et al.*, 2012).

Host range and symptoms

PepMV is reported to infect most members of the Solanaceae family as well as *Cucumis sativus* (Salomone & Roggero, 2002), *Amaranthus* spp, *Malva* spp, *Nicotiana* spp, and *Sonchus* spp (Jordá, *et al*., 2001) are also prone to infection by the virus. Symptoms vary depending on the species infected with the virus, with symptoms including growth reduction, leaf bubbling, leaf chlorosis, veinal necrosis, vein banding, leaf deformation, necrotic lesions, yellow spots, and in some cases, infection has been reported to cause no symptoms (Blystad, *et al*., 2015).

Aims of the project

The aims of the project comprised three parts:

1) To determine if the diseased material supplied to the university by a local commercial glasshouse was caused by viral infection. The material presented with mosaic symptoms which were consistent with infection by an unknown viral species.

2) Identification of the infectious agent in the tomato material and an attempt to acquire a full genome sequence of any virus present. Obtaining a full genome sequence would allow direct comparison with other viral genomes to confirm the virus responsible for the infection.

3) The development of a rapid test to identify infectious agents in plant material.

Chapter 2: Methods and Materials

Diseased tissue

The diseased material was sent to the university by a local commercial glasshouse, and as such the identity of the glasshouse in question is commercially sensitive data. The diseased material from was *Solanum lycopersicum* (*S. lycopersicum*) leaves which presented with mosaic symptoms.

Indicator Plants

The virus was maintained in four tobacco species, as different tobacco species have been shown to display differential symptoms when infected with Pepino mosaic virus (Hasiów-Jaroszewska, *et al*., 2009), as well as a tomato species (*Solanum lycopersicum*), which were grown at the University in temperature and light controlled glasshouses. The indicator plants were manually inoculated with extracts from diseased *S. lycopersicum* leaf tissue which was sent to the university by a local commercial glasshouse, and was stored at -70°C. The leaf tissue was ground in 0.1M phosphate buffer and rubbed onto the leaves which were dusted with carborundum powder to lyse the cells facilitating the transfer of viral particles into the leaves. The species of tobacco used were *Nicotiana glutinosa* (*N. glutinosa*), *Nicotiana benthamiana* (*N. benthamiana*), *Nicotiana tabacum* Cv. Xanthi (*N. tabacum* 'Xanthi'), and *Nicotiana tabacum* Cv. Samson (*N. tabacum* 'samson') and chosen due to the relative ease to grow in a short time frame, and the symptoms displayed by potexviruses in previous studies (Hasiów-Jaroszewska, *et al.*, 2009).

Software programs utilised

Sequence analysis, comparisons, and phylogenetic analysis was performed with the MEGA7 (Kumar *et al*, 2015) and UGENE (Okonechnikov *et al*, 2012) software.

MEGA7 was used to align sequences for primer design and sequences obtained from DNA sequencing, and to analyse the phylogeny of sequences. UGENE was primarily used to examine the sequences and their associated chromatographs.

RNA Isolation

Total RNA was extracted from 100 mg of plant tissue from the tomato and tobacco leaves by grinding frozen leaves in liquid nitrogen using a pestle and using the RNeasy Mini kit (Qiagen, Germany), following the manufacturer's instructions. The total RNA concentration was assessed using a Qubit fluorometer (Invitrogen, USA), and the RNA was stored at -70°C to be used later in further downstream applications.

Reverse transcription

The synthesis of cDNA from total RNA was performed using the RNA to cDNA Ecodry premix Double primed kit from Takara (Japan). The concentration of RNA added to each reaction ranged from 0.48 µl to 19.6 µl according to the concentration found after Qubit analysis of the RNA extraction procedure (**Table. 2.1**). The reactions were incubated in a Techne Prime thermal cycler at 42°C for 60 minutes, and the reaction was stopped by heating to 72°C for 10 minutes.

Mechanically inoculated infection	**RNA added (µl)**
N. glutinosa	0.93
N. tabacum 'Xanthi'	1.16
N. benthamiana	10.59
N. tabacum 'Samson'	2.65
Systemic infection	
N. glutinosa	0.58
N. tabacum 'Xanthi'	0.48
N. benthamiana	19.6
N. tabacum 'Samson'	1.78

Table 2.1. Relative RNA concentrations (µl) used in reverse transcriptase reaction for the synthesis of cDNA as obtained by Qubit analysis from the RNA extraction of both mechanically inoculated leaves and systemically infected leaves.

Polymerase chain reaction conditions

RT-PCR was performed on 50 µl reactions consisting of 5 µl x10 PCR buffer, 5 µl dNTPs (25 µM), 4 µl $MgSO_4$ (50 mM), 1 µl *Taq* polymerase, 1µl of each primer (10 µM), 1 µl cDNA, and brought up to 50 µl with distilled water, with the reactions carried out in PCR strip tubes and mixed by gentle stirring. The PCR conditions were as follows: 1 cycle of 105°C to preheat the lid, followed by 1 cycle of 94°C for 5 minutes for initial denature, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 10 minutes, with the fragments analysed on a 0.8% agarose gel. The positive control in the reactions was either Tomato Actin or Tobacco Actin, depending on which species was being examined during PCR, while the negative control included all previously mentioned PCR components excluding the cDNA, which was replaced with distilled water.

TA cloning method

To clone the PCR fragments, a TA vector cloning kit (TA Cloning™ Kit, with pCR™2.1 Vector and One Shot™ TOP10 Chemically Competent *E. coli*) from Invitrogen was used. The ligation reactions were incubated for 25 minutes at room temperature. A 50 µl vial of frozen OneShot® Competent Cells was thawed on ice, and once thawed, 2 µl of ligation reaction was pipetted into competent cells and incubated on ice for 30 minutes. The cells were subjected to a heat shock step for 45 seconds at 42°C and returned to ice. After adding 250 µl of room temperature LB broth to cells they were placed in vials horizontally at 37°C for 45 minutes in a shaking incubator at 225 RPM. Two LB agar plates containing IPTG & X-gal, and Ampicillin (120 µg/ml) with 50 µl and 250 µl of the cells were used for the transformation step, incubated for 24 hours at 37°C, and placed at 4°C for 1 hour to allow for colour development. Colonies were picked and place in vials containing 10 ml LB broth, and 10 µl Ampicillin (120

μg/ml) and incubated at 37°C for 20 hours in a shaking incubator at 225 RPM. Plasmid samples were prepared using the QIAprep Spin Miniprep kit from QIAgen (Germany) following standard protocols.

To evaluate the fragment inserts in the plasmid samples collected, 2 μl EcoRI buffer, 1.5 Units *Eco*RI enzyme, 1 μl DNA, and 5.5 μl distilled water were mixed in a 1.5 ml centrifuge tube, and incubated for 1 hour at 37°C, and the product was analysed on a 0.8% agarose gel.

Plasmid samples which showed bands cut at the expected size were excised and sent to DBS Genomics, Durham University, for sequencing using the M13 primers, forward and reverse.

Rapid Amplification of cDNA ends (RACE) method

For the RACE experiment, the Gene specific primers (GSP) were designed using the sequence information obtained from the cloned PCR fragments according to the protocol specifications included in the RACE experiment kit from Takara Bio (Japan).

First-Strand cDNA synthesis was performed using total RNA. A Buffer mix was prepared by combining 4 μl of 5X First-Strand Buffer, 0.5 μl DTT (100mM), and 1 μl dNTPs (20mM). For the 5'-Race-Ready cDNA, 5 μl of total RNA, 1 μl 5'-CDS Primer A, and 5 μl Sterile H_2O were combined giving a total volume of 11 μl, and for the 3'-Race-Ready cDNA, 5 μl of total RNA, 1 μl 3'-CDS Primer A, and 7 μl Sterile H_2O were combined giving a total volume of 12 μl. The contents from each tube were mixed and briefly centrifuged, then incubated at 72°C for 3 minutes, and 42°C for 2 minutes in a thermal cycler, after which the tubes were centrifuged for 20 seconds at 9000 RPM, and 1 μl of SMARTer II A Oligonucleotide was added to the 5'-RACE cDNA

synthesis reaction only. A master mix was prepared for the reactions by combining 11 µl of the Buffer mix, 1 µl RNase Inhibitor (40 U/ µl), and 4 µl SMARTScribe Reverse Transcriptase (100 U) giving a final volume of 16 µl, with 8 µl being decanted into both the 5' and 3' reactions giving a total volume of 20 µl per reaction. The reaction tubes were then mixed via pipetting and centrifuged for 20 seconds, and incubated in a thermal cycler for 90 minutes at 42°C, and 10 minutes at 70°C. The cDNA synthesis reactions were then diluted using 90 µl of Tricine-EDTA Buffer.

A PCR Master Mix was prepared by combining 31 µl PCR-Grade H_2O, 50 µl 2X SeqAmp Buffer, and 2 µl SeqAmp DNA polymerase, to give a total volume of 83 µl, which can be used for both the 5' and 3' RACE reactions. The PCR reactions were then prepared by adding 2.5 µl 5' and 3'-RACE-Ready cDNA, 5 µl 10X UPM, 1 µl 5' and 3' GSP (10 µM) and 41.5 µl of the PCR master mix, into their respective 0.5ml PCR tubes, and mixed. Because the melting temperatures of the GSPs were below 70°C, the thermal cycler was set to 20 cycles, 94°C seconds, 68°C 30 seconds, and 72°C 4 minutes. After the initial PCR step was complete, the PCR reactions were then subjected to the same program as stated in the protocol for a further 10 cycles, and the reactions run on an agarose gel for product characterisation.

The bands corresponding to the expected fragment size were excised from the agarose gel and placed into 1.5 ml centrifuge tubes, in which 200 µl Buffer NTI was added. The samples were incubated at 50°C for 10 minutes and vortexed regularly. Following this, 700 µl of the samples were transferred to a NucleoSpin Gel and PCR Clean-Up Column, and centrifuged for 30 seconds at 11,000 RPM, with the flow-through discarded. 700 µl Buffer NT3 were added to each column and centrifuged again for 30 seconds with the flow-through discarded, and the columns were then

centrifuged for 2 minutes, to ensure removal of Buffer NT3. To ensure total removal of ethanol was achieved, the columns were also incubated for 5 minutes at 70°C prior to elution. To elute the DNA, the columns were placed into clean 1.5 ml centrifuge tubes, and 15 µl Buffer NE was added to each column. Because the expected DNA fragments were over 1,000 bp, the columns were incubated at 70°C for 5 minutes, and centrifuged for 1 minute, with the same procedure repeated using fresh Buffer NE, to give a total elution volume of 30 µl.

To clone the RACE products, the In-Fusion HD Cloning Kit from Takara Bio (Japan) was used. The ligation was performed by combining 1 µl Linearized pRACE vector, 7 µl RACE product, and 2 µl In-Fusion HD Master Mix, and incubated for 15 minutes at 50°C. A 100 µl vial of Stellar Competent Cells were thawed on ice, before 50 µl of the cells were transferred to 1.5 ml centrifuge tubes. 2.5 µl of the In-Fusion mixture containing the RACE products were added to each centrifuge tube, and placed on ice for 30 minutes, after which they were subjected to a heat shocks stage for 45 seconds at 42°C and returned to ice for 2 minutes. 1/100-1/5 of the transformations were transferred to separate tubes and brought to a volume of 100 µl with SOC medium, with the 100 µl spread on LB agar plates containing 100 µl/ml of Ampicillin. The remainder of the transformation reactions were centrifuged at 6,000 RPM for 5 minutes and the pellets were resuspended in 100 µl of SOC medium and spread on LB agar plates, with all plates incubated overnight at 37°C. The picking of colonies and isolation of plasmid DNA followed the method previously described when cloning the PCR fragments, and the characterisation of the DNA by restriction enzyme digestion utilised the *Eco*RI and *Hin*dIII enzymes.

Plasmid samples were sent to DBS Genomics, Durham University for sequencing and a Poly-T primer which was held on file by DBS Genomics was used for sequencing the samples.

Dot Blot method

For the dot blot, an unpublished protocol was used, which was developed by N. Beddoe (Personal communication) for RNA viruses. To prepare samples, small amounts of frozen tissue from *N. tabacum* 'Xanthi', *N. tabacum* 'Samsun', and *L. esculentum* were ground in 700 µl of nucleic acid extraction buffer (10 mM NaCl, 20 mM Tris HCl pH 8.0, 1 mM EDTA (Fan & Gulley, 2001)) in a 1.5 ml centrifuge tube, and 700 µl of phenol/chloroform mixture was added. The samples were centrifuged at maximum speed for 10 minutes, and 400 µl of the supernatant were transferred to a fresh tube and precipitated overnight at -20°C with 40 µl of ammonium acetate and 800 µl of 100% ethanol which had been chilled on ice. The samples were then centrifuged (Thermo Scientific) for 20 minutes at maximum speed and the supernatant discarded, with the pellets washed in 70% ethanol and re-suspended. The re-suspended pellets were centrifuged at 13,300 RPM for 10 minutes, with the supernatant discarded, and the pellets were air dried for 15 minutes before being re-suspended in 30 µl molecular biology grade H_2O.

Plasmid concentrations were diluted to 98.5 ng/µl, and denatured plasmid was prepared by thermal cycling a plasmid sample at 100°C for 9 minutes. The two plasmid samples were diluted to 100ng, 10ng, 1ng, and 100pg aliquots and spotted onto the dot blot membrane. Phenol/chloroform and sap samples prepared from the tissue samples were diluted twice for 4 dilutions and spotted onto the membrane and UV cross-linked for 180s using a Bio-rad Gel Doc (USA).

The blot membrane was pre-hybridised at 42°C for 1 hour in 20 ml of Ultrahyb buffer which had been preheated at 68°C. The probe was denatured in boiling water for 10 minutes and then snap frozen on ice for 4 minutes, and 2 µl of the denatured probe added to the Ultrahyb in the hybridisation cylinder, and hybridised overnight at 42°C. The membrane was subjected to four wash steps (2 x 15 minutes, and 2 x 30 minutes) and a blocking step, and finally subjected to a detection buffer and examined every 30 minutes for 2 hours, and again after 24 hours.

Chapter 3: Identification of partial viral sequences

Introduction

To determine if the chlorotic tomato material was infected with a virus, series of RT-PCR were conducted, however relevant primers were first required to be designed for the appropriate PCR reactions. Degenerate primers designed for *Potyvirus* (J. Abad USDA, personal communication with Monger, W) and *Potexvirus* (van der Vlugt & Berendsen, 2002) species were already available, and degenerate primers were designed to enable the amplification of all PepMV strains. The degenerate primers for *Potyvirus* and *Potexvirus* used targeted conserved portions of the genome across the viral species (respectively) as to ensure the amplification of any *Potyvirus* or *Potexvirus* which may be present within the infected tissue. Furthermore, primers used in the control PCR reactions also were required to be designed for TMV, as TMV presents similar symptoms when infecting tomato. Primers used for the positive control were also designed for the Tomato Actin gene, and Tobacco Actin gene.

Primer design

To design degenerate primers for PepMV, 17 PepMV whole genome sequences which covered all known strains of PepMV were acquired from Genkbank and aligned with their evolutionary relationship inferred by phylogenetic analysis using the Maximum likelihood method (based on the Tamura-Nei method) with all sites used and bootstrapped to 100 replications (**Figure 3.1**) highlighting the differing strain groups. From the data it could be seen that the TGB and Coat Protein portions of the genome were highly conserved between strains of PepMV, however, there are differences within these segments of the genome, which allows for differentiation of strains (**Figure 3.2**). When designing primers for TMV, a degenerate was not

required as a conserved region between the genomes analysed showed no variation in the targeted primer sequences. Two further primers were designed to be used as positive control reactions in the PCR reactions: Actin for tomato, and Actin for tobacco. The tobacco Actin primer was designed against an Actin mRNA sequence for *N. tabacu*m 'Xanthi' (**Table 3.1**).

Results from preliminary RT-PCR

RT-PCR was performed using the primer sets on the cDNA synthesised from the frozen *S. lycopersicum* tissue, with the products resolved on an agarose gel (**Figure 3.3**). Lane 1 shows the control for the RT-PCT reaction which amplified a fragment of the correct size (~1kb) for the primers designed to the actin cDNA sequence. Lane 3 shows a band of approximately 750bp which is correct for the degenerate POTX primers, and the same is true for lane 4 which is the degenerate POTY primers, also producing a band of the expected size. Lane 5 is the degenerate PepMV primer set which shows a strong band for the product of the correct size. From the PCR, there is evidence to suggest there are potexviruses (POTX), potyviruses (POTY), and PepMV present in the leaf sample.

After concluding there was a strong possibility of viral material in the tomato tissue, the PCR bands of the POTX, POTY and PepMV resolutions were extracted and sent for sequencing. Unfortunately, due to the relative concentration of the POTX and POTY fragments on the agarose gel, there was not enough DNA to complete the

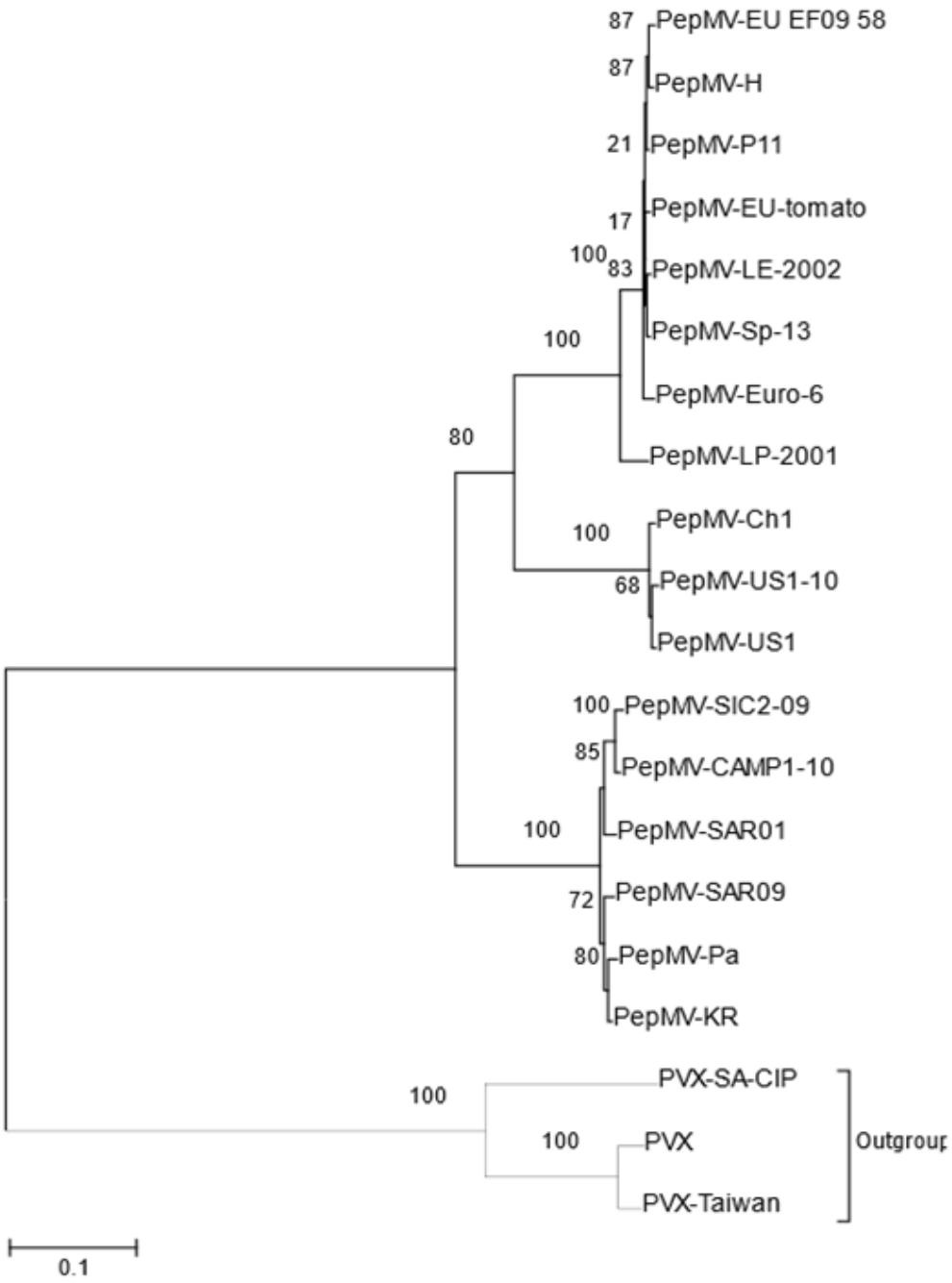

Figure 3.1. Phylogenetic relationship of multiple strains of PepMV used to design degenerate primers for the TGB portion of the genome based on complete genome sequences from Genbank: PepMV-EU EF09 58 (JQ314459.1), PepMV-H (AM491606.1), PepMV-P11 (JN133846.1), PepMV-EU-tomato (FJ940223.1), PepMV-LE-2002 (AJ606360.1), PepMV-SP-13 (AF484251.1), PepMV-Euro-6 (KF718832.1), PepMV-LP-2001 (AJ606361.1), PepMV-Ch1 (DQ000984.1), PepMV-US1-10 (KF718832.1), PepMV-US1 (FJ940225.1), PepMV-SIC2-09 (HQ663892.1), PepMV-CAMP1-10 (HG976946.1), PepMV-SAR01 (HQ663891.1), PepMV-SAR09 (HQ663892.1), PepMV-Pa (FJ612601.1), PepMV-KR (DI370279.1). The phylogenetic tree was inferred by using the Maximum Likelihood method base on the Tamura-Nei model bootstrapped to 100 replications. Potato virus X was used as an outgroup: PVX-SA-CIP (KJ534601.1), PVX (M38480.1), PVX-Taiwan (AF272736.2).

Forward

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PepMV-SP-13          5130 CTCCTAGAGCTGACCTCACTGACAC 5154
PepMV-LE-2002        5060 CTCCTAGAGCTGACCTCACTGACAC 5084
PepMV-LP-2001        5128 CTCCTAGAGCTGACCTCACTGACAC 5152
PepMV-H              5060 CTCCTAGAGCTGACCTCACTGACAC 5084
PepMV-KR             4996 CTCCTAGAGCTGATCTTACTGACAC 5020
PepMV-Ch1            5127 CTCCTAGAGCTGACCTCACTGACAC 5151
PepMV-Pa             5126 CTCCTAGAGCTGATCTTACTGACAC 5150
PepMV-EU-tomato      5130 CTCCTAGAGCTGACCTCACTGACAC 5154
PepMV-US1            5128 CTCCTAGAGCTGACCTCACTGACAC 5152
PepMV-CAMP1-10       5126 CTCCTAGAGCCGATCTTACTGACAC 5150
PepMV-SAR09          5126 CTCCTAGAGCTGATCTTACTGACAC 5150
PepMV-SIC2-09        5126 CTCCTAGAGCCGATCTTACTGACAC 5150
PepMV-SAR01          5126 CTCCTAGAGCTGATCTTACTGACAC 5150
PepMV-P11            5160 CTCCTAGAGCTGACCTCACTGACAC 5184
PepMV-EU EF09 58     5133 CTCCTAGAGCTGACCTCACTGACAC 5157
PepMV-Euro-6         5127 CTCCTAGAGCTGACCTCACTGACAC 5151
PepMV-US1-10         5057 CTCCTAGAGCTGACCTCACTGACAC 5081
PepMV-FWD                 CTCCTAGAGGYGAYCTYACTGACAC
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Reverse

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PepMV-SP-13          6200 GCTGCTCACTCCGTA GCTAAGTACG 6224
PepMV-LE-2002        6129 GCTGCTCACTCCGTA GCTAAGTACG 6153
PepMV-LP-2001        6198 GCTGCTCACTCAGTA GCTAAATATG 6222
PepMV-H              6130 GCTGCTCACTCCGTA GCTAAGTACG 6154
PepMV-KR             6067 GCTGCTCACTCGGTT GCTAAATATG 6091
PepMV-Ch1            6199 GCTGCTCACTCAGTT GCTAAGTATG 6223
PepMV-Pa             6197 GCTGCTCACTCGGTT GCTAAATATG 6221
PepMV-Eu-tomato      6200 GCTGCTCACTCCGTA GCTAAGTACG 6224
PepMV-US1            6200 GCTGCTCACTCAGTT GCTAAGTATG 6224
PepMV-CAMP1-10       6197 GCTGCTCACTCGGTT GCTAAATATG 6221
PepMV-SAR09          6195 GCTGCTCACTCGGTT GCTAAATATG 6219
PepMV-SIC2-09        6197 GCTGCTCACTCGGTT GCTAAATATG 6221
PepMV-SAR01          6297 GCTGCTCACTCGGTT GCTAAATATG 6221
PepMV-P11            6130 GCTGCTCACTCCGTA GCTAAGTACG 6154
PepMV-EU EF09 58     6203 GCTGCTCACTCCGTA GCTAAGTACG 6227
PepMV-Euro-6         6198 GCTGCTCACTCCGTA GCTAAGTACG 6222
PepMV-US1-10         6129 GCTGCTCACTCAGTT GCTAAGTATG 6153
PepMV-Rev                 GCTGCTCACTCRGTWGCTAARTAYG
```

Figure 3.2. The alignment of the 17 PepMV isolates and their relative positions within their Genbank sequences; PepMV-EU EF09 58 (JQ314459.1), PepMV-H (AM491606.1), PepMV-P11 (JN133846.1), PepMV-EU-tomato (FJ940223.1), PepMV-LE-2002 (AJ606360.1), PepMV-SP-13 (AF484251.1), PepMV-Euro-6 (KF718832.1), PepMV-LP-2001 (AJ606361.1), PepMV-Ch1 (DQ000984.1), PepMV-US1-10 (KF734961.1), PepMV-US1 (FJ940225.1), PepMV-SIC2-09 (HQ663892.1), PepMV-CAMP1-10 (HG976946.1), PepMV-SAR01 (HQ663893.1), PepMV-SAR09 (HQ663890.1), PepMV-Pa (FJ612601.1), PepMV-KR (DI370279.1)., highlighting the differences between the isolates and the primers which were designed against these sequences. The designed primers for PepMV are the last in each section and underlined.

	Primer	*Position*	*5'- 3' Primer sequences*	*Amplicon length (bp)*
Potexvirus	Potex-5 (forward)	3265-3284	CAY CAR CAR GCM AAR GAY CA	737
	Potex-1RC (reverse)	4001-3982	TCA GTR TTD GCR TCR AAR GT	
Potyviridae	PV2 (forward)	7962-7981	GGB AAY AAY AGY GGD CAR CC	1,014
	POT1 (reverse)	8952-8976	GAC TGG ATC CAT TBT CDA TTC ACC A	
Pepino mosaic virus	PEPMV-1 (forward)	5133-5157	CTC CTA GAG CYG AYC TYA CTG ACA C	1,096
	PEPMV-2 (reverse)	6205-6229	CGA CGA GTG AGY CAW CGA TTY ATR C	
Tobacco mosaic virus	TMV-1 (forward)	5485-5505	AGA TGA GTT CAT GGA AGA TGT C	778
	TMV-2 (reverse)	6242-6263	GAC GCA TGC TAT TCC GTA TCA CT	
Tomato Actin	Actin-1 (forward)	142-163	GCA TAA GAT GGC AGA CGG AGA G	1,050
	Actin-2 (reverse)	1169-1192	CAG ACC TAA CCT CCG AGA TAG AAC	
Tobacco Actin(Xanthi)	Tb-Actin-1 (forward)	148-167	CAC GAG GTC CGG AGA AGG TC	831
	Tb-Actin 2 (reverse)	961-979	GCG ACA TAC ATA GCA GGA G	

Table 3.1. Primers used in the study with their positions of the genome as well as their expected amplicon length.

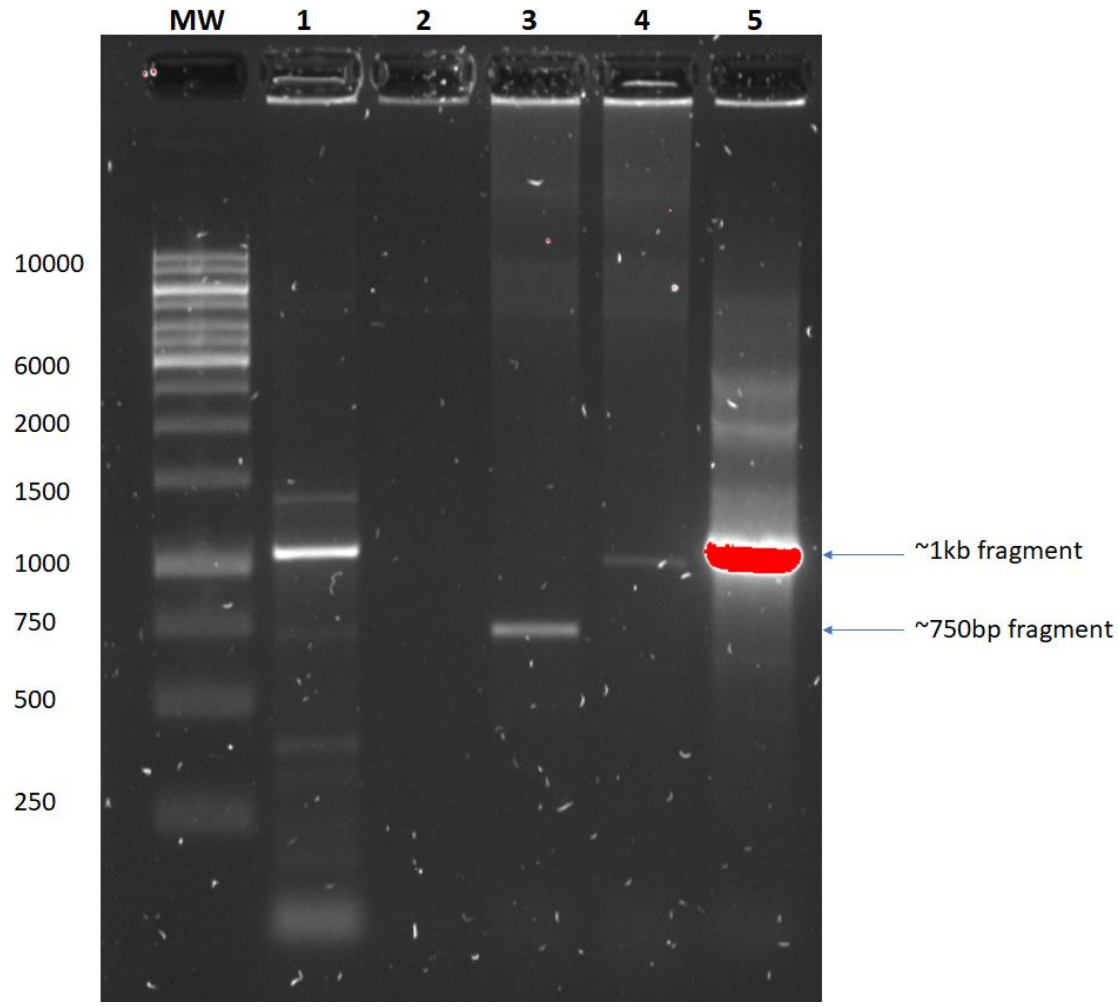

Figure 3.3. Preliminary PCR analysis of possible viral cDNA extracted from *S. lycopersicum* leaves. Lane 1 is a positive control (Actin, ~1kb), lane 2 is a negative control, lane 3 is a POTX degenerate primer (~750bp), lane 4 is a POTY degenerate primer (~1kb), and lane 5 is a *Pepino mosaic virus* degenerate primer (~1.1kb).

sequencing reactions. Attempts to rectify the issue by repeating the PCR reactions with additional DNA were performed, but the loss of DNA fragments during the clean-up stage resulted in failed sequencing reactions. However, the PepMV fragment was successfully sequenced, and sequence data was analysed using BLAST and showed sequence homology with PepMV of an identity of 94% with the isolates Pa, P19, P22, CH2, Bpo160, Bpo162, and Bpo158 (**Appendix I**).

Identification Conclusion

The results of the experiments performed for the identification of the suspected virus show there is evidence to suggest there is a virus present in the infected tomato material which was sent to the university. This is evident in the RT-PCR (**Figure 3.3**) which was performed which shows PepMV, POTX, and POTY fragments of the expected size for the primers used. When the PepMV fragment was extracted and cloned, the results from the BLAST report showed strong homology with known sequences of PepMV that corresponded with the TGB, confirming that PepMV was present in the material.

Chapter 4: Confirmation of infectious agent

Introduction

The RT-PCR results as mentioned in the previous chapter suggest there was an infectious virus present within the tomato material. To confirm this, various experiments were performed to isolate the cause of the infection.

Indicator plant inoculation

Tobacco cultivars were used as indicator plants to assess the symptoms caused by the viruses as well as to test if the viruses could be mechanically transmitted. The indicator plants were subjected to mechanical damage and inoculated with sap taken from the infected *S. lycopersicum* tissue which had been crushed in 0.1M phosphate buffer. Symptoms and severity were variable between species (**Table 4.1**), with all indicators showing symptoms to some degree, while conversely, all indicators also had instances of either no infection or symptomless infection, and all four species of indicator plant showed symptoms of infection (**Figure 4.1**).

	Number of replicates	***Symptomatic***	***Non-symptomatic***	***Symptoms***
N. glutinosa	29	25	4	M, Ch, Vc
N. benthamiana	33	21	12	Ch
N. tabacum 'Xanthi'	39	26	13	M, Ch, Nl*
N. tabacum 'Samsun'	20	15	5	M, Vc

Table 4.1. The indicator plants that were subjected to infection with the sap of the infected tomato material with the relative data showing the number of symptomatic and non-symptomatic replicates with the associated symptoms for each species and cultivar. Symptoms: M= Mosaic, Ch=Chlorosis, Vc=Vein clearing, Nl=Necrotic Lesions.

*Necrotic lesions did not appear on leaves until at least four weeks post inoculation.

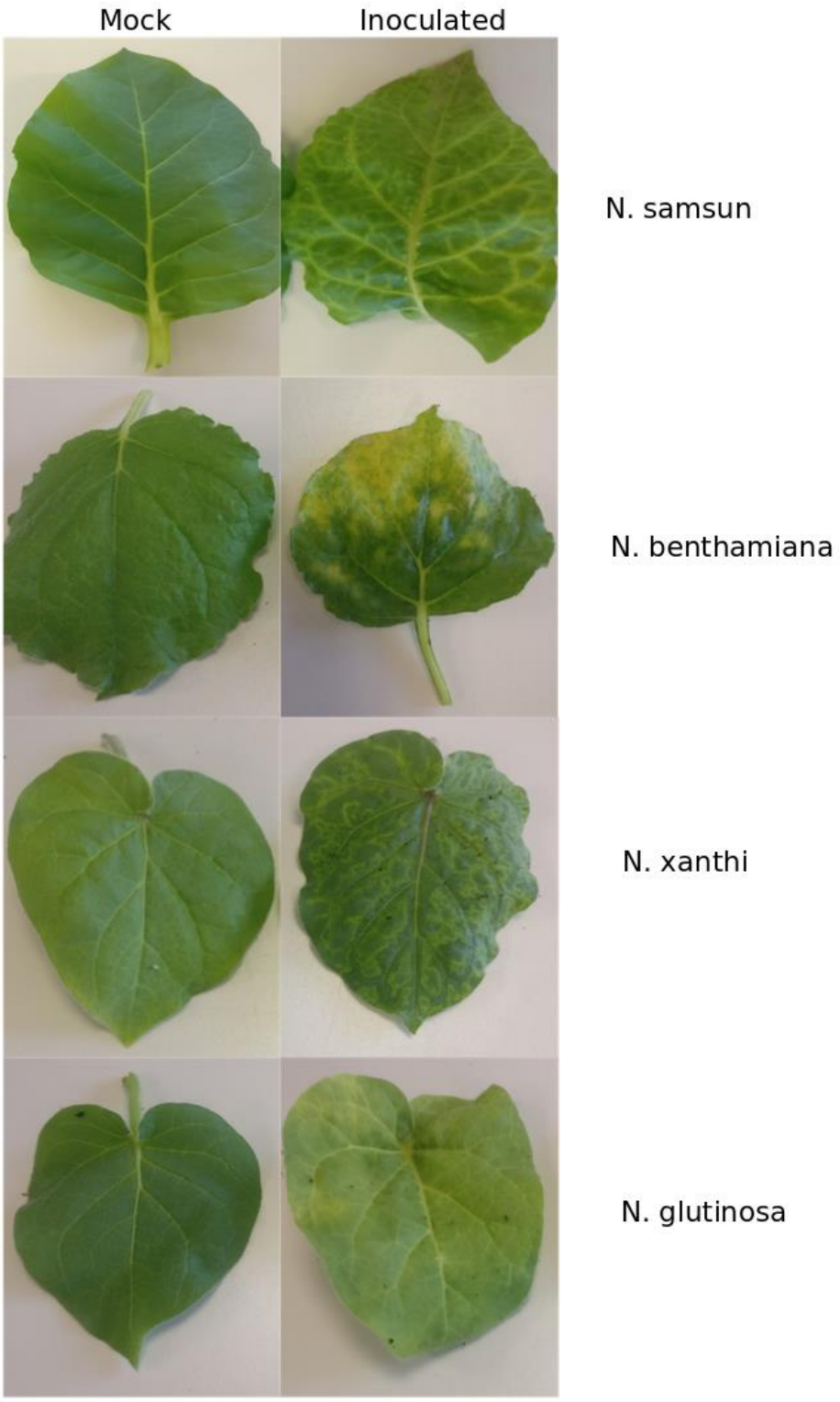

Figure 4.1. Symptoms displayed by systemically infected indicator plants three weeks post inoculation with sap obtained from infected tomato material. The mock leaves were inoculated using only 0.1M phosphate buffer, while the infected leaves were inoculated by crushing infected leaves with a pestle and mortar with the addition of 0.1M phosphate buffer.

The indicator plants were maintained for a period of six weeks post inoculation to document the changes in symptoms exhibited over time. While many of the symptoms did not change, there was a development of necrotic lesions on the systemically infected leaves of *N. tabacum* 'Xanthi' (**Figure 4.2**).

Figure 4.2. Necrotic lesions appeared on systemically infected *N. tabacum* 'Xanthi' leaves, which were infected with sap obtained from infected tomato material, four weeks post inoculation.

S. lycopersicum was also used as an indicator plant, however, the number of replicates was smaller, and was used to test the transmission route in tomato. These were maintained for eight weeks and did not show signs of infection. PCR was performed on the cDNA derived from RNA in the tomato leaves which had been inoculated with the same sap as the indicator plants. The resolved gel showed an abundant fragment of the correct size for PepMV, and the actin control (**Figure 4.3**).

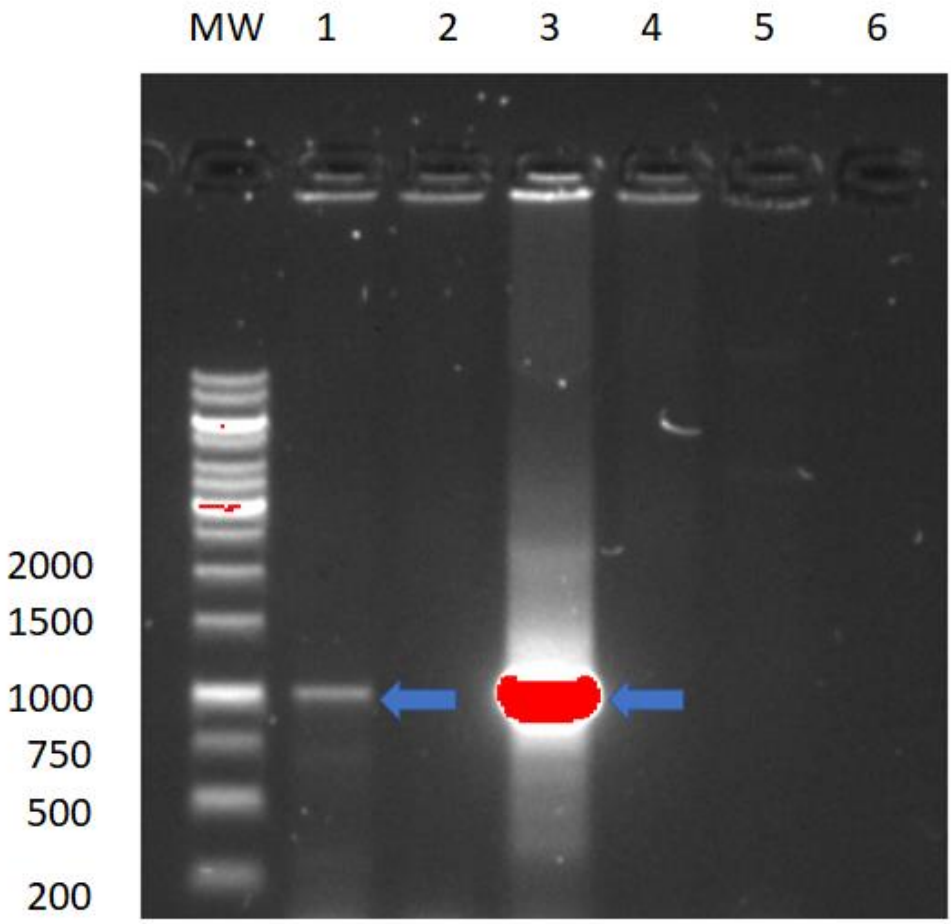

Figure 4.3. Agarose gel resolution from PCR reactions on inoculated tomato leaves with sap from the infected tomato material. Lane 1 is the control of ~1kb with the tomato actin primers, Lane 2 is the TMV primers showing no fragment, Lane 3 is the PepMV primers showing a fragment of ~1.1kb, Lane 4 is the POTY primers showing no fragment, Lane 5 is the POTX primers also showing no fragment, and Lane 6 is the negative control.

PCR results from indicator plants

RT-PCR reactions performed on cDNA synthesised from the RNA extracted from the bioindicators also shows evidence of viral infection (**Figures 4.4** & **4.5**).

The agarose gel resolution from *N. glutinosa* (**Figure 4.4,** A) shows transmission of PepMV, POTX, and POTY in the inoculated leaves, however in the systemically infected leaves (**Figure 4.4,** B), only the PepMV was detected.

N. tabacum 'Xanthi' displayed infection by PepMV in both the inoculated and systemically infected leaves, with the systemically infected leaves returning a higher concentration of amplified DNA than in the inoculated leaves (**Figure 4.4,** C & D).

N. benthamiana inoculated leaves showed amplification of both PepMV and POTY fragments, however, in the systemically infected leaves, neither of these was detected and a POTX fragment was amplified (**Figure 4.5,** E & F). The fragments obtained from *N. tabacum* 'Samsun' also showed amplified fragments corresponding to the PepMV and POTY primers (**Figure 4.5** G & H).

The PCRs also included the primers designed for TMV to ensure there was no cross contamination of TMV, and all PCRs were clear of any TMV amplicons.

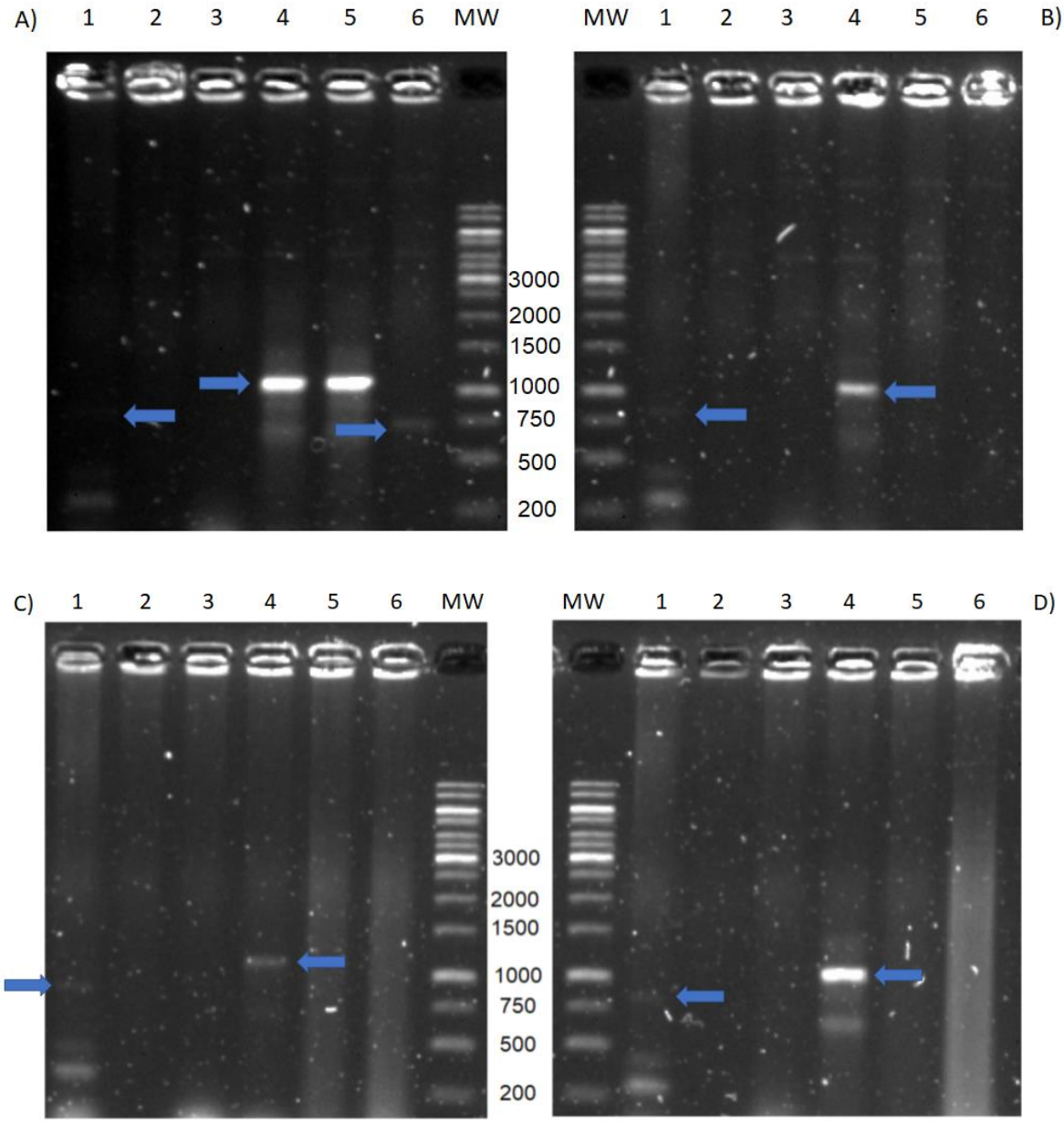

Figure 4.4. Agarose gel resolutions from PCR reactions on the indicator plants. **A)** *N. glutinosa* inoculated leaf extraction PCR reaction resolution, Lane 1 is the control reaction showing a faint expected band of ~750bp, Lane 2 is the negative control, Lane 3 is the TMV reaction, Lane 4 is the PepMV reaction using the PepMV degenerate producing an expected band of ~1.1kb, Lane 5 is the POTY degenerate with an expected band of 1kb, and Lane 6 is the POTX degenerate showing an expected band of ~750bp. **B)** *N. glutinosa* systemtic infection leaf extraction PCR reaction resolution, Lane 1 is the control again showing a faint band of ~750bp, Lane 2 is the negative control, Lane 3 is the TMV reaction, Land 4 is the PepMV degenerate producing a band of ~1.1kb, Lane 5 is the POTY degenerate, and Lane 6 is the POTX degenerate. **C)** *N. tabacum* 'Xanthi' inoculated leaf extraction PCR reaction resolution, Lane 1 shows the control fragment of ~750bp, Lane 2 is the negative control, Lane 3 is the TMV reaction, Lane 4 is the PepMV degenerate, Lane 5 is the POTX degenerate, and Lane 6 is the POTY degenerate. **D)** *N. tabacum* 'Xanthi' systemic leaf extraction PCR reaction resolution, Lane 1 shows the control fragment of ~750bp, Lane 2 is the negative control, Lane 3 is the TMV reaction, Lane 4 is the PepMV degenerate, Lane 5 is the POTX degenerate, and Lane 6 is the POTY degenerate.

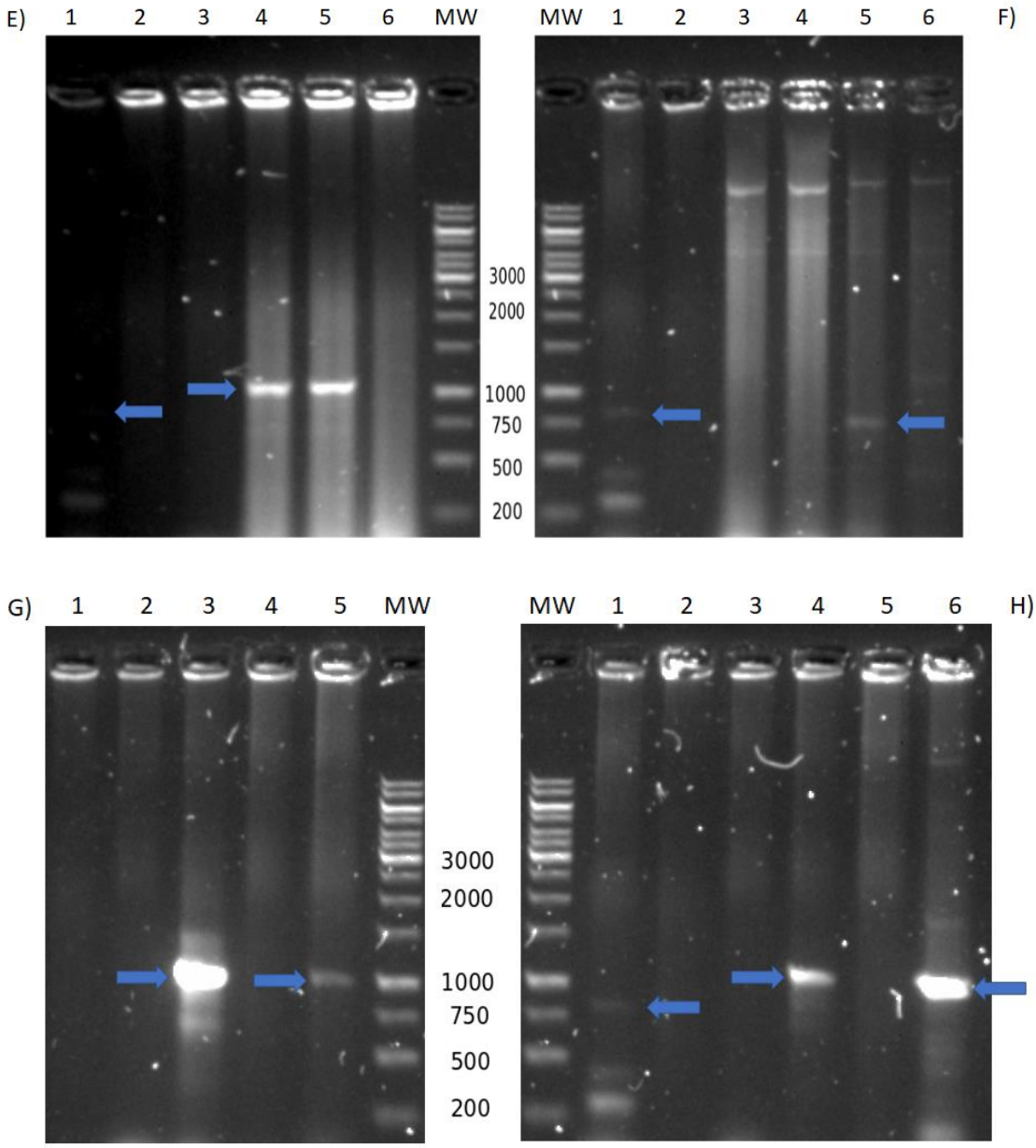

Figure 4.5. Agarose gel resolutions from PCR reactions on the indicator plants. **E)** *N. benthamiana* inoculated leaf extraction PCR reaction resolution, Lane 1 is the control reactions showing a faint expected band of ~750bp, Lane 2 is the negative control, Lane 3 is the TMV reaction, Lane 4 is the PepMV degenerate producing an expected band of ~1.1kb, Lane 5 is the POTY degenerate with an expected band of 1kb, and Lane 6 is the POTX degenerate which shows no clear fragments. **F)** *N. benthamiana* systemic infection leaf extraction PCR reaction resolution. Lane 1 is the control, Lane 2 is the negative control, Lane 3 is the TMV, Lane 4 is the PepMV, Lane 5 is the POTX showing a fragment of ~750bp, and Lane 6 is the POTY degenerate. **G)** *N. tabacum* 'Samsun' inoculated leaf extraction PCR reaction resolution, Lane 1 is the negative control, Lane 2 is the TMV, Lane 3 is the PepMV degenerate showing an intense band of ~1.1kb, Lane 4 is the POTX degenerate, and Lane 5 is the POTY degenerate showing a fragment of ~1kb. **H)** *N. tabacum* 'Samsun' systemic infection leaf extraction PCR reaction resolution, Lane 1 is the positive control showing an expected band of ~750bp, Lane 2 the negative control, Lane 3 is the TMV, Lane 4 is the PepMV degenerate showing an expected fragment of ~1.1kb, Lane 5 is the POTX degenerate, and Lane 6 is the POTY degenerate showing an expected band of 1kb.

Sequencing of amplified viral genomes

As the cloning protocol suggested using fresh PCR fragments to ensure successful cloning, an additional PCR was performed. For this *N. tabacum* 'Xanthi' was chosen to amplify the viral genome. While in the previous section it is described that there was no POTX product from N. tabacum 'Xanthi', during the second PCR, a fragment corresponding to the correct size was isolated (**Figure 4.6**).

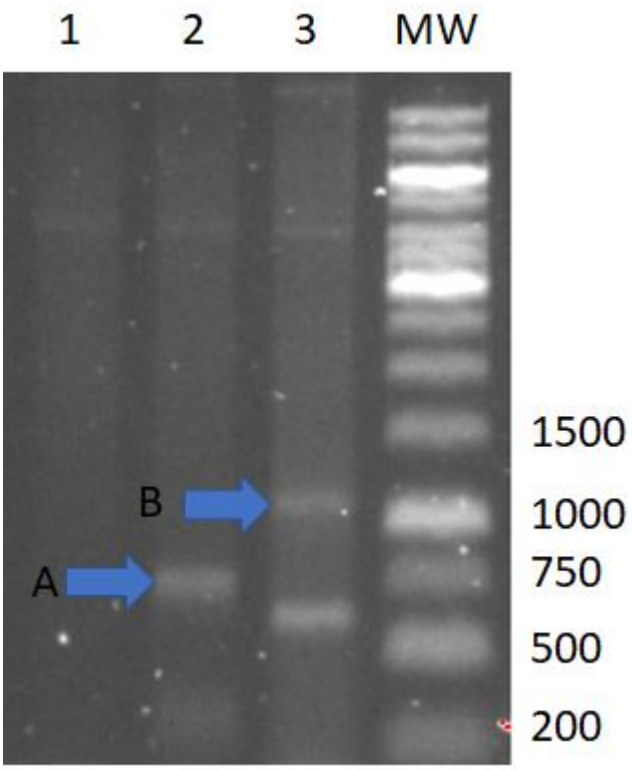

Figure 4.6. Additional PCR resolution for fragments used in cloning. Lane 1 is the negative control, Lane 2 is the POTX degenerate showing a fragment of ~750bp, and Lane 3 is the PepMV degenerate showing a fragment of ~1.1kb.

The POTX fragment and a PepMV fragment, were extracted from the agarose gel and cloned using the method set out in **Chapter 2**. The clones were then sent for sequencing, and while the PepMV sequencing reaction failed, the POTX degenerate sequencing was successful, and returned a sequence which corresponded with pepino mosaic virus. The clone was sequenced with both forward and reverse

reactions, and the sequence data showed few aberrations between the reactions. When analysed using BLAST, the cloned sequence showed sequence homology with the original Peruvian strain of PepMV with an identity of 98 % (MF4229614.1, MF422612.1, MF422616.1, AM109896.1, AJ606361.1) (**Appendix II**).

Confirmation of infectious agent conclusion

By using indicator plants, it was possible to show the transmission of the virus through mechanical inoculation. *N. glutinosa* showed signs of mild mosaic and chlorosis, which have been observed in previous studies. However, in *N. glutinosa* PepMV typically presents as severe mosaic and local chlorotic lesions (Jones, *et al*., 1980; Pospieszny, *et al*., 2008). In *N. tabacum* 'Xanthi' there was severe mosaic on all replicates which showed symptomatic infection. Past experiments indicate that the symptoms exhibited on the indicator plant vary depending on the strain of PepMV (Pospieszny, *et al*., 2008) and in some cases even result in non-symptomatic infection (Hasiów-Jaroszewska, *et al*., 2009). Occasionally, necrotic lesions were also observed (**Figure 5**). *N. benthamiana* displayed only with mild chlorosis, while *N. tabacum* 'Samsun' displayed mosaic and vein clearing.

Transmission of PepMV was also shown to be possible through mechanical transmission between the same species of tomato, however the transmission of a possible *Potyvirus* was not achieved. Despite PCR results showing a positive infection of PepMV in the tomato tissue, there were no symptoms observed, which may correspond with the Chilean 2 variety of the virus. A previous study had also found a similar result in which tomato plants were infected with the virus but showed neither local, nor systemic symptoms, despite ELISA tests positively confirming the presence of PepMV (Blystad, *et al*., 2015).

It was also shown that the RNA could be extracted from these indicator plants and cloned following cDNA synthesis. The viral fragments found in the indicator plants varied between species. The agarose gel resolutions from the indicator plants also indicate that the *Potyvirus* was also mechanically transmitted to *N. glutinosa, N. benthamiana,* and *N. tabacum* 'Samsun', but not *N. tabacum* 'Xanthi'. Furthermore, the DNA sequencing of the POTX fragment amplified with degenerate primers also detected the presence of PepMV in the indicator plants.

Chapter 5: Acquisition of full length PepMV sequence

Introduction

Despite the positive identification of Pepino mosaic virus infection being obtained as described in previous chapters, it was deemed necessary to attempt to obtain a full length sequence for the virus. By acquiring the full length sequence, in depth analysis of the virus could be performed, by examining the all gene encoding regions to determine any variations between the acquired sequence and known reference sequences.

Results

For the acquisition of the full-length sequence, the rapid amplification of cDNA ends (RACE) protocol was utilised, in which two Gene Specific Primers (GSP) were designed based on the partial sequence information obtained in previous experiments (**Chapter 4**). The POTX clone fragment which targeted the RNA-dependent RNA polymerase domain (RdRP), was selected to be used for the primer design for the RACE experiment, from the sequences obtained by DNA sequencing (**Appendix II**). The GSPs were designed to obtain two fragments which encompassed the full genome of PepMV, and sites were selected on homologous regions between the cloned fragments and the reference strain they corresponded to (**Figure 5.1**). Along with the universal primers in the RACE kit, the GSPs were designed to amplify fragments of 3891bp from the 5' end (GSP1) and 3145bp from the 3' end (GSP2). The RACE experiment was performed according to the protocol set out in **Chapter 2**. Two fragments were resolved on an agarose gel and the GSP2 fragment was of the correct size, the GSP1 fragment was not the correct size and was short by approximately 1000bp (**Figure 5.2**).

```
LP_BPO163    3891 CATTATGAGGTTGTCTGGTGAAGGTCC
POTX-FWD          CATTATGAGGTTGTCGGGTGAAGGTCC
POTX-REV          CATTATGAGGTTGTCGGGTGAAGGTCC
GSP1              CATTATGAGGTTGTCGGGTGAAGGTCC

LP_BPO_163   3263 CAACTCCGGAAGCCAATTTACGAG
POTX-FWD          CAACTCCGGAAGCCAATTTACGAG
POTX-REV          CAACTCCGGAAGCCAATTTACGAG
GSP2              CAACTCCGGAAGCCAATTTACGAG
```

Figure 5.1. Alignment of Gene specific primers (GSP) with the reference strain of PepMV and the POTX clone sequences.

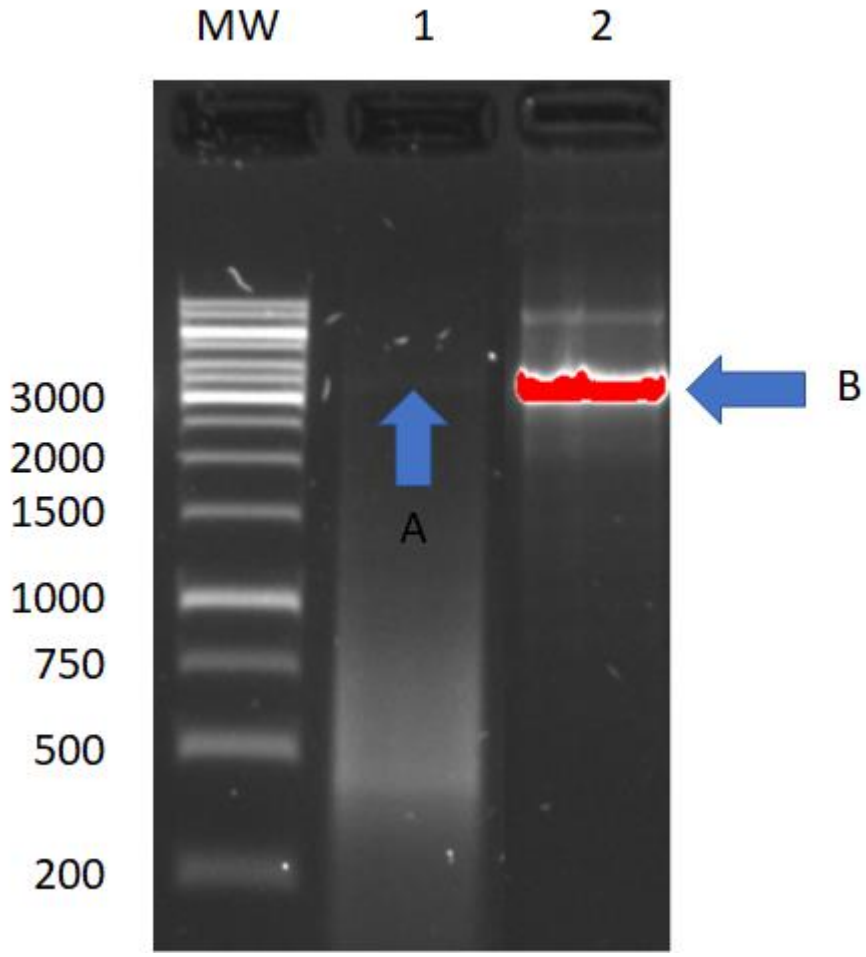

Figure 5.2. The results of the resolved RACE fragments on a 0.8% agarose gel. Lane 1 is the GSP1 fragment which is weakly stained and is of the incorrect size predicted for the primers used (~3,000bp), and Lane 2 is the GSP2 fragment which is of the correct size (~3100bp).

While the GSP2 fragment returned a strongly stained band, the GSP1 was faint (**Figure 5.2**). Despite this, both fragments were isolated, cloned and sent for DNA sequencing. Unfortunately, the GSP1 clone did not generate sequence, however, the GSP2 clones were successfully sequenced with a Poly-T primer. The sequences were base called to exclude incorrect base calling and when subjected to a BLAST search, were found to show homology with PepMV (**Table 5.1**). Of the 10 clones sent for sequencing, eight generated data, one failed to produce sequences, and one recorded partial coat protein encoding sequence.

	Base pairs	Identity match (%)	Accession match
Clone 2	967	99	MF422614.1
Clone 3	862	99	MF422614.1
Clone 4	1104	98	MF422614.1
Clone 5	1285	94	AM109896.1
Clone 6	929	99	MF422614.1
Clone 8	779	99	MF422614.1
Clone 9	937	99	MF422614.1
Clone 10	927	99	MF422614.1

Table 5.1. Results of a BLAST search for the clones of the GSP2 fragment with the size of the sequenced data returned, the identity match percentage with reference strains, and the strain of the highest similarity. Clone 1 and clone 7 were excluded due to a failed reaction and insufficient data, respectively. The accession numbers shown are all of the Peruvian strain.

The sequences were analysed to find regions of homology with the reference strains, and it was found that the clones aligned for the coat protein gene of PepMV. The coat protein sequence for the clones corresponded with the original Peruvian strain of PepMV, showing 100% identity with four known full-length genome sequences (Genbank: MF422616.1, MF422614.1, MF422612.1, AM109896.1, AJ606361.1) (**Appendix III**). Clone 5 showed the most complete sequence return out of the 10 isolates sent for DNA sequencing. As a result, it was possible to find the TGB3

sequence within this data, which showed a sequence identity with the Peruvian strain of the virus (**Appendix IV**). However, the PCR fragment which was sequenced from the PepMV-1 and PepMV-2 primers returned the TGB3 portion of the genome, and a partial coat protein. The TGB3 gene coding sequence for PCR fragment did not correspond to the Peruvian strain, and instead, matched the Chilean 2 strain with an identity of 100% with known Chilean strains of the virus (Genbank: MF422615.1, MF4226131.1, MF422611.1, HQ650560.2) (**Appendix V**). As a summary of the acquired sequences and their relationship with various strains of PepMV was analysed phylogenetically (**Figure 5.3**).

The single point mutations within the clone sequence data changed the amino acid codons (**Appendix VI**). For the coat protein of clone 2 there is an amino acid change at residue 122, S vs P, clone 3 sees a substitution at residue 105, V vs A, and clone 5 at residue 206, P vs T. There is also a 17 amino acid sequence at the 3' end of the gene which is different from the reference sequences (**Figure 5.4**).

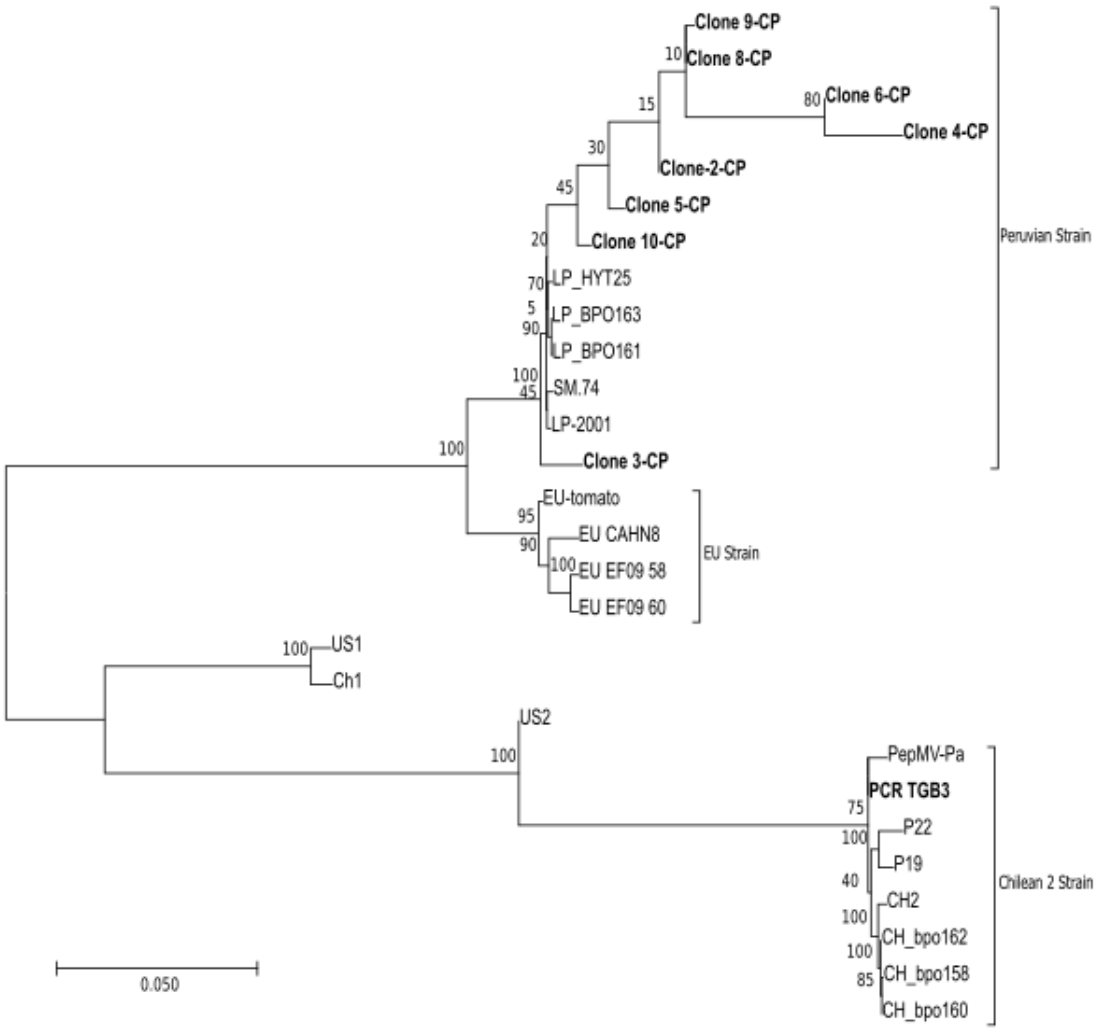

Figure 5.3. Phylogenetic analysis of the coat protein clones from the 3'RACE experiment and the TGB3 sequence from the PepMV sequenced fragment, which shows the evolutionary relationship between the two results, with the clones and PCR results showing in **bold**. The tree shows the 3'RACE coat protein sequences to be closely related to the original Peruvian strain of the virus, whereas the TGB3 sequence aligns more closely to the Chilean 2 strain, which indicates there are multiple strains of the virus present in the infected tomato material. The sequences were aligned with whole genome sequences from Genbank: LP_HYT25 (MF422616.1), LP_BPO163 (MF422614.1), LP_BPO161 (MF422612.1), SM.74 (AM109896.1), LP-2001 (AJ606361.1), EU-tomato (FJ940223.1), EU CAHN8 (JQ314457.1), EU EF09 58 (JQ314459.1), EU EF09 60 (JQ314461.1), US1 (AY509926.1), Ch1 (DQ000984.1), US2 (AY509926.1), PepMV-Pa (FJ612601.1), P22 (HQ650560.2), P19 (HQ650559.1), CH2 (JN835466.1), CH_BPO162 (MF422613.1), CH_BPO158 (MF422615.1), CH_BPO160 (MF422611.1), and a Maximum likelihood tree was constructed using the Tamura-Nei model and bootstrapped to 100 replications.

```
LP-BPO163-AA     208 NYITTLGEVTRGHMGGANTMYAIDAPPEL
LP_HYT25-AA      208 NYITTLGEVTRGHMGGANTMYAIDAPPEL
LP_BPO161-AA     208 NYITTLGEVTRGHMGGANTMYAIDAPPEL
Clone-2-AA       208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-3-AA       208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-4-AA       208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-5-AA       208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-6-AA       208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-8-AA       208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-9-AA       208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-10-AA      208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
```

Figure 5.4. Highlighted are the differences found between the reference amino acid sequence for known PepMV isolates, and the amino acid sequence for the clones of the coat protein which were derived from the 3' RACE experiment.

Conclusion

From the data gathered and analysed, there is reason to believe that the virus infecting the tomato material is PepMV. From the coat protein it can be suggested that the Peruvian strain is infecting the tomato material (**Appendix III**). There is also reason to believe that there is a coinfection of two PepMV strains within the infected tomato material. This may explain the symptoms exhibited in the indicator plants, as previous studies have suggested that when *N. glutinosa* and *N. tabacum* 'Samsun' are infected with a single strain of PepMV they are non-symptomatically infected, however when coinfected with the Peruvian and European strains, symptoms were present (Gómez, *et al.*, 2009).

The amino acid substitutions in clones 2, 3 and 5 could be due to a PCR generated error in the nucleotide sequence or this could be a genuine substitution and as a result may alter the interaction between the virus and the host of which it infects as may the 3' end of the coat protein sequence. It is unclear at this time if these are genuine substitutions or erroneous, but it is certainly an area for future research.

Chapter 6: Rapid detection of viral RNA

Introduction

PepMV is a highly infectious virus which is easily spread among crops by mechanical transmission (Soler, *et al*., 2002), and is capable of infecting multiple species within the *Solanaceae* family as well as some species from other families (Córdoba, *et al*., 2004). While there are several methods in place to detect the virus, alternative methods may also prove beneficial to controlling the impact of the virus on agricultural crops.

Current methods of viral detection

Current methods of screening for PepMV use RT-PCR, real time RT-PCR and ELISA methods. While using PCR methods are advantageous due to the specificity, screening may take considerable time due to isolating RNA and synthesizing cDNA, and are prone to contamination resulting in false positives, as well as the resources required for sequencing and analysis (Webster, *et al*, 2004).

Using the ELISA technique allows for rapid detection of viral presence within tissue material, with results obtainable within as little as four hours (Thermo Scientific, 2010). However, ELISA requires the application of known antigens, which may increase the overall time and cost in detecting viral presence in large numbers of seedlings.

Results

The method utilised the PepMV cloned plasmid data from **Chapter 2**. The probe bound to the degenerate *Potexvirus* plasmid on the membrane, and clearly bound to the tomato sap, after 30 minutes of residing in the detection buffer. It can also be seen that the probe does not bind non-specifically, as after 24 hours of residing in

detection buffer, the mock sap samples were not highlighted, confirming the experiment was successful, as well as a weak signal for *N. tabacum* 'Samsun' (**Figure 6.1**)., of which the infection was confirmed in **Chapter 4**. The phenol/chloroform extracted samples did not show on the blot, and while there does appear to be a weak blot, this is thought to be attributed to mechanical damage when applying the samples to the blot membrane.

Conclusion

While this experiment was only briefly run and without replicates due to time constraints, it may be possible to use this method as a means of rapidly assessing if a seedling may be infected with PepMV. The experiment also showed that in order to detect viral presence with this method, a clean sample is not required and can be performed by simply crushing tissue samples and applying directly onto the blot membrane. Additionally, the probe used in the experiment was from a degenerate *Potexvirus* sequence, which may provide a basis for detecting other potexviruses with the same probe, there by limiting the number of reference sequences required for the rapid detection of viral infection in seedlings. Further optimization would need to take place for this specific virus, as the protocol was based on a nerine virus.

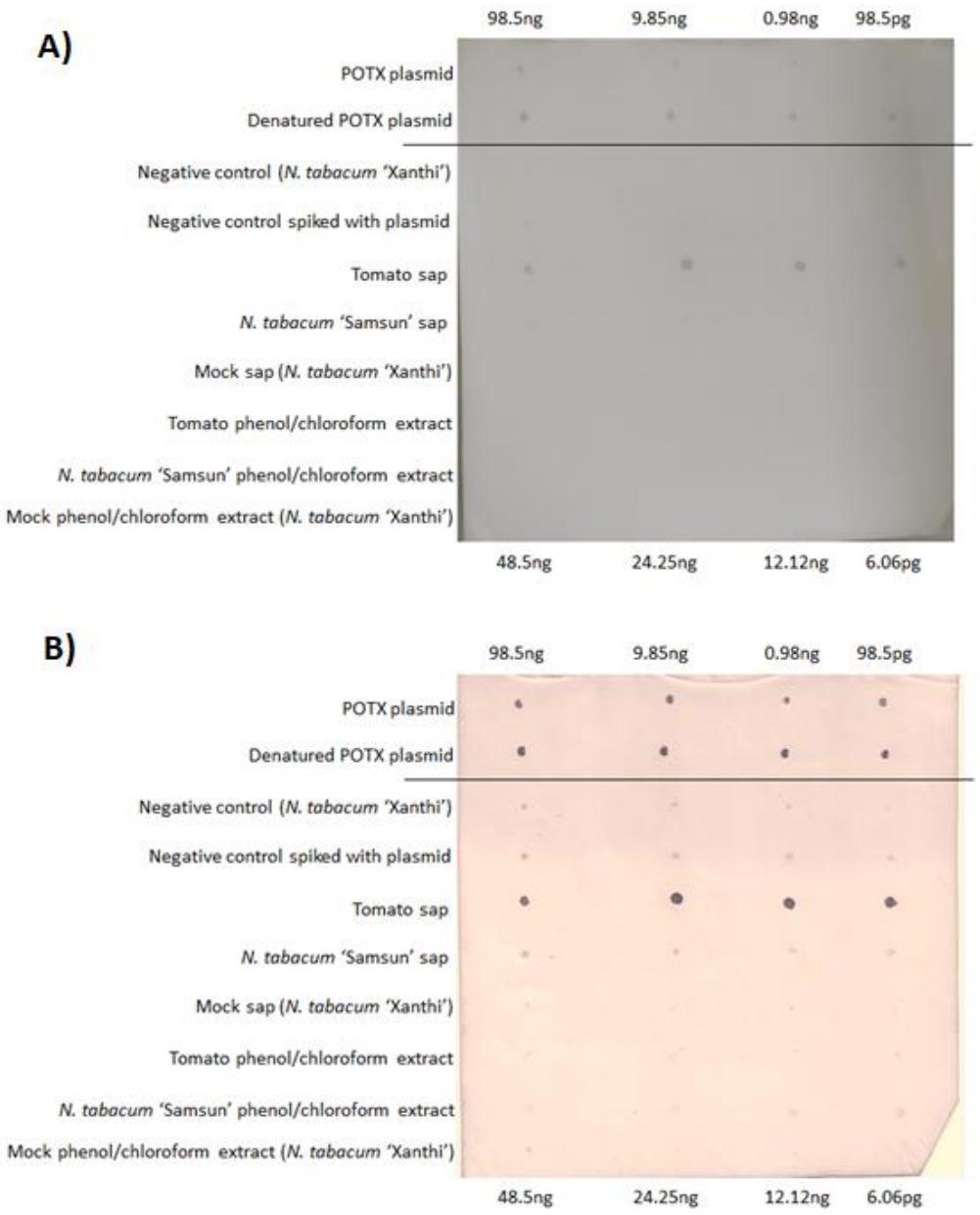

Figure 6.1. Results from the Dot blot using the POTX plasmid, Mock (*N. tabacum* 'Xanthi'), original infected tomato material, and *N. tabacum* 'Samsun'. **A)** The results of the dot blot after incubating in detection buffer for 30 minutes shows non-specific binding of the probe with the plasmid spots, and the tomato sap. **B)** 24 hours of residing in detection buffer. The plasmid samples and tomato sap show a strong signal for the viral RNA, while the other samples show a weak signal, and the probe did not bind to the mock phenol/chloroform extracts.

Chapter 7: Discussion

The university were sent tomato leaves which appeared to be diseased. The project aimed to identify the cause of the infection in the tomato material, and obtain a full-length sequence of the genome of any virus present, as well as to develop a method for the rapid detection of the agent causing the infection.

Identification and Sequence acquisition

Through a series of experiments, the results indicate that the cause of the infection of the tomato material is PepMV. As described previously, PepMV has been found in tomato crops in glasshouses throughout the world, and is currently spreading through commercial glasshouses in Europe, with Spain (Pagán, *et al.*, 2006) and Italy (Tiberini, *et al.*, 2011) reporting widespread infection.

The virus itself is capable of being transmitted mechanically, which is of concern as the spread of the virus throughout a glasshouse could occur rapidly, resulting in significant crop quality degradation and loss of revenue for the glasshouse (Spence, *et al.*, 2006).

The experiments examined not only the presence of PepMV within the tomato leaves, but also whether the virus was infectious. This was achieved using indicator plants, where leaves were infected with the sap of the original sample, to see if symptoms were produced. In all species of indicator plant used symptoms did appear, but the symptoms varied somewhat between each replicate, with some replicates showing clear symptoms, and others presenting as asymptomatic (**Chapter 4**).

The results not only showed that the cause of the infection was PepMV, but also that possibly two strains of the virus were present within the original material. Coat

protein and TGB3 analysis from the RACE experiment indicated that one form of the virus is that of the Peruvian strain. A previous study suggested that it is possible to identify the species and genus of potviruses by examining the coat protein sequence (Shukla & Ward, 1989). While this claim has not been made in regard to potexviruses, the phylogenetic tree in **Chapter 5** seems to suggest that the same may be possible for potexviruses, with the possibility of the TGB3 sequence also as a candidate for identification.

While the study set out to obtain a full-length sequence of the virus, it was only possible to obtain a partial sequence. This corresponded with the 3' end of the genome which encompasses the coat protein and TGB3. The gene specific primers which targeted the 5' end of the genome, while producing a fragment, were not of the correct size, nor was it possible to sequence this fragment. It would be useful to redesign the primers used for this part of the experiment and repeat the RACE for the 5' end to attempt to resolve the issue.

Rapid detection method

As PepMV is mechanically transmitted and has been shown to readily infect *Solanaceae* crops (Gómez, *et al*., 2009), it is imperative to develop a rapid and inexpensive system of screening seedlings entering a commercial greenhouse for the virus. While the virus can be isolated and identified through conventional methods such as PCRs and indicator plants, this is a time-consuming process.

The study used the dot blot method to assess the suitability of the technique for the rapid detection of PepMV. While the experiment was successful in that the probe bound to the virus in the infected tomato sap, plasmid, and denatured plasmid, and displayed clear results within 30 minutes, the membrane was required to be

submerged in the detection buffer for 24 hours before further reactions were visible, notably, the *N. tabacum* 'Samsun'.

Previous studies which have looked at the rapid detection of plant viruses have focused on the use of universal primers to amplify a selected conserved region throughout viruses of a specific genus (van der Vlugt & Berendsen, 2002). While this method is useful, and has been shown to produce the results required, it also involves the extraction of RNA and synthesis of cDNA from the infected plant, which can be time consuming in the case of a glasshouse where bulk quantities of seedlings may be introduced at any one time. The method described in this study however, indicates that a large number of seedlings may be screened in a shorter amount of time and require fewer additional steps, as the virus has been shown to be detectable from the sap extracts of infected material (**Figure 14**).

While the method was not optimised for the detection of PepMV and was originally designed for detecting Nerine latent virus (N. Beddoe, Personal communication), the results show promise and would need further optimising for use with PepMV.

Additional findings

PepMV has been described as infecting glasshouse crops in the United Kingdom previously, however it does not appear to have been reported in the country since 2001. Previous studies have shown that PepMV has been isolated in glasshouses in the United Kingdom previously involving six UK isolates with the authors concluding that the strains present were different from either the Peruvian or Chilean 2 strains (Mumford & Metcalfe, 2001). A study two years later, also collecting UK isolates, only sought to identify the virus and did not report on the strain present (Verhoeven, *et al*., 2003), as such, at this time it is not possible to

determine whether the introduction of the Peruvian and Chilean 2 strain to the United Kingdom is the first instance of tomato crops being infected with these strains.

It has been noted previously that the virus is capable of not only reducing the yield of fruits in tomato plants, but also adversely affecting the quality of the fruits as well (Spence, *et al*., 2006). The study did use tomato for inoculation, and while only a limited sample size was used, a few interesting results were obtained. It could be seen between the infected tomato plants and the tomato plants used as mocks, there was a clear reduction in fruit yield, where infected plants would produce one or two fruits, and the mock plants six or seven. These plants were maintained for six weeks under the same constant conditions and was found that while the fruits from the infected plants were not adversely infected in terms of quality, they did mature at an increased rate than those produced by the mock plants and were larger in size (personal observation).

Unknown Potyvirus

It is possible that an uncharacterised *Potyvirus* was also present within the tomato material. This was shown by PCR data from not only the original tissue but also several indicator plants which displayed fragments of the correct size expected to be amplified by the degenerate *Potyvirus* primers used in the study. Because it was not possible to sequence the fragments produced in the PCRs it is not possible to say whether this is an actual *Potyvirus* or erroneous data. While no previous reports of coinfection between *Potyvirus* species and PepMV have been found, there have been reports which suggest that potyviruses, when coinfecting a host with PVX exhibit synergistic effects which increase the severity of the symptoms produced (Vance, *et*

al., 2005), and this could explain partially the severity of the symptoms found on the *N. tabacum* 'Samsun', and *N. tabacum* 'Xanthi' indicator plants.

Conclusion

The study set out to answer the question of whether the tomato material was infected with a virus, and whether a rapid detection method could be developed for this. The study found that the material was infected with PepMV, as well as a possible unknown Potyvirus. Further to this it is possible that two strains of PepMV were coinfecting the original sample and this may have contributed, as well as the possible Potyvirus, to the symptoms displayed when indicator plants were inoculated with the sap from the sample. A partial sequence for PepMV was obtained encompassing the TGB3 and coat protein, which was linked to the Peruvian strain of the virus. Analysis of the PCR fragment which was sent for DNA sequencing revealed the possibility of the second strain belonging to the Chilean 2 strain. A rapid detection method was also examined, and while the results were mixed, there is a possibility of the development of this system, as the dot blot used showed the detection of PepMV in the tomato sap after incubation in detection buffer for 30 minutes.

While some objectives were not fully achieved, the study does lay the groundwork for future research, in particular, obtaining a full-length sequence of the virus, and optimising the dot blot for the detection of PepMV from sap. Further research could also examine the use of using specific gene sequences of potexviruses to determine if these are suitable for the characterisation of viral species and strains.

References

Abou-Jawdah, Y., Sobh, H. & Saad, A., (2001). Incidence of potato virus diseases and their significance for a seed certification program in Lebanon. *Phytopathologia Mediterranea,* 40(2), pp. 113-118. Doi: 10.14601/Phytopathol_Mediterr-1600

Adkins, S., (2001). Tomato spotted wilt virus-positive steps towards negative success. *Molecular Plant Pathology*, 1(3), pp. 151-257. Doi: 10.1046/j.1364-3703.2000.00022.x

Aguilar, J. M., Hernández-Gallardo, M, D., Cenis, J, L., Lacasa, A. & Aranda, M, A., (2002). Complete sequence of the Pepino mosaic virus RNA genome. *Archives of Virology,* 147(10), pp. 2009-2015. Doi: 10.1007/s00705-002-0848-9

Alemu, T., Hamacher, J. & Dehne, H. W., (2002). *The role of some weeds as hosts of Capsicum viruses in the rift valley parts of Ethiopia.* s.l., Mededelingen - Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent, pp. 283-289.

Alfaro-Fernández, A., del Carmen Córdoba-Sellés, M., Herrera-Vásquez, J, A., del Carmen Cebrián, M. & Jordá, C., (2010). Transmission of Pepino mosaic virus by the Fungal Vector *Olpidium virulentus*. *Journal of Phytopathology,* 158(4), pp. 217-226. Doi: 10.1111/j.1439-0434.2009.01605.x

Aramburu, J., Galipienso, L. & Matas, M., (2006). Characterization of potato virus Y isolates from tomato crops in northeast Spain. *European Journal of Plant Pathology,* 115(2), pp. 247-258. Doi: 10.1007/s10658-006-9003-x

Atabekov, J., Dobrov, E., Karpova, O. & Rodionova, N., (2007). Potato virus X: structure, disassembly and reconstitution. *Molecular Plant Pathology,* 8(5), pp. 667-675. Doi: 10.1111/j.1364-3703.2007.00420.x

Balique, F., Colson, P. & Raoult, D., (2012). Tobacco mosaic virus in cigarettes and saliva of smokers. *Journal of Clinical Virology,* 55(4), pp. 347-376. Doi: 10.1016/j.jcv.2012.08.012

Baltimore, D., (1971). Expression of animal virus genomes. *Bacteriological Reviews,* 35(3), pp. 236-241.

Baracsi, É., Tóth, E, K., Kriston, É., Takács, A., Pribék, D. & Horváth, J., (2002). Study of viruses of *Petunia* in Hungary. *International Journal of Horticultural Science,* 8(3/4), pp. 55-59.

Ben Moussa, A., Makni, M. & Marrakchi, M., (2000). Identification of the principle viruses infecting tomato crops in Tunisia. *Bulletin OEPP,* 30(2), pp. 293-296. Doi: 10.1111/j.1365-2338.2000.tb00898.x

Blystad, D.-R., van der Vlugt, R., Alfaro-Fernández, A., del Carmen Córdoba, M., Bese, G., Hristova, D., Pospieszny, H., Mehle, N., Ravnikar, M., Tomassoli, L., Varveri, C. & Nielsen, S, L., (2015). Host range and symptomatology of Pepino mosaic virus strains

occurring in Europe. *European Journal of Plant Pathology,* 143(1), pp. 43-56. Doi: 10.1007/s10658-015-0664-1

Boguel, S., Ameline, A. & Giordanengo, P., (2011). Assessing aphids potato virus Y-transmission efficiency: A new approach. *Journal of Virological Methods,* 178(1-2), pp. 63-67. Doi: 10.1016/j.jviromet.2011.08.013

Bostan, H. & Haliloglu, K., (2004). Distribution of PLRV, PVS, PVX and PVY (PVY^N, PVY^O and PVY^c) in the seed potato tubers in Turkey.. *Pakistan Journal of Biological Sciences,* 7(7), pp. 1140-1143. Doi: 10.3923/pjbs.2004.1140.1143

Broadbent, L. & Fletcher, J, T. (1963) The epidemiology of tomato mosaic. *Annals of Applied Biology*, 52(2), pp. 233-241. Doi: 10.1111/j.1744-7348.1963.tb03747.x

Carmichael, D. J., Rey, M, E, C., Naidoo, S., Cook, G. & van Heerden, S, W., (2011). First Report of Pepino mosaic virus Infecting Tomato in South Africa. *Plant Disease,* 95(6), pp. 767. Doi: 10.1094/PDIS-01-11-0036

Chang, K.-C., Chang, L-T., Huang, Y-W., Lai, Y-C., Lee, C-W., Liao, J-T., Lin, N-S., Hsu, Y-H. & Hu, C-C., (2017). Transmission of Bamboo mosaic virus in Bamboos Mediated by Insects in the Order Diptera. *Frontiers in Microbiology,* 8(870), pp. 1-13. Doi: 10.3389/fmicb.2017.00870

Chiunga, E. & Valkonen, J. P. T., (2013). First Report of Five Viruses Infecting Potatoes in Tanzania. *Plant Disease,* 97(9), pp. 1260. Doi: 10.1094/PDIS-02-13-0143-PDN

Clausen, A. M., Colavita, M., Butzonitch, I. & Valeria Carranza, A., (2005). A Potato Collecting Expedition in the Province of Jujuy, Argentina and Disease Indexing of Virus and Fungus Pathogens in Andean Cultivars. *Genetic Resources and Crop Evolution,* 52(8), pp. 1099-1109. Doi: 10.1007/s10722-004-6131-z

Córdoba, M, C., Martínez-Priego, ll. & Jordá, C., (2004). New Natural Hosts of *Pepino mosaic virus* in Spain. *Plant Disease*, 88(8), pp. 906. Doi: 10.1094/PDIS.2004.88.8.906D

Cotillon, A. -C., Girard, M. & Ducouret, S., (2002). Complete nucleotide sequence of the genomic RNA of a French isolate of Pepino mosaic virus (PepMV). *Archives of Virology,* 147(11), pp. 2231-2238. Doi: 10.1007/s00705-002-0873-8

Davenport, G. F. & Baulcombe, D. C., (1997). Mutation of the GKS motif of the RNA-dependent RNA polymerase from potato virus X disables or eliminates virus replication. *Journal of General Virology,* 78(6), pp. 1247-1251. Doi: 10.1099/0022-1317-78-6-1247

del Carmen Códoba-Sellés, M., García-Rández, A., Alfaro-Fernández, A. & Jordá-Gutiérrez, C., (2007). Seed Transmission of Pepino mosaic virus and Efficacy of Tomato Seed Disinfection Treatments. *Plant Disease,* 91(10), pp. 1250-1254. Doi: 10.1094/PDIS-91-10-1250

del Toro, F., Fernández F, T., Tilsner, J., Wright, K, M., Tenllado, F., Chung, B, N., Praveen, S. & Canto, T., (2014). Potato virus Y HCPro Localization at Distinct,

Dynamically Related and Environment Influenced Structures in the Cell Cytoplasm. *Molecular Plant-Microbe Interactions,* 27(12), pp. 1331-1343. Doi: 10.1094/MPMI-05-14-0155-R

Dougherty, W. G. & Carrington, J. C., (1988). Expression and Function of Potyviral Gene Products. *Annual Review of Phytopathology,* 26, pp. 123-143. Doi: 10.1146/annurev.py.26.090188.001011

Efthimiou, K. E., Gatsios, A, P., Aretakis, K, C., Papyiannis, L, C. & Katis, N, I., (2010). First report of Pepino mosaic virus infecting greenhouse cherry tomatoes in Greece. *Plant Disease,* 95(1), pp. 78. Doi: 10.1094/PDIS-09-10-0643

El-Araby, W. S., Ibrahim, I, A., Hemeida, A, A., Mahmoud, A., Soliman, A, M., El-Attar, A, K. & Mazyad, H, M., (2009). Biological, serological and molecular diagnosis of three major potato viruses in Egypt. *International Journal of Virology,* 5(2), pp. 77-88. Doi: 10.3923/ijv.2009.77.88

Eurostat, (2017) Agriculture, forestry and fishing statistics – 2017 edition. *Statistical books,* pp. 69-70. Doi: 10.2785/570022

Fageria, M., Nie, X., Gallagher, A. & Singh, M., (2015). Mechanical Transmission of Potato Virus Y (PVY) Through Seed Cutting and Plant Wounding. *American Journal of Potato Research,* 92(143), pp. 143-147. Doi: 10.1007/s12230-014-9418-4

Fakhro, A., Von Bargen, S., Bandte, M. & Büttner, C., (2010). Pepino mosaic virus, a first report of a virus infecting tomato in Syria. *Phytopathologia Mediterranea,* 49(1), pp. 99-101.

Fan, H. & Gulley, M. L., (2001). *Molecular Pathology Protocols.* Totowa(New Jersey): Humann Press Inc. Doi: 10.1385/1592590810

Fletcher, J. T. & MacNeill, B. H., (1971). Influence of environment, cultivar and virus strain on the expression of tobacco mosaic virus symptoms in tomato. *Canadian Journal of Plant Science,* 51(2), pp. 101-107. Doi: 10.4141/cjps71-022

Forray, A., Tüske, M. & Gáborjányi, R., (2004). First report on the occurrence of Pepino mosaic virus in Hungary. *Növényvédelem,* 40(9), pp. 471-473.

Fox, A., Collins, L, E., Macarthur, R., Blackburn, L, F. & Northing, P., (2017). New aphid vectors and efficiency of transmission of Potato virus A and strains of Potato virus Y in the UK. *Plant Pathology,* 66(2), pp. 325-335. Doi: 10.1111/ppa.12561

French, C. J., Bouthillier, M., Bernardy, M., Ferguson, G., Sabourin, M., Johnson, R, C., Masters, C., Godkin, S. & Mumford, R., (2001). First Report of Pepino mosaic virus in Canada and the United States. *Plant Disease,* 85(10), p. 1121. Doi: PDIS.2001.85.10.1121B

French, C. J., Bunckle, A. & Ferguson, G., (2005). Complete sequencing and phylogenetic analysis of tomato isolates of Pepino mosaic virus from Canada and other geographic regions. *Phytopathology,* 95(6), p. S31. Doi: 10.1094/PHYTO.2005.95.6.S1

Gilbert, G., Caron, J., Dallaire, C., Hamel, D., Morais, D. & Vézina, L., (2010). Maladies Diagnostiquées sur des Échantillons de Cultures Commerciales Soumis au Laboratoire de Diagnostic en Phytoprotection du Mapaq en 2009. *Canadian Plant Disease Survey,* 90, pp. 30-52.

Gómez, P., Sempere, R. N., Elena, S. F. & Aranda, M. A., (2009). Mixed Infections of Pepino mosaic virus Strains Modulate the Evolutionary Dynamics of this Emergent Virus. *American Society for Microbiology,* 83(23), pp. 12378-12387. Doi: 10.1128/JVI.01486-09

Guo, S., Kierzek, E., Chen, G., Zhou, Y-J. & Wong, S-M., (2015). TMV mutants with poly(A) tracts of different lengths demonstrate structural variations in 3'UTR affecting viral RNAs accumulation and symptom expression. *Scientific Reports,* 5(18412). Doi: 10.1038/srep18412

Hampson, M. C. & Haard, N. F., (1980). Pathogenesis of *Synchytrium endobioticum*: 1. Infection responses in potato and tomato. *Canadian Journal of Plant Pathology,* 2(3), pp. 143-147. Doi: 10.1080/07060668009501430

Hanssen, I. M., Paeleman, A., Wittemans, L., Goen, K., Lievens, B., Bragard, C., Vanachter, A, C, R, C. & Thomma, B, P, H, J., (2008). Genetic characterization of Pepino mosaic virus isolates from Belgian greenhouse tomatoes reveals genetic recombination. *European Journal of Plant Pathology,* 121(2), pp. 121-131. Doi: 10.1007/s10658-007-9255-0

Hasiów-Jaroszewska, B., Borodynko, N. & Pospieszny, H., (2009). Infectious RNA transcripts derived from cloned cDNA of a pepino mosaic virus isolate. *Archives of Virology,* 154, pp. 853-856. Doi: 10.1007/s00705-009-0368-y

Hasiów-Jaroszewska, B., Pospieszny, H. & Borodynko, N., (2009). New Necrotic Isolates of Pepino mosaic virus Representing the Ch2 Genotype. *Journal of Phytopathology,* 157(7-8), pp. 494-496. Doi: 10.1111/j.1439-0434.2008.01496.x

Huisman, M. J., Linthorst, H. J. M., Bol, J. F. & Cornelissen, B. J. C., (1988). The Complete Nucleotide Sequence of Potato Virus X and Its Homologies at the Amino Acid Level with Various Plus-stranded RNA Viruses. *Journal of General Virology,* 69, pp. 1789-1798. Doi: 10.1099/0022-1317-69-8-1789

Hu, J. S., Ferreira, S., Xu, M, Q., Lu, M., Iha, M., Pflum, E. & Wang, M., (1994). Transmission, movement and inactivation of cymbidium mosaic and odontoglossum ringspot viruses. *Plant Disease,* 78(6), pp. 633-636. Doi: 10.1094/PD-78-0633

Imane, B., (2016). Survey and molecular characterization. Pepino mosaic virus (PepMV) infecting tomato crops in Morocco. *Arab and Near East Plant Protection Newsletter,* 68, p. 15.

Jenner, C. E., Wang, X., Tomimura, K., Ohshima, K., Ponz, F. & Walsh, J, A., (2003). The Dual Role of the Potyvirus P3 Protein of Turnip mosaic virus as a Symptom and Avirulence Determinant in Brassicas. *Molecular Plant-Microbe Interactions,* 16(9), pp. 777-784. Doi: 10.1094/MPMI.2003.16.9.777

Jing, Q., ChangXiang, Z., FuJiang, W., XingQi, G. & YunZhi, S., (2003). Coat protein gene analysis and identification of an isolate of potato virus X. *Acta Phytophylacica Sinica,* 30(4), pp. 358-364.

Johnson, J., (1925). Transmission of viruses from apparently healthy potatoes. *Bulletin of the Wisconsin Agricultural Experiment Station,* 68, p. 12.

Jones, R. A. C., Koenig, R. & Lesemann, D. E., (1980). Pepino mosaic virus, a new potexvirus from pepino (*Solanum muricatum*). *Annals of Applied Biology,* 94(1), pp. 61-68. Doi: 10.1111/j.1744-7348.1980.tb03896.x

Jordá, C., Lázaro Pérez, A. & Martínez Culebras, P. V., (2001). First Report of Pepino mosaic virus on Natural Hosts. *Plant Disease,* 85(12), p. 1292. Doi: 10.1094/PDIS.2001.85.12.1292D

Kagiwada, S., Yamaji, Y., Nakabayashi, H., Ugaki, M. & Namba, S., (2002). The complete nucleotide sequence of Potato virus X strain OS: the first complete sequence of a Japanese isolate. *Journal of General Plant Pathology,* 68(1), pp. 94-98. Doi: 10.1007/PL00013061

Karasev, A. V., Hu, X., Brown, C, J., Kerlan, C., Nikolaeva, O, V., Crosslin, J, M. & Gray, S, M., (2011). Genetic Diversity of the Ordinary Strain of Potato virus Y (PVY) and Origin of Recombinant PVY Strains. *Phytopathology,* 101(7), pp. 778-785. Doi: 10.1094/PHYTO-10-10-0284

Kendall, A., Bian, W., Maris, A., Azzo, C., Groom, J., Williams, D., Shi, J., Stewart, P, L., Wall, J, S. & Stubbs, G., (2013). A common structure for the potexviruses. *Virology,* 436(1), pp. 173-178. Doi: 10.1016/j.virol.2012.11.008

Khakvar, R., Shamsbakhsh, M. & Pourrahim, R., (2005). Status of six potato viruses in Khuzestan province. *Applied Entomology and Phytopathology,* 73(1), pp. en9-en11.

Khan, M. A. & Saif-ur-Rehman, M., (2002). *Detection of major potato viruses from spring crop grown at three locations in the Punjab.* Faisalabad, Pakistan Phytopathology Society, pp. 113-115.

Klein, C., Fritsch, C., Briand, J, P., Richards, K, E., Jonard, G. & Hirth, L., (1976). Physical and functional heterogeneity in TYMV RNA: evidence for the existence of an independent messenger coding for coat protein. *Nucleic Acids Research,* 3(11), pp. 3043-3061.

Kumar, S., Stecher, G. & Tamura, K., (2015) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. *Molecular Biology and Evolution*. 33, pp. 1870-1874.

Lacasa, A., Guerrero, M, M., Hita, I., Martínez, M, A., Jordá, C., Bielza, P., Contreras, J., Alcázar, A. & Cano, A., (2003). Implicaciones de los abejorros (*Bombus* spp.) en la dispersiín del virus del mosaico del pepino dulce (Pepino mosaic virus) en cultivos de tomate. *Boletín de Sanidad Vegetal - Plagas,* 29(3), pp. 393-403.

Lambert, S. J., Hay, F. S., Pethybridge, S. J. & Wilson, C. R., (2007). Spatiotemporal spread of Potato virus S and Potato virus X in seed potato in Tasmania, Australia. *Plant Health Progress.*

Lavandero, B., Rojas, P., Ramirez, C, C., Salazar, M. & Caligari, P, D, S., (2012). Genetic structure of the aphid, *Chaetosiphon fragaefolii*, and its role as a vector of the Strawberry Yellow Edge Virus to a native strawberry, *Fragaria chiloensis* in Chile. *Journal of Insect Science,* 12(1). Doi: 10.1673/031.012.11001

Ling, K.-S., (2007). Molecular characterization of two Pepino mosaic virus variants from imported tomato seed reveals high levels of sequence identity between Chilean and US isolates. *Virus Genes,* 34(1), pp. 1-8. Doi: 10.1007/s11262-006-0003-x

Ling, K.-S., (2008). Pepino mosaic virus on Tomato Seed: Virus Location and Mechanical Transmission. *Plant Disease,* 92(12), pp. 1701-1705. Doi: 10.1094/PDIS-92-12-1701

Ling, K. S., Wintermantel, W. M. & Bledsoe, M., (2008). Genetic composition of Pepino mosaic virus population in North America greenhouse tomatoes. *Plant Disease,* 92(12), pp. 1683-1688. Doi: 10.1094/PDIS-92-12-1683

Ling, K. -S. & Zhang, W., (2011). First Report of Pepino mosaic virus Infecting Tomato in Mexico. *Plant Disease,* 95(8), p. 1035. Doi: 10.1094/PDIS-04-11-0334

Ling, Q., LingYun; B., ChangYong, Z., ShuiYing, Y. & XianChao, S., (2010). ELISE detection of infectious pathogenic viruses and variation of CMV from squash in Chongqing. *Acta Horticulturae Sinica,* 37(3), pp. 405-412.

Li, R., Gao, S., Hernandez, A, G., Wechter, W, P., Fei, Z. & Ling, K-S., (2012). Deep Sequencing of Small RNAs in Tomato for Virus and Viroid Identification and Strain Differentiation. *PLOS One,* 7(5), p. e37127. Doi: 10.1371/journal.pone.0037127

Li, W., Zhang, Y., Zhang, C., Pei, X., Wang, Z. & Jia, S., (2014). Presence of poly(A) and poly(A)-rich tails in a positive-strand RNA virus known to lack 3' poly(A) tails. *Virology,* 454-455, pp. 1-10. Doi: 10.1016/j.virol.2014.02.002

Li, X. H., Valdez, P., Olvera, R. E. & Carrington, J. C., (1997). Functions of the tobacco etch virus RNA polymerase (NIb): subcellular transport and protein-protein interaction with VPg/proteinase (NIa). *Journal of Virology,* 71(2), pp. 1598-1607.

Li, X. S., Cao, Y, Y., Cheng, Y, Q., Li, H, F. & Fan, Z, F., (2008). *Orychophragmus violaceus*, a new host of Potato virus X, repoted from China. *Plant Pathology,* 57(2), p. 395. Doi: 10.1111/j.1365-3059.2007.01758.x

Lockhart, B. E., (2007). Charcterization of a tymovirus occurring in greenhouse-grown tomatoes in Minnesota. *Phytopathology,* 97(7), p. S67. Doi: 10.1094/PHYTO.2007.97.7.S1

Manzer, F. E. & Merriam, D., (1961). Field transmission of the potato spindle tuber virus and virus X by cultivating and hilling equipment. *American Potato Journal,* 38(10), pp. 346-352. Doi: 10.1007/BF02862243

Maroon-Lango Guaragna, M. A., Jordan, R. L., Bandla, M. & Marquardt, S., (2003). Detection and characterization of a US isolate of Pepino mosaic *Potexvirus*. *Phytopathology,* 93(6), p. S57. Doi: 10.1094/PHYTO.2003.93.6.S1

Maroon-Lango, C. J., Guaragna, M, A., Jordan, R, L., Hammond, L., Bandla, M. & Marquardt, S, K., (2005). Two unique US isolates of Pepino mosaic virus from a limited source of pooled tomato tissue are distinct from a third (European-like) US isolate. *Archives of Virology,* 150(6), pp. 1187-1201. Doi: 10.1007/s00705-005-0495-z

Massumi, H., Shaabanian, M., Pour, A, H., Heydarnejad, J. & Rahimian, H., (2009). Incidence of Viruses Infecting Tomato and Their Natural Hosts in the Southeast and Central Regions of Iran. *The American Phytopathological Society,* 93, pp. 67-72. Doi: 10.1094 / PDIS-93-1-0067

McLean, B. G., Zupan, J. & Zambryski, P. C., (1995). Tobacco mosaic virus movement protein associates with the cytoskeleton in tobacco cells. *The Plant Cell,* 7(12), pp. 2101-2114. Doi: 10.1105/tpc.7.12.2101

Mehle, N., Gutiérrez-Aguirre, I., Prezelj, N., Delić, D., Vidic, U. & Ravnikar, M., (2013). Survival and Transmission of Potato Virus Y, Pepino Mosaic Virus, and Potato Spindle Tuber Viroid in Water. *Applied and Environmental Microbiology,* 80(4), pp. 1455-1462. Doi: 10.1128/AEM.03349-13

Moreno-Pérez, M. G., Pagán, I., Aragón-Caballero, L., Cáceres, F., Fraile, A. & García-Arenal, F., (2014). Ecological and Genetic Determinants of Pepino Mosaic Virus Emergence. *Journal of Virology,* 88(6), pp. 3359-3368. Doi: 10.1128/JVI.02980-13

Mumford, R. A. & Metcalfe, E. J., (2001). The partial sequencing of the genomic RNA of a UK isolate of Pepino mosaic virus and the comparison of the coat protein sequence with other isolates from Europe and Peru. *Archives of Virology,* 146(12), pp. 2455-2460. Doi: 10.1007/s007050170015

Nechadi, S., Benddine, F., Moumen, A. & Kheddam, M., (2002). Etat des maladies virales de la tomate en stratégie de lutte en Algérie. *Bulletin OEPP,* 32(1), pp. 21-24. Doi: 10.1046/j.1365-2338.2002.d01-21.x

Neergaard, E., Munk, L. & Nielsen, S. L., (2014). First report of Potato leafroll virus, Potato virus A, Potato virus X and Potato virus Y in potato in Greenland. *New Disease Reports,* 30, p. 20. Doi: 10.5197/j.2044-0588.2014.030.020

Nienhaus, F. & Stille, B., (1965). Übertragung des Kartoffel-X-Virus durch Zoosporen von *Synchytrium endobioticum*. *Journal of Phytopathology,* 54(4), pp. 335-337. Doi: 10.1111/j.1439-0434.1965.tb04104.x

Okonechnikov, K., Golosova, O. & Fursov, M., (2012) Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics*. 28, pp. 1166-1167. Doi: 10.1093/bioinformatics/bts091

Özdemir, S., (2010). First report of Pepino mosaic virus in tomato in Turkey. *Journal of Plant Pathology,* 92(4, Suppl), p. S4.107. Doi: 10.4454/jpp.v92i4sup.348

Pagán, I., Córdoba-Sellés, M, C., Martínez-Priego, L., Fraile, A., Malpica, J, M., Jordá, C. & García-Arenal, F., (2006). Genetic structure of the population of Pepino mosaic virus infecting tomato crops in Spain. *Phytopathology,* 96(3), pp. 274-279. Doi: 10.1094/PHYTO-96-0274

Papayiannis, L. C., Kokkinos, C. D. & Alfaro-Fernández, A., (2012). Detection, characterization and host range studies of Pepino mosaic virus in Cyprus. *European Journal of Plant Pathology,* 132(1), pp. 1-7. Doi: 10.1007/s10658-011-9854-7

Park, M.-R., Jeong, R.-D. & Kim, K.-H., (2014). Understand the intracellular trafficking and intercellular transport of potexviruses in their host plants. *Frontiers in Plant Science,* 5, pp. 1-12. Doi: 10.3389/fpls.2014.00060

Petrzik, K., (2009). Occurrence of Potato virus X on hybrid dock in Czech Republic. *Acta Virologica,* 53(1), pp. 49-52. Doi: 10.4149/av_2009_01_49

Pospieszny, H. & Borodynko, N., (2002). Pepino mosaic virus. *Progress in Plant Protection,* 42(1), pp. 83-87.

Pospieszny, H., Hasiów, B. & Borodynko, N., (2008). Characterization of two distinct Polish isolates of Pepino mosaic virus. *European Journal of Plant Pathology,* 122, pp. 443-445. Doi: 10.1007/s10658-008-9280-7

Pospieszny, H. & Borodynko, N., (2006). New Polish isolate of Pepino mosaic virus highly distinct from European Tomato, Peruvian and US2 strains. *Plant Disease,* 90(8), p. 1106. Doi: 10.1094/PD-90-1106C

Raikhy, G. & Tripathi, D., (2017). Leading Molecular Aspects of Plant Viruses. *Journal of Bacteriology & Mycology: Open Access,* 5(2), p. 00128. Doi: 10.15406/jbmoa.2017.05.00128

Rakitina, D. V., Kantidze, O, L., Leshchiner, A, D., Solovyev, A, G., Novikov, V, K., Morozov, S, Y. & Kalinina, N, O., (2005). Coat proteins of two filamentous plant viruses display NTPase activity in vitro. *FEBS Letters,* 579(22), pp. 4955-4960. Doi: 10.1016/j.febslet.2005.07.083

Ramel, M., Gugerli, P. & Gilli, C., (2007). La mosaïque du pépino sur tomate. *Revue suisse de Viticulture, Arboriculture, Horticulture,* 39(3), pp. 211-212.

Ramirez, G. & Bustamante, P., (2001). *Deteccion del Pepino mosaic virus (PepMV) en plantas de tomate (Lycopersicon esculentum) mediante tecnicas serlogica.* Santa Cruz, Chile, s.n.

Rezende, J. A. M., Camelo-García, V, M., Andrade, S, C, S., Buriolla, J., El Kitajima, E, W. & Duarte, L, M, L., (2017). Biological and molecular characterization of a putative new potexvirus infecting *Senna occidentalis. Archives of Virology,* 162(2), pp. 529-533. Doi: 10.1007/s00705-016-3114-2

Riechmann, J. L., Laín, S. & García, J. A., (1992). Highlights and prospects of potyvirus molecular biology. *Journal of General Virology,* 73, pp. 1-16. Doi: 10.1099/0022-1317-73-1-1

Robaglia, C., Durand-Tardif, M., Tronchet, M., Boudazin, G., Astier-Manifacier, S. & Casse-Delbart, F., (1989). Nucleotide Sequence of Potato Virus Y (N Strain) Genomic RNA. *Journal of General Virology,* 70, pp. 935-947. Doi: 10.1099/0022-1317-70-4-935

Robertson, N. L., Smeenk, J. & Anderson, J. M., (2011). Molecular characterization of Potato leafroll virus, Potato virus A, and Potato virus X isolates from potatoes in Alaskan cities and villages. *Plant Health Progress.* Doi: 10.1094/PHP-2011-0209-01-BR

Rodríguez-Cerezo, E., Ammar, E. D., Pirone, T. P. & Shaw, J. G., (1993). Association of the non-structural P3 viral protein with cylindrical inclusions in potyvirus-infected cells. *Journal of General Virology,* 74, pp. 1945-1949. Doi: 10.1099/0022-1317-74-9-1945

Roossinck, M, J., (1997) Mechanisms of Plant Virus Evolution. *Annual Review of Phytopathology*. 35, pp. 191-209. Doi: 10.1146/annurev.phyto.35.1.191

Saboruin, M., Blaser, C., Cook, S., Waud, M., Renaud, M, A. & Wegener, L., (2007). Diseases Diagnosed on Crops Submitted to the Pest Diagnostic Clinic in Ontario in 2006. *Canadian Plant Disease Survey,* 87, pp. 27-34.

Saito, T., Meshi, T., Takamatsu, N. & Okada, Y., (1987). Coat protein gene sequence of tobacco mosaic virus encodes a host response determinant. *Proceedings of the National Academy of Sciences,* 84(17), pp. 6074-6077. Doi: 10.1073/pnas.84.17.6074

Salomone, A. & Roggero, P., (2002). Host Range, Seed Transmission and Detection by ELISA and Lateral Flow of an Italian Isolate of Pepino mosaic virus. *Journal of Plant Pathology,* 84(1), pp. 65-68.

Samuels, T. D., Ju, H-J., Ye, C-M., Motes, C, M., Blancaflor, E, B. & Verchot-Lubicz, J., (2007). Subcellular targeting and interactions among the Potato virus X TGB proteins. *Virology,* 367(2), pp. 375-389. Doi: 10.1016/j.virol.2007.05.022

Santanu, P. & Amitava, K., (2003). Incidence of viral diseases and insect vectors on potato in indo-gangetic plains of West Bengal. *Journal of the Indian Potato Association,* 30(1/2), pp. 127-128.

Scholthof, K-B, G., (2005) Tobacco mosaic virus. *The Plant Health Instructor*. Doi: 10.1094/PHI-I-2000-1010-01

Scholthof, K-B, G., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., Hohn, B., Saunders, K., Candresse, T., Ahlquist, P., Hemenway, C. & Foster, G, D., (2011) Top 10 plant viruses in moleculat plant pathology. *Molecular Plant Pathology*, 12(9), pp. 938-954. Doi: 10.1111/j.1364-3703.2011.00752.x

Schwarz, D., Beuch, U., Bandte, M., Fakhro, A., Büttner, C. & Obermeier, C., (2010). Spread and interaction of Pepino mosaic virus (PepMV) and *Pythium aphanidermatum* in a closed nutrient solution recirculation system: effects on

tomato growth and yield. *Plant Pathology,* 59(3), pp. 443-452. Doi: 10.1111/j.1365-3059.2009.02229.x

Sharma, K. K. & Kang, G. S., (2003). Appraisal of rate of degreneration in some potato cultivars. *Journal of the Indian Potato Association,* 30(1/2), pp. 123-124.

Short, N. M. & Davies, J. W., (1987). Host ranges, symptoms and amino acid compositions of eight potexviruses. *Annals of Applied Biology,* 110(1), pp. 213-219. Doi: 10.1111/j.1744-7348.1987.tb03250.x

Shukla, D. D. & Ward, C. W., (1989). Identification and classification of potyviruses on the basis of coat protein sequence data and serology. *Archives of Virology,* 106(3-4), pp. 171-200. Doi: 10.1007/BF01313952

Silva, O. de A., Figueira, A, dos R., Boari, A de J., Pinto, C, A, B, P. & Boni, R, R., (2005). Caracterização biológica de onze isolados de PVX (Potato virus X) do Brasil. *Ciência e Agrotecnologia,* 29(3), pp. 521-527. Doi: 10.1590/S143-705420050000300003

Skryabin, K. G., Kraev, A, S., Morozov, SYu., Rozanov, M, N., Chernov, B, K., Lukasheva, L, I. & Atabekov, J, G., (1988). The nucleotide sequence of potato virus X RNA. *Nucleic Acids Research,* 16(22), pp. 10929-10930.

Smith, K. D., (1931). On the Composite Nature of Certain potato Viruse Diseases of the Mosaic Group as Revealed by the use of Plant Indicators and Selective Methods of Transmission. *Proceedings of the Royal Society B,* 109(762), pp. 251-267. Doi: 10.1098/rspb.1931.0080

Šneideris, D., ŽiŽyte, M., Zitikaite, I., Urbanavičiené, L. & Staniulis, J., (2013). First report of two distinct strains of Pepino mosaic virus infecting tomatoes in greenhouses in Lithuania. *Journal of Plant Pathology,* 95(1), pp. 217-218. Doi: 10.4454/JPP.V95I1.024

Soler, S., López, C. & Nuez, F., (2005). Natural occurrence of viruses in *Lycopersicon* spp. in Ecuador. *Plant Disease,* 89(11), p. 1244. Doi: 10.1094/PD-89-1244C

Soler, S., Prohens, J., Díez, M. J. & Nuez, F., (2002). Natural Occurence of Pepino mosaic virus in *Lycopersicon* Species in Central and Southern Peru. *Journal of Phytopathology,* 150(2), pp. 49-53. Doi: 10.1046/j.1439-0434.2002.00712.x

Sonenberg, N., Shatkin, A, J., Ricciardi, R, P., Rubin, M. & Goodman, R, M., (1978). Analysis of terminal structures of RNA from potato virus X. *Nucleic Acids Research,* 5(7), pp. 2501-2512.

Spence, N. J., Basham, K., Mumford, R, A., Hayman, G., Edmondson, R. & Jones, D, R., (2006). Effect of Pepino mosaic virus on the yield and quaility of glasshouse-grown tomatoes in the UK. *Plant Pathology,* 55(5), pp. 595-606. Doi: 10.1111/j.1365-3059.2006.01406.x

Spetz, C. & Valkonen, J. P. T., (2004). Potyviral 6K2 Protein Long-Distance Movement and Symptom-Induced Functions are Independent and Host-Specific. *Molecular Plant-Microbe Interactions,* 17(5), pp. 502-510. Doi: 10.1094/MPMI.2004.17.5.502

Stäubli, A., (2004). Faits marquants dans la recherche 2004. Nouvelle virose sur tomate. *Revue suisse de Viticulture, Arboriculture, Horticulture,* 37(3), p. 150.

Stevens, W, A., (1983) Plant Virus Disease Control. In: Virology of Flowering Plants. Tertiary Level Biology. Springer, Boston, MA. Doi: 10.1007/978-1-4757-1251-3_6

Strange, R. N. & Scott, P. R., (2005). Plant Disease: A Threat to Global Food Security. *Annual Review of Phytopathology,* 43, pp. 83-116. Doi: 10.1146/annurev.phyto.43.113004.133839

Thermo Fisher Scientific, (2010) ELISA technical guide and protocols. *TECH TIP #65*

Tiberini, A., Davino, S., Davino, M. & Tomassoli, L., (2011). Complete Sequence, Genotyping and Comparative Analysis of Pepino Mosaic Virus Isolates From Italy. *Journal of Plant Pathology,* 93(2), pp. 437-442. Doi: 10.4454/jpp.v93i2.1199

Turpen, T., (1989). Molecular Cloning of a Potato Virus Y Genome: Nucleotide Sequence Homology in Non-coding Regions of Potyviruses. *Journal of General Virology,* 70, pp. 1951-1960. Doi: 10.1099/0022-1317-70-8-1951

Vance, V, B., Berger, P, H., Carrington, J, C., Hunt, A, G. & Shi, X, M., (1995) 5' Proximal Potyviral Sequences Mediate Potato Virus X/Potyviral Synergistic Disease in Transgenic Tobacco. *Virology*. 206, pp. 583-590.

van der Vlugt, R. A. A. & Berendsen, M., (2002). Development of a General *Potexvirus* Detection Method. *European Journal of Plant Pathology,* 108(4), pp. 367-371. Doi: 10.1023/A:1015644409484

van der Vlugt, R. A. A., Stijger, C. C. M. M., Verhoeven, J. T. J. & Lesemann, D. -E., (2000). First Report of Pepino Mosaic Virus on Tomato. *Plant Disease,* 84(1), p. 103. Doi: 10.1094/PDIS.2000.84.1.103C

Verchot-Lubicz, J., Ye, C.-M. & Bamunusinghe, D., (2007). Molecular biology of potexviruses: recent advances. *Journal of General Virology,* 88, pp. 1643-1655. Doi: 10.1099/vir.0.82667-0

Verhoeven, J. T. J., van der Vlugt, R. A. A. & Roenhorst, J. W., (2003). High Similarity Between Tomato Isolates of Pepino mosaic Virus Suggests a Common Origin. *European Journal of Plant Pathology,* 109(5), pp. 419-425. Doi: 10.1023/A:1024261121468

Waltermann, A. & Maiss, E., (2006). Detection of 6K1 as a mature protein of 6kDa in plum pox virus-infected *Nicotiana benthamiana*. *Journal of General Virology,* 87, pp. 2381-2386. Doi: 10.1099/vir.0.81873-0

Walters, H. J., (1952). Some relationships of three plant viruses to the differential grasshopper, *Melanoplus differentialis* (Thos.).. *Phytopathology,* 42(7), pp. 355-362.

Webster, G, C., Wylie, S, J. & Jones, M, G, K., (2004) Diagnosis of plant viral pathogens. *Current Science*, 86(12), pp. 1604-1607

Wright, D. & Mumford, R., (1999). Pepino Mosaic *Potexvirus* (PepMV): First Records in Tomato in the United Kingdom. *Plant Disease Notice,* Volume 89.

Yamaji, Y., Kobayashi, T., Hamada, K., Sakurai, K., Yoshii, A., Suzuki, M., Namba, S. & Hibi, T., (2006). In vivo interaction between Tobacco mosaic virus RNA-dependent RNA polymerase and host translation elongation factor 1A. *Virology,* 347(1), pp. 100-108. Doi: 10.1016/j.virol.2005.11.031

Yang, L. J., Hidaka, M., Masaki, H. & Uozumi, T., (1998). Detection of Potato Virus Y P1 Protein in Infected Cells and Analysis of Its Cleavage Site. *Bioscience, Biotechnology, and Biochemistry,* 62(2), pp. 380-382. Doi: 10.1271/bbb.62.380

Yanten Carreno, Y., Sandoval Briones, C. & Lolas Caneo, M., (2005). *Determinacion de la presencia de pepino mosaic potexvirus -PepMV en tomate en la zona Central de Chile,* Talca: Universidad de Talca.

YaoLiang, Z., ZhongJian, S., Jiang, Z., XiaoLi, L., Gong, C. & RuDuo, L., (2003). Preliminary characterization of Pepino Mosaic Virus Shanghai isolate (PepMV-Sh) and its detection with ELISA. *Acta Agriculturae Shanghai,* 19(3), pp. 90-92.

ZhongKang, W., YuXian, X., Qing, Y. W. T. & YouPing, Y., (2005). Detection of mix-infected potato viruses with multiplex RT-PCR. *Acta Phytopatholofica Sinica,* 35(2), pp. 109-115.

Zimmern, D., (1975). The 5' end group of tobacco mosaic virus RNA is m7G5 ppp5' Gp. *Nucleic Acids Research,* 2(7), pp. 1189-1201.

Appendix I

PCR sequence data aligned with known strains of PepMV acquire from Genbank: Pa (FJ612601.1), P19 (HQ650559.1), P22 (HQ650560.2), CH2 (JN835466.1), CH_BPO160 (MF422611.1), CH_BPO162 (MF422613.1), CH_BPO158 (MF422615.1).

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Pa          5163 TGCTATAGCCTTTCTACTGTCAGCTTGCATTTACTTCCAAAACAGTCATTATCA
P19         5163 TGCTATAGCCTTTCTACTGTCAGCTTGCATTTACTTCCAAAACAGTCATTATCA
P22         5163 TGCTATAGCCTTTCTACTGTCAGCTTGCATTTACTTCCAAAACAGTCATTATCA
CH2         5163 TGCTATAGCCTTTCTACTGTCAGCTTGCATTTACTTCCAAAACAGTCATTATCA
CH_BPO160   5163 TGCTATAGCCTTTCTACTGTCAGCTTGCATTTACTTCCAAAACAGTCATTATCA
CH_BPO162   5163 TGCTATAGCCTTTCTACTGTCAGCTTGCATTTACTTCCAAAACAGTCATTATCA
CH_BPO158   5163 TGCTATAGCCTTTCTACTGTCAGCTTGCATTTACTTCCAAAACAGTCATTATCA
PCR              TGCTATTGCCTTTCTGCTGTCAGCTTGCATTTACTTCCAAAACAGTCATTATCA

Pa          5217 ACCAGTTGCAGGTGATAATTTGCACAGACTACCCTTTGGTGGTCAGTATCAAGA
P19         5217 ACCAGTTGCAGGTGATAATTTGCACAGACTACCCTTTGGTGGTCAGTATCAAGA
P22         5217 ACCAGTTGCAGGTGATAATTTGCACAGACTACCCTTTGGTGGTCAGTATCAAGA
CH2         5217 ACCAGTTGCAGGTGATAATTTGCACAGACTACCCTTTGGTGGTCAGTATCAAGA
CH_BPO160   5217 ACCAGTTTCAGGTGATAATTTGCACAGACTACCCTTTGGTGGTCAGTATCAAGA
CH_BPO162   5217 ACCAGTTGCAGGTGATAATTTGCACAGACTACCCTTTGGTGGTCAGTATCAAGA
CH_BPO158   5217 ACCAGTTTCAGGTGATAATTTGCACAGACTACCCTTTGGTGGTCAGTATCAAGA
PCR              ACCAGTTGCAGGTGATAATTTGCACAGACTGCCCTTTGGTGGTCAGTATCAAGA

Pa          5283 GGAACTAAGAAGATTTCTTACTTTCCGCAACAACAATCCTACTTTCACTCAGGA
P19         5283 GGAACTAAGAAGATTTCTTACTTTCCGCAACAACAATCCTACTTTCACTCAGGA
P22         5283 GGAACTAAGAAGATTTCTTACTTTCCGCAGCAACAATCCTACTTTCACTCAGGA
CH2         5283 GGAACTAAGAAGATTTCTTACTTTCCGCAGCAACAATCCTACTTTCACTCAGGA
CH_BPO160   5283 GGAACTAAGAAGATTTCTTACTTTCCGCAGCAACAATCCTACTTTCACTCAGGA
CH_BPO162   5283 GGAACTAAGAAGATTTCTTACTTTCCGCAGCAACAATCCTACTTTCACTCAGGA
CH_BPO158   5283 GGAACTAAGAAGATTTCTTACTTTCCGCAGCAACAATCCTACTTTCACTCAGGA
PCR              GGAACTAAGAAGATTTCTTACTTTCCGCAGCAACAATCCTACTTTCACTCAGGA

Pa          5271 ACACAAGCTTAATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCT
P19         5271 ACACAAGCTTAATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCT
P22         5271 ACACAAGCTTAATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCT
CH2         5271 ACACAAGCTTAATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCT
CH_BPO160   5271 ACACAAGCTTAATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCT
CH_BPO162   5271 ACACAAGCTTAATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCT
CH_BPO158   5271 ACACAAGCTTAATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCT
PCR              ACACAAGCTTAATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCT

Pa          5325 CACCAATAAATTTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTA
P19         5325 CACCAATAAATTTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTA
P22         5325 CACCAATAAATTTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTA
CH2         5325 CACCAATAAATTTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTA
CH_BPO160   5325 CACCAATAAATTTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTA
CH_BPO162   5325 CACCAATAAATTTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTA
CH_BPO158   5325 CACCAATAAATTTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTA
PCR              CACCAATAAATTTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTA
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Pa          5379 CAACACACATTCTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACG
P19         5379 CAACACACATTCTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACG
P22         5379 CAACACACATTCTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACG
CH2         5379 CAACACACATTCTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACG
CH_BPO160   5379 CAACACACATTCTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACG
CH_BPO162   5379 CAACACACATTCTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACG
CH_BPO158   5379 CAACACACATTCTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACG
PCR              CAACACACATTCTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACG

Pa          5433 GTGCAGCCATAGTTATAACAAATTGTCCAAACACACCCGAAGTTCTTGAAGCAA
P19         5433 GTGCAGCCATAGTTATAACAAATTGTCCAAACACACCCGAAGTTCTTGAAGCAA
P22         5433 GTGCAGCCATAGTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAA
CH2         5433 GTGCAGCCATAGTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAA
CH_BPO160   5433 GTGCAGCCATAGTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAA
CH_BPO162   5433 GTGCAGCCATAGTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAA
CH_BPO158   5433 GTGCAGCCATAGTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAA
PCR              GTGCAGCCATAGTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAA

Pa          5487 TCAACTTCTCCCCTTGGAACGGGTTAAGTTTTCCTCAATTGTGAAATTATATTT
P19         5487 TCAACTTCTCCCCTTGGAACGGGTTAAGTTTTCCTCAATTGTGAAATTATATTT
P22         5487 TCAACTTCTCCCCTTGGAACGGGTTAAGTTTTCCTCAATTGTGAAATTATATTT
CH2         5487 TCAACTTCTCCCCTTGGAACGGGTTAAGTTTTCCTCAATTGTGAAATCATATTT
CH_BPO160   5487 TCAACTTCTCCCCTTGGAACGGGTTAAGTTTTCCTCAATTGTGAAATCATATTT
CH_BPO162   5487 TCAACTTCTCCCCTTGGAACGGGTTAAGTTTTCCTCAATTGTGAAATCATATTT
CH_BPO158   5487 TCAACTTCTCCCCTTGGAACGGGTTAAGTTTTCCTCAATTGTGAAATCATATTT
PCR              TCAACTTCTCCCCTTGGAACGGGTTAAGTTTTCCTCAATTGTGAAATTATATTT

Pa          5541 GTTATCTAGTTAAATTCAAACAATTTAACTCAACTATGGAAAACCAACCTACAG
P19         5541 GTTATCTAGTTAAATTCAAACAATTTAACTCAACTATGGAAAACCAACCTACAG
P22         5541 GTTATCTAGTTAAATTCAAACAATTTAACTCAACTATGGAAAACCAACCTACAG
CH2         5541 GTTATCTAGTTAAATTCAAACAATTTAACTCAACTATGGAAAACCAACCTACAG
CH_BPO160   5541 GTTATCTAGTTAAATTCAAACAATTTAACTCAACTATGGAAAACCAACCTACAG
CH_BPO162   5541 GTTATCTAGTTAAATTCAAACAATTTAACTCAACTATGGAAAACCAACCTACAG
CH_BPO158   5541 GTTATCTAGTTAAATTCAAACAATTTAACTCAACTATGGAAAACCAACCTACAG
PCR              GTTAGCTAGTTAAATTCAAACTATTTAACTCACCTATGGGAAACCAACCTAGAG

Pa          5595 CTTCTAACCCATCAGATGCACCACCAACTGCTGCTCAAGCTGGTGCCCAGAGCC
P19         5595 CTTCTAACCCATCAGATGCACCACCAACTGCTGCTCAAGCTGGTGCCCAGAGCC
P22         5595 CTTCTAACCCATCAGATGCACCACCAACTGCTGCTCAAGCTGGTGCCCAGAGCC
CH2         5595 CTTCTAACCCATCAGATGCACCACCAACTGCTGCTCAAGCTGGTGCCCAGAGCC
CH_BPO160   5595 CTTCTAACCCATCAGATGCACCACCAACTGCTGCTCAAGCTGGTGCCCAGAGCC
CH_BPO162   5595 CTTCTAACCCATCAGATGCACCACCAACTGCTGCTCAAGCTGGTGCCCAGAGCC
CH_BPO158   5595 CTTCTAACCCATCAGATGCACCACCAACTGCTGCTCAAGCTGGTGCCCAGAGCC
PCR              GTTGCAACCCATCATATGCACCACCAACTGCTGCACAAGCTGGTGCCCAGAGCC

Pa          5649 CAGCCGACTTCTCAAATCCTAATACAGCTCCTTCCCTAAGTGATTTGAAGAAGA
P19         5649 CAGCCGACTTCTCAAATCCTAATACAGCTCCTTCCCTAAGTGATTTGAAGAAGA
P22         5649 CAGCCGACTTCTCAAATCCTAATACAGCTCCTTCCCTAAGTGATTTGAAGAAGA
CH2         5649 CAGCCGACTTCTCAAATCCTAATACAGCTCCTTCCCTAAGTGATTTGAAGAAGA
CH_BPO160   5649 CAGCCGACTTCTCAAATCCTAATACAGCTCCTTCCCTAAGTGATTTGAAGAAGA
CH_BPO162   5649 CAGCCGACTTCTCAAATCCTAATACAGCTCCTTCCCTAAGTGATTTGAAGAAGA
CH_BPO158   5649 CAGCCGACTTCTCAAATCCTAATACAGCTCCTTCCCTAAGTGATTTGAAGAAGA
PCR              CAGCAGACTTCTCAAATCCTAATACAGCTCCTTCCCTAAGTGATTTGAAGAAGA
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Pa          5703 TCAAATACGTGTCAACTGTCACTTCAGTTGCCACGCCTGCTGAAATTGAGGCCC
P19         5703 TCAAATACGTGTCAACTGTCACTTCAGTTGCCACGCCTGCTGAAATTGAGGCCC
P22         5703 TCAAATACGTGTCAACTGTCACTTCAGTTGCCACGCCTGCTGAAATTGAGGCCC
CH2         5703 TCAAATACGTGTCAACTGTCACTTCAGTTGCCACGCCTGCTGAAATTGAGGCCC
CH_BPO160   5703 TCAAATACGTGTCAACTGTCACTTCAGTTGCCACGCCTGCTGAAATTGAGGCCC
CH_BPO162   5703 TCAAATACGTGTCAACTGTCACTTCAGTTGCCACGCCTGCTGAAATTGAGGCCC
CH_BPO158   5703 TCAAATACGTGTCAACTGTCACTTCAGTTGCCACGCCTGCTGAAATTGAGGCCC
PCR              TCATATACCTGTCAACTGTCACTTCAGTTGCCACGCCTGCAGAAATTGACGCCC

Pa          5757 TTGGCAAGATCTTTACTGCCATGGGTTTAGCAGCCAATGAGACCGGACCTGCCA
P19         5757 TTGGCAAGATCTTTACTGCCATGGGTTTAGCAGCCAATGAGACCGGACCTGCCA
P22         5757 TTGGCAAGATCTTTACTGCCATGGGTTTAGCAGCCAATGAGACCGGACCTGCCA
CH2         5757 TTGGCAAGATCTTTACTGCCATGGGTTTAGCAGCCAATGAGACCGGACCTGCCA
CH_BPO160   5757 TTGGCAAGATCTTTACTGCCATGGGTTTAGCAGCCAATGAGACCGGACCTGCCA
CH_BPO162   5757 TTGGCAAGATCTTTACTGCCATGGGTTTAGCAGCCAATGAGACCGGACCTGCCA
CH_BPO158   5757 TTGGCAAGATCTTTACTGCCATGGGTTTAGCAGCCAATGAGACCGGACCTGCCA
PCR              TTGGAAAGATCTTTACTGCCGTGGGTTGAGCACCCAATGACACCGGACCTGCCG

Pa          5811 TGTGGGACCTCGCTCGTGCTTATGCTGATGTGCAAAGTTCAAAATCTGCACAAC
P19         5811 TGTGGGACCTCGCTCGTGCTTATGCTGATGTGCAAAGTTCAAAATCTGCACAAC
P22         5811 TGTGGGACCTCGCTCGTGCTTATGCTGACGTGCAAAGTTCAAAATCTGCACAAC
CH2         5811 TGTGGGACCTCGCTCGTGCTTATGCTGATGTGCAAAGTTCAAAATCTGCACAAC
CH_BPO160   5811 TGTGGGACCTCGCTCGTGCTTATGCTGATGTGCAAAGTTCAAAATCTGCACAAC
CH_BPO162   5811 TGTGGGACCTCGCTCGTGCTTATGCTGATGTGCAAAGTTCAAAATCTGCACAAC
CH_BPO158   5811 TGTGGGACCTCGCTCGTGCTTATGCTGATGTGCAAAGTTCAAAATCTGCACAAC
PCR              TGTGAGACCTCTCTCGCGCTTATGCTGATGTGCAAAGTTCAAAATCTGCACAAC

Pa          5865 TTATAGGTGCCACACCATCCAACCCTGCTTTGTCTAGACGTGCACTTGCTGCAC
P19         5865 TTATAGGTGCCACACCATCCAACCCTGCTTTGTCTAGACGTGCACTTGCTGCAC
P22         5865 TTATAGGTGCCACACCATCCAACCCTGCTTTGTCTAGACGTGCACTTGCTGCAC
CH2         5865 TTATAGGTGCCACACCATCCAACCCTGCTTTGTCTAGACGTGCACTTGCTGCAC
CH_BPO160   5865 TTATAGGTGCCACACCATCCAACCCTGCTTTGTCTAGACGTGCACTTGCTGCAC
CH_BPO162   5865 TTATAGGTGCCACACCATCCAACCCTGCTTTGTCTAGACGTGCACTTGCTGCAC
CH_BPO158   5865 TTATAGGTGCCACACCATCCAACCCTGCTTTGTCTAGACGTGCACTTGCTGCAC
PCR              ATTGGAGTGCCCCCCTATCCAACCGTGCTTTGTCTAGAAGTGCATGTGCTGCAC

Pa          5919 AGTTTGATCGTATCAATATCACACCCAGACAATTCTGCATGT
P19         5919 AGTTTGATCGTATCAATATCACACCCAGACAATTCTGCATGT
P22         5919 AGTTTGATCGTATCAATATCACACCCAGACAATTCTGCATGT
CH2         5919 AATTTGATCGTATCAATATCACACCCAGACAATTCTGCATGT
CH_BPO160   5919 AGTTTGATCGTATCAATATCACACCCAGACAATTCTGCATGT
CH_BPO162   5919 AGTTTGATCGTATCAATATCACACCCAGACAATTCTGCATGT
CH_BPO158   5919 AGTTTGATCGTATCAATATCACACCCAGACAATTCTGCATGT
PCR              AGTGTGATCGTATCAATATCCCCCCGGGACAATTCTGCGTGT
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Appendix II

POTX degenerate cloned fragments aligned with strains which showed genetic homology acquired from Genbank: LP-2001 (AJ606361.1), SM.74 (AM109896.1), LP_BPO161 (MF422612.1), LP_BPO163 (MF422614.1), LP_HYT25 (MF422616.1).

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LP-2001      3239 CAATAGAAGCTAGACTTTCTATTACAACTCCGGAAGCCAATTTACGAGAATTT
SM.74        3239 CAATAGAAGCTAGACTTTCTATTACAACTCCGGAAGCCAATTTACGAGAATTT
LP_BPO161    3239 CAATAGAAGCTAGACTTTCTATTACAACTCCGGAAGCCAATTTACGAGAATTT
LP_BPO163    3239 CAATAGAAGCTAGACTTTCTATTACAACTCCGGAAGCCAATTTACGAGAATTT
LP_HYT25     3239 CAATAGAAGCTAGACTTTCTATTACAACTCCGGAAGCCAATTTACGAGAATTT
POTX-FWD          CAATAGAAGCTAGACTTTCTATTACAACTCCGGAAGCCAATTTACGAGAATTT
POTX-REV          CAATTGAAGGTAGACTTTCTATTACAACTCCGGAAGCCAATTTACGAGAATTT

LP-2001      3292 GTGCTGAAGAAAGATGTTGGAGACATCTTGTTTTTCAACTACCACAATGCGAT
SM.74        3292 GTGCTGAAGAAAGATGTTGGAGACATCTTGTTTTTCAACTACCACAATGCGAT
LP_BPO161    3292 GTGCTGAAGAAAGATGTTGGAGACATCTTGTTTTTCAACTACCACAATGCGAT
LP_BPO163    3292 GTGCTGAAGAAAGATGTTGGAGACATCTTGTTTTTCAACTACCACAATGCGAT
LP_HYT25     3292 GTGCTGAAGAAAGATGTTGGAGACATCTTGTTTTTCAACTACCACAATGCGAT
POTX-FWD          GTGCTGAAGAAAGATGTTGGAGACATCTTGTTTTTCAACTACCACAATGCGAT
POTX-REV          GTGGTGAAGAAAGATGTTGGAGACATCTTGTTTTTCAACTACCACAATGCGAT

LP-2001      3345 GTGCTTACCCGCTGATCCAGTGGACTTTGAGCCAAGAACATGGGAAATATGTG
SM.74        3345 GTGCTTACCCGCTGATCCAGTGGACTTTGAGCCAAGAACATGGGAAATATGTG
LP_BPO161    3345 GTGCTTACCCGCTGATCCAGTGGACTTTGAGCCAAGAACATGGGAAATATGTG
LP_BPO163    3345 GTGCTTACCCGCTGATCCAGTGGACTTTGAGCCAAGAACATGGGAAATATGTG
LP_HYT25     3345 GTGCTTACCCGCTGATCCAGTGGACTTTGAGCCAAGAACATGGGAAATATGTG
POTX-FWD          GTGCTTACCCGCTGATCCAGTGGACTTTGAGCCAAGAACATGGGAAATATGTG
POTX-REV          GTGCTTACCCGCTGATCCAGTGGACTTTGAGCCAAGAACATGGGAAATATGTG

LP-2001      3398 CTGCTGAAGTTAAAAATACTTACTTAGCCAAACCAATGGCTAACCTGATCAAT
SM.74        3398 CTGCTGAAGTTAAAAATACTTACTTAGCCAAACCAATGGCTAACCTGATCAAT
LP_BPO161    3398 CTGCTGAAGTTAAAAATACTTACTTAGCCAAACCAATGGCTAACCTGATCAAT
LP_BPO163    3398 CTGCTGAAGTTAAAAATACTTACTTAGCCAAACCAATGGCTAACCTGATCAAT
LP_HYT25     3398 CTGCTGAAGTTAAAAATACTTACTTAGCCAAACCAATGGCTAACCTGATCAAT
POTX-FWD          CTGCTGAAGTTAAAAATACTTACTTAGCCAAACCAATGGCTAACCTGATCAAT
POTX-REV          CTGCTGAAGTTAAAAATACTTACTTAGCCAAACCAATGGCTAACCTGATCAAT

LP-2001      3451 GCTGCTAGCAGACAATCTCCTGATTTTGATACTAATAAAATTTCCCTGTTCTT
SM.74        3451 GCTGCTAGCAGACAATCTCCTGATTTTGATGCTAATAAAATTTCCCTGTTCTT
LP_BPO161    3451 GCTGCTAGCAGACAATCTCCTGATTTTGATGCTAATAAAATTTCCCTGTTCTT
LP_BPO163    3451 GCTGCTAGCAGACAATCTCCTGATTTTGATGCTAATAAAATTTCCCTGTTCTT
LP_HYT25     3451 GCTGCTAGCAGACAATCTCCTGATTTTGATGCTAACAAAATTTCCCTGTTCTT
POTX-FWD          GCTGCTAGCAGACAATCTCCTGATTTTGATGCTAATAAAATTTCCCTGTTCTT
POTX-REV          GCTGCTAGCAGACAATCTCCTGATTTTGATGCTAATAAAATTTCCCTGTTCTT
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LP-2001      3504 AAAATCTCAATGGGTCAAAAAAGTGGAAAAATTAGGTGCTGTCAAATCAAAGC
SM.74        3504 AAAATCTCAATGGGTCAAAAAAGTGGAAAAATTAGGTGCTGTCAAATCAAAGC
LP_BPO161    3504 AAAATCTCAATGGGTCAAAAAAGTGGAAAAATTAGGTGCTGTCAAATCAAAGC
LP_BPO163    3504 AAAATCTCAATGGGTCAAAAAAGTGGAAAAATTAGGTGCTGTCAAATCAAAGC
LP_HYT25     3504 AAAATCTCAATGGGTCAAAAAAGTGGAAAAATTAGGTGCTGTCAAATCAAAGC
POTX-FWD          AAAATCTCAATGGGTCAAAAAAGTGGAAAAATTAGGTGCTGTCAAATCAAAGC
POTX-REV          AAAATCTCAATGGGTCAAAAAAGTGGAAAAATTAGGTGCTGTCAAATCAAAGC

LP-2001      3557 CTGGCCAGACCATCGCAGCTTTCATGCAACAAACAGTGATGTTGTATGGGACC
SM.74        3557 CTGGCCAGACTATCGCAGCTTTCATGCAACAAACAGTGATGTTGTATGGGACC
LP_BPO161    3557 CTGGCCAGACCATCGCAGCTTTCATGCAACAAACAGTGATGTTGTATGGGACC
LP_BPO163    3557 CTGGCCAGACCATCGCAGCTTTCATGCAACAAACAGTGATGTTGTATGGGACC
LP_HYT25     3557 CTGGCCAGACCATCGCAGCTTTCATGCAACAAACAGTGATGTTGTATGGGACC
POTX-FWD          CTGGCCAGACCATCGCAGCTTTCATGCAACAAACAGTGATGTTGTATGGGACC
POTX-REV          CTGGCCAGACCATCGCAGCTTTCATGCAACAAACAGTGATGTTGTATGGGACC

LP-2001      3610 ATGGCCAGATACCTCAGAAAGATGAGACAAAGATTCCAACCAAAGCATATTTT
SM.74        3610 ATGGCCAGATACCTCAGAAAGATGAGACAAAGATTCCAACCAAAGCATATTTT
LP_BPO161    3610 ATGGCCAGATACCTCAGAAAGATGAGACAAAGATTCCAACCAAAGCATATTTT
LP_BPO163    3610 ATGGCCAGATACCTCAGAAAGATGAGACAAAGATTCCAACCAAAGCATATTTT
LP_HYT25     3610 ATGGCCAGATACCTCAGAAAGATGAGACAAAGATTCCAACCAAAGCATATTTT
POTX-FWD          ATGGCCAGATACCTCAGAAAGATGAGACAAAGATTCCAACCAAAGCATATTTT
POTX-REV          ATGGCCAGATACCTCAGAAAGATGAGACAAAGATTCCAACCAAAGCATATTTT

LP-2001      3663 CATTAATTGTGAGACAACAACGGATGATCTGAACCAATTTGTTAAACAAGGTT
SM.74        3663 CATTAATTGTGAGACAACAACGGATGATCTGAACCAATTTGTTAAACAAGGTT
LP_BPO161    3663 CATTAATTGTGAGACAACAACGGATGATCTGAACCAATTTGTTAAACAAGGTT
LP_BPO163    3663 CATTAATTGTGAGACAACAACGGATGATCTGAACCAATTTGTTAAACAAGGTT
LP_HYT25     3663 CATTAATTGTGAGACAACAACGGATGATCTGAACCAATTTGTTAAACAAGGTT
POTX-FWD          CATTAATTGTGAGGCAACAACGGAAGATCTGAACCAATTTGTTAAACAAGGTT
POTX-REV          CATTAATTGTGAGGCAACAACGGAAGATCTGAACCAATTTGTTAAACAAGGTT

LP-2001      3716 GGAACTTTAACAGAACTGCTCAGACAAATGATTTCACAGCTTTTGACCAATCA
SM.74        3716 GGAACTTTAACAGAACGGCTCAGACAAATGATTTCACAGCTTTTGACCAATCA
LP_BPO161    3716 GGAACTTTAACAGAACGGCTCAGACAAATGATTTCACAGCTTTTGACCAATCA
LP_BPO163    3716 GGAACTTTAACAGAACGGCTCAGACAAATGATTTCACAGCTTTTGACCAATCA
LP_HYT25     3716 GGAACTTTAACAGAACGGCTCAGACAAATGATTTCACAGCTTTTGACCAATCA
POTX-FWD          GGAACTTTAACAGAACGGCTCACACAAATGATTTCACAGCTTTTGACCAATCA
POTX-REV          GGAACTTTAACAGAACGGCTCAGACAAATGATTTCACAGCTTTTGACCAATCA

LP-2001      3769 CAAGATGGTGCAATGCTTCAATTTGAAGTCATGAAGGCAAAATTCTTCAATAT
SM.74        3769 CAAGATGGTGCAATGCTTCAAATTGAAGTCATGAAGGCAAAATTCTTCAATAT
LP_BPO161    3769 CAAGATGGTGCAATGCTTCAATTTGAAGTCATGAAGGCAAAATTCTTCAATAT
LP_BPO163    3769 CAAGATGGTGCAATGCTTCAATTTGAAGTCATGAAGGCAAAATTCTTCAATAT
LP_HYT25     3769 CAAGATGGTGCAATGCTTCAATTTGAAGTCATGAAGGCAAAATTCTTCAATAT
POTX-FWD          CAAGATGGTGCAATGCTTCAATTTGAAGTCATGAAGGCAAAATTCTTCAATAT
POTX-REV          CAAGATGGTGCAATGCTTCAATTTGAAGTCATGAAGGCAAAATTCTTCAATAT
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LP-2001     3822 CCCTGCTGACATTATTGAAGGATACATCAATATCAAATTGAACGCCAAAATTT
SM.74       3822 CCCTGCTGACATTATTGAAGGATACATCAATATCAAATTGAACGCCAAAATTT
LP_BPO161   3822 CCCTGCTGACATTATTGAAGGATACATCAATATCAAATTGAACGCCAAAATTT
LP_BPO163   3822 CCCTGCTGACATTATTGAAGGATACATCAATATCAAATTGAACGCCAAAATTT
LP_HYT25    3822 CCCTGCTGACATTATTGAAGGATACATCAATATCAAATTGAACGCCAAAATTT
POTX-FWD         CCCTGCTGACATTATTGAAGGATACATCAATATCAATTTGAACGCCAAAATTT
POTX-REV         CCCTGCTGACATTATTGAAGGATACATCAATATCAAATTGAACGCCAAAATTT


LP-2001     3875 TCCTTGGTACATTGTCCATTATGAGGTTGTCTGGTGAAGGTCCAACTTTTG
SM.74       3875 TCCTTGGTACATTGTCCATTATGAGGTTGTCTGGTGAAGGTCCAACTTTTG
LP_BPO161   3875 TCCTTGGTACATTGTCCATTATGAGGTTGTCTGGTGAAGGTCCAACTTTTG
LP_BPO163   3875 TCCTTGGTACATTGTCCATTATGAGGTTGTCTGGTGAAGGTCCAACTTTTG
LP_HYT25    3875 TCCTTGGTACATTGTCCATTATGAGGTTGTCTGGTGAAGGTCCAACTTTTG
POTX-FWD         TCCTTGGTACATTGTCCATTATGAGGTTGTCGGGTGAAGGTCCAACTTTTG
POTX-REV         TCCTTGGTACATTGTCCATTATGAGGTTGTCTGGTGAAGGTCCAACTTTTG
```

Appendix III

Coat protein gene sequences from the Isolates of the 3' RACE experiment (Clones 2-10) aligned with known sequences of PepMV (Genbank: LP_HYT25 (MF422616.1), LP_BPO163 (MF422614.1). LP_BPO161 (MF422612.1), SM.74 (AM109896.1), LP-2001 (AJ606361.1) showing homology between the sequences.

```
LP_HYT25    5631 ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
LP_BPO163   5631 ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
LP_BPO161   5631 ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
SM.74       5631 ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
LP-2001     5631 ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
Clone-2-CP       ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
Clone-3-CP       ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
Clone-4-CP       ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
Clone-5-CP       ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
Clone-6-CP       ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
Clone-8-CP       ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
Clone-9-CP       ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA
Clone-10-CP      ATGCCTGACACAACACCTGTTGCTGCCACTTCAAGTGCACCACCTACAGCCAAA

LP_HYT25    5685 GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
LP_BPO163   5685 GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
LP_BPO161   5685 GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
SM.74       5685 GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
LP-2001     5685 GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
Clone-2-CP       GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
Clone-3-CP       GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
Clone-4-CP       GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
Clone-5-CP       GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
Clone-6-CP       GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
Clone-8-CP       GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
Clone-9-CP       GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT
Clone-10-CP      GATGCTGGTGCCAAAGCTCCTTCTGACTTCTCAAATCCCAATACAGCTCCTAGT

LP_HYT25    5739 CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
LP_BPO163   5739 CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
LP_BPO161   5739 CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
SM.74       5739 CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
LP-2001     5739 CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
Clone-2-CP       CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
Clone-3-CP       CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
Clone-4-CP       CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
Clone-5-CP       CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
Clone-6-CP       CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
Clone-8-CP       CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
Clone-9-CP       CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
Clone-10-CP      CTCAGTGATTTGAAGAAAGTCAAGTATGTCTCCACAGTGACTTCCGTGGCCACA
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LP_HYT25    5793 CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
LP_BPO163   5793 CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
LP_BPO161   5793 CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
SM.74       5793 CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
LP-2001     5793 CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
Clone-2-CP       CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
Clone-3-CP       CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
Clone-4-CP       CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
Clone-5-CP       CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
Clone-6-CP       CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
Clone-8-CP       CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
Clone-9-CP       CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC
Clone-10-CP      CCAGCTGAAATTGAAGCCCTAGGCAAAATCTTCACCGCTATGGGCCTTGCCGCC

LP_HYT25    5847 AATGAGACTGGTCCAGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
LP_BPO163   5847 AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
LP_BPO161   5847 AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
SM.74       5847 AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
LP-2001     5847 AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
Clone-2-CP       AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
Clone-3-CP       AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
Clone-4-CP       AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
Clone-5-CP       AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
Clone-6-CP       AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
Clone-8-CP       AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
Clone-9-CP       AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG
Clone-10-CP      AATGAGACTGGTCCGGCGATGTGGGATCTAGCTCGTGCGTATGCTGATGTGCAG

LP_HYT25    5901 AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
LP_BPO163   5901 AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
LP_BPO161   5901 AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
SM.74       5901 AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
LP-2001     5901 AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
Clone-2-CP       AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
Clone-3-CP       AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGTATTATCA
Clone-4-CP       AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
Clone-5-CP       AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
Clone-6-CP       AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
Clone-8-CP       AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
Clone-9-CP       AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA
Clone-10-CP      AGCTCTAAATCCGCACAGCTGATTGGTGCTACCCCTTCCAACCCTGCATTATCA

LP_HYT25    5955 CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATTAATATAACACCCAGGCAATTT
LP_BPO163   5955 CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
LP_BPO161   5955 CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
SM.74       5955 CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
LP-2001     5955 CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
Clone-2-CP       CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACATCCAGGCAATTT
Clone-3-CP       CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
Clone-4-CP       CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
Clone-5-CP       CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
Clone-6-CP       CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
Clone-8-CP       CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
Clone-9-CP       CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
Clone-10-CP      CGCCGAGCCCTTGCTGCTCAGTTTGATCGAATCAATATAACACCCAGGCAATTT
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LP_HYT25    6009 TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
LP_BPO163   6009 TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
LP_BPO161   6009 TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
SM.74       6009 TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
LP-2001     6009 TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
Clone-2-CP       TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
Clone-3-CP       TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
Clone-4-CP       TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
Clone-5-CP       TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
Clone-6-CP       TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
Clone-8-CP       TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
Clone-9-CP       TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA
Clone-10-CP      TGCATGTACTTTGCTAAAGTTGTTTGGAACATCCTTCTCGACAGCAATATTCCA


LP_HYT25    6063 CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
LP_BPO163   6063 CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
LP_BPO161   6063 CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
SM.74       6063 CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
LP-2001     6063 CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
Clone-2-CP       CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
Clone-3-CP       CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
Clone-4-CP       CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
Clone-5-CP       CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
Clone-6-CP       CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
Clone-8-CP       CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
Clone-9-CP       CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT
Clone-10-CP      CCAGCAAATTGGGCTAAACTTGGTTACCAAGAAGATACAAAATTTGCTGCATTT


LP_HYT25    6117 GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
LP_BPO163   6117 GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
LP_BPO161   6117 GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
SM.74       6117 GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
LP-2001     6117 GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
Clone-2-CP       GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
Clone-3-CP       GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
Clone-4-CP       GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
Clone-5-CP       GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
Clone-6-CP       GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
Clone-8-CP       GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
Clone-9-CP       GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC
Clone-10-CP      GACTTCTTCGATGGAGTCACCAACCCTGCCAGCCTGCAGCCTGCTGATGGTCTC


LP_HYT25    6171 ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
LP_BPO163   6171 ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
LP_BPO161   6171 ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
SM.74       6171 ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
LP-2001     6171 ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
Clone-2-CP       ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
Clone-3-CP       ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
Clone-4-CP       ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
Clone-5-CP       ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
Clone-6-CP       ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
Clone-8-CP       ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
Clone-9-CP       ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
Clone-10-CP      ATCAGGCAACCAAATGAGAAAGAACTAGCTGCTCACTCAGTAGCTAAATATGGC
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LP_HYT25    6225 GCCTTGGCTAGGCAAAAGATCTCCACAGGCAATTATATTACCACACTTGGAGAA
LP_BPO163   6225 GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA
LP_BPO161   6225 GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA
SM.74       6225 GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA
LP-2001     6225 GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA
Clone-2-CP       GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA
Clone-3-CP       GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA
Clone-4-CP       GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA
Clone-5-CP       GCCTTGGCTAGGCAAAAGATCTCCCCAGGTAATTATATTACCACACTTGGAGAA
Clone-6-CP       GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA
Clone-8-CP       GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA
Clone-9-CP       GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA
Clone-10-CP      GCCTTGGCTAGGCAAAAGATCTCCACAGGTAATTATATTACCACACTTGGAGAA

LP_HYT25    6279 GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
LP_BPO163   6279 GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
LP_BPO161   6279 GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
SM.74       6279 GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
LP-2001     6279 GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
Clone-2-CP       GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
Clone-3-CP       GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
Clone-4-CP       GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
Clone-5-CP       GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
Clone-6-CP       GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
Clone-8-CP       GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
Clone-9-CP       GTCACACGTGGACAAATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC
Clone-10-CP      GTCACACGTGGACACATGGGTGGAGCTAACACCATGTACGCGATCGACGCACCC

LP_HYT25    6333 CCTGAACTTTAA
LP_BPO163   6333 CCTGAACTTTAA
LP_BPO161   6333 CCTGAACTTTAA
SM.74       6333 CCTGAACTTTAA
LP-2001     6333 CCTGAACTTTAA
Clone-2-CP       CCTGAACTTTAA
Clone-3-CP       CCTGAACTTTAA
Clone-4-CP       CCTGAACTTTAA
Clone-5-CP       CCTGAACTTTAA
Clone-6-CP       CCTGAACTTTAA
Clone-8-CP       CCTGAACTTTAA
Clone-9-CP       CCTGAACTTTAA
Clone-10-CP      CCTGAACTTTAA
```

Appendix IV

Sequence of the TGB3 from the 3'RACE aligned with Peruvian strain isolates which show homology. Aligned sequences were obtained from Genbank: LP_HYT25 (MF422616.1), LP_BPO163 (MF422614.1), LP_BPO161 (MF422612.1), SM.74 (AM109896.1).

```
LP_HYT25    5338 ATGTCCTCATACTTATCTTCATTCTCACATTGGGTATTGTCCTCACCAATAAAT
LP_BPO163   5338 ATGTCCTCATACTTATCTTCATTCTCACATTGGGTATTGTCCTCACCAATAAAT
LP_BPO161   5338 ATGTCCTCATACTTATCTTCATTCTCACATTGGGTATTGTCCTCACCAATAAAT
SM.74       5338 ATGTCCTCATACTTATCTTCATTCTCACATTGGGTATTGTCCTCACCAATAAAT
Clone-5-TGB3     ATGTCCTCATACTTATCTTCATTCTCACATTGGGTATTGTCCTCACCAATAAAT


LP_HYT25    5392 TTAGTTTTAGCTTTAGTCGTACTACTCACCAGCATTCTTGCTATAACACACATT
LP_BPO163   5392 TTAGTTTTAGCTTTAGTCGTACTACTCACCAGCATTCTTGCTATAACACACATT
LP_BPO161   5392 TTAGTTTTAGCTTTAGTCGTACTACTCACCAGCATTCTTGCTATAACACACATT
SM.74       5392 TTAGTTTTAGCTTTAGTCGTACTACTCACCAGCATTCTTGCTATAACACACATT
Clone-5-TGB3     TTAGTTTTAGCTTTAGTCGTACTACTCACCAGCATTCTTGCTATAACACACATT


LP_HYT25    5446 CAGCAACCAACAACACACAACCATTGTCAGGTCATCATTGACGGTGCTGCAATA
LP_BPO163   5446 CAGCAACCAACAACACACAACCATTGTCAGGTCATCATTGACGGTGCTGCAATA
LP_BPO161   5446 CAGCAACCAACAACACACAACCATTGTCAGGTCATCATTGACGGTGCTGCAATA
SM.74       5446 CAGCAACCAACAACACACAACCATTGTCAGGTCATCATTGACGGTGCTGCAATA
Clone-5-TGB3     CAGCAACCAACAACACACAACCATTGTCAGGTCATCATTGACGGTGCTGCAATA


LP_HYT25    5500 GTCATAACAAATTGTGAGAACACACCAGAAGTACTTAAGGCAATCAACTTCTCC
LP_BPO163   5500 GTCATAACAAATTGTGAGAACACACCAGAAGTACTTAAGGCAATCAACTTCTCC
LP_BPO161   5500 GTCATAACAAATTGTGAGAACACACCAGAAGTACTTAAGGCAATCAACTTCTCC
SM.74       5500 GTCATAACAAATTGTGAGAACACACCAGAAGTACTTAAGGCAATCAACTTCTCC
Clone-5-TGB3     GTCATAACAAATTGTGAGAACACACCAGAAGTACTTAAGGCAATCAACTTCTCC


LP_HYT25    5554 CCCTGGAACGGGTTAAGTTTTCCTAAGTTTGAAAATCAATATTGA
LP_BPO163   5554 CCCTGGAACGGGTTAAGTTTTCCTAAGTTTGAAAATCAATATTGA
LP_BPO161   5554 CCCTGGAACGGGTTAAGTTTTCCTAAGTTTGAAAATCAATATTGA
SM.74       5554 CCCTGGAACGGGTTAAGTTTTCCTAAGTTTGAAAATCAATATTGA
Clone-5-TGB3     CCCTGGAACGGGTTAAGTTTTCCTAAGTTTGAAAATCAATATTGA
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Appendix V

Sequence of the TGB3 from the PCR using the degenerate PepMV primers showing homology with TGB3 sequences from the Chilean 2 strain. Aligned sequences were obtained from Genbank: CH_BPO158 (MF422615.1), CH_BPO162 (MF422613.1), CH_BPO160 (MF422611.1), P22 (HQ650560.2).

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CH_BPO158   5336 ATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCTCACCAATAAAT
CH_BPO162   5336 ATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCTCACCAATAAAT
CH_BPO160   5336 ATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCTCACCAATAAAT
P22         5336 ATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCTCACCAATAAAT
PCR-TGB3         ATGTCCTCATACTTATCTTCATTCTTACACTGGGTATTGTCCTCACCAATAAAT


CH_BPO158   5390 TTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTACAACACACATT
CH_BPO162   5390 TTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTACAACACACATT
CH_BPO160   5390 TTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTACAACACACATT
P22         5390 TTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTACAACACACATT
PCR-TGB3         TTAGTTTTAGCATTAGCCGTAATACTCACCAGCATCATTGCTACAACACACATT


CH_BPO158   5444 CTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACGGTGCAGCCATA
CH_BPO162   5444 CTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACGGTGCAGCCATA
CH_BPO160   5444 CTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACGGTGCAGCCATA
P22         5444 CTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACGGTGCAGCCATA
PCR-TGB3         CTGCAACCCAAACAGGTCAATCAGTGCCAGGTCATCATTGACGGTGCAGCCATA


CH_BPO158   5498 GTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAATCAACTTCTCC
CH_BPO162   5498 GTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAATCAACTTCTCC
CH_BPO160   5498 GTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAATCAACTTCTCC
P22         5498 GTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAATCAACTTCTCC
PCR-TGB3         GTTATAACAAATTGTCCAAACACACCCGAAGTTCTTAAAGCAATCAACTTCTCC


CH_BPO158   5552 CCTTGGAACGGGTTAAGTTTTCCTCAATTGTGA
CH_BPO162   5552 CCTTGGAACGGGTTAAGTTTTCCTCAATTGTGA
CH_BPO160   5552 CCTTGGAACGGGTTAAGTTTTCCTCAATTGTGA
P22         5552 CCTTGGAACGGGTTAAGTTTTCCTCAATTGTGA
PCR-TGB3         CCTTGGAACGGGTTAAGTTTTCCTCAATTGTGA
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Appendix VI

The cloned coat protein translated sequence alignment with reference isolates of the Peruvian strain. The reference sequences were acquired from Genbank: LP_BPO163 (MF422614.1), LP_HYT25 (MF422616.1), LP_BPO161 (MF422612.1).

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LP_BPO163-AA      1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV
LP_HYT25          1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV
LP_BPO161         1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV
Clone-2-AA        1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV
Clone-3-AA        1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV
Clone-4-AA        1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV
Clone-5-AA        1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV
Clone-6-AA        1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV
Clone-8-AA        1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV
Clone-9-AA        1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV
Clone-10-AA       1 MPDTTPVAATSSAPPTAKDAGAKAPSDFSNPNTAPSLSDLKKVKYVSTVTSV

LP_BPO163-AA     52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN
LP_HYT25         52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN
LP_BPO161        52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN
Clone-2-AA       52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN
Clone-3-AA       52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN
Clone-4-AA       52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN
Clone-5-AA       52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN
Clone-6-AA       52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN
Clone-8-AA       52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN
Clone-9-AA       52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN
Clone-10-AA      52 ATPAEIEALGKIFTAMGLAANETGPAMWDLARAYADVQSSKSAQLIGATPSN

LP_BPO163-AA    104 PALSRRALAAQFDRINITPRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED
LP_HYT25        104 PALSRRALAAQFDRINITPRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED
LP_BPO161       104 PALSRRALAAQFDRINITPRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED
Clone-2-AA      104 PALSRRALAAQFDRINITSRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED
Clone-3-AA      104 PVLSRRALAAQFDRINITPRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED
Clone-4-AA      104 PALSRRALAAQFDRINITPRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED
Clone-5-AA      104 PALSRRALAAQFDRINITPRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED
Clone-6-AA      104 PALSRRALAAQFDRINITPRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED
Clone-8-AA      104 PALSRRALAAQFDRINITPRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED
Clone-9-AA      104 PALSRRALAAQFDRINITPRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED
Clone-10-AA     104 PALSRRALAAQFDRINITPRQFCMYFAKVVWNILLDSNIPPANWAKLGYQED

LP_BPO163-AA    156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISTG
LP_HYT25        156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISTG
LP_BPO161       156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISTG
Clone-2-AA      156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISTG
Clone-3-AA      156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISTG
Clone-4-AA      156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISTG
Clone-5-AA      156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISPG
Clone-6-AA      156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISTG
Clone-8-AA      156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISTG
Clone-9-AA      156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISTG
Clone-10-AA     156 TKFAAFDFFDGVTNPASLQPADGLIRQPNEKELAAHSVAKYGALARQKISTG
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LP-BPO163-AA    208 NYITTLGEVTRGHMGGANTMYAIDAPPEL
LP_HYT25-AA     208 NYITTLGEVTRGHMGGANTMYAIDAPPEL
LP_BPO161-AA    208 NYITTLGEVTRGHMGGANTMYAIDAPPEL
Clone-2-AA      208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-3-AA      208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-4-AA      208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-5-AA      208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-6-AA      208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-8-AA      208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-9-AA      208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
Clone-10-AA     208 NYITTLGEVTRGQWVELTPCTRSTHPLNF
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