The genetic diversity of Turnip yellows virus in oilseed rape (*Brassica napus*) in Europe, pathogenic determinants, new sources of resistance and host range.

by

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Thesis

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Declarations

This thesis is presented in accordance with the regulations for the degree of Doctor of Philosophy. It has been written by myself and has not been submitted in any previous application for any degree. The work in this thesis has been undertaken by myself except where otherwise stated.

Max John Newbert

Abstract

The aphid transmitted *Polerovirus* Turnip yellows virus (TuYV) was found to be widespread with high incidences in oilseed rape (OSR) across Europe. UK, France, Germany and Poland all having >90% TuYV incidence in some OSR crops. From the 179 whole TuYV genomes sequenced in this study the phylogenetic analyses indicated three distinct genetic groups in the UK, two of which were also detected in Europe. These three genotypes were also distinct from the original sequenced TuYV-FL. These groups are proposed to be distinct species due to their genetic distance based on the most variable gene ORF5 and phylogenetic analyses of ORF1, ORF3, ORF4 and ORF5. Mixed TuYV infection was uncommon and only two plant samples had genetically distinct isolates. Whole genome analysis also provided valuable information on two recombination hotspots located within TuYV genes ORF3 and ORF5. Investigation into the epidemiology of TuYV revealed many weed and crop species as hosts, including sugar beet, which it was previously thought not to infect. TuYV isolates detected infecting weed plants in the UK were successfully transmitted to OSR. Previously undescribed hosts, verbascum, geranium, teasel, spear thistle, dock and previously described hosts in the *Brassicaceae*, *Compositae* and *Lepidium* families were found in the UK. A full-length infectious clone of a UK isolate of TuYV has been produced, this will allow further assessment of TuYV in the future. The infectious clone was able to cause systemic infection of TuYV and was aphid transmissible. The *Arabidopsis thaliana* gene knock-out study did not reveal a single eIF gene or gene linked to virus movement or silencing that could provide extreme broad-spectrum resistance. The gene *eIF(iso)4G.1* was able to give a broad-spectrum quantitative resistance, and the potential of *eIF3D.2* as well as sucrose symporters *SUC1* and *SUC2* as candidates for extreme TuYV resistance were discovered. This understanding of the epidemiology and diversity of TuYV is being used to develop strategies for control.

Abbreviations

Common Abbreviations

35S	Cauliflower mosaic virus 35S promotor
A_{405nm}	absorbance at wavelength 405 nm
AIC	akaike information criterion
AKT	phosphorylation gated potassium channel
ASK	apoptosis signal-regulating kinase
ATP	adenosine triphosphate
BChV	Beet chlorosis virus
BLAST	Basic Local Alignment Search Tool
BMYV	Beet mild yellowing virus
bp	base pair
BrYV	Brassica yellows virus
BSA	bovine serum albumin
BWYV	Beet western yellows virus
CaMV	Cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CP	coat protein

cm	centimetre
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dH_2O	distilled water
dsDNA	double stranded deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
eIF	eukaryotic translation initiation factors
g	gram
G	gravitational force
GFP	green fluorescent protein
GTP	guanosine triphosphate
H_2O	water
IgG	Immunoglobulin G
IRES	Internal ribosomal entry site
kHa	kilo hectares (1000 hectares)
kb	kilobase
L	litre
LB	left border
LSD	least significant difference
M	molar (moles per litre)
mDA	megadalton
mg	milligram
ml	millilitre
mM	millimolar

mm	millimetre
MP	movement protein
mRNA	messenger ribonucleic acid
NASC	Nottingham Arabidopsis Stock Centre
NCBI	National Centre for Biotechnology Information
nCBP	novel cap binding protein
nm	nanometres
nos	Nopaline synthase
nt	nucleotide
ORF	open reading frame
PAPK	plasmodesmal-associated protein kinase
PCR	polymerase chain reaction
pH	$-\log_{10}[H^+]$
PLRV	Potato leaf roll virus
PTGS	post-transcriptional gene silencing
QTL	quantitative trait loci
R	resistance gene
RB	right border
RISC	RNA-induced silencing complex
REML	residual maximum likelihood
RTD	read through domain
RTP	read through protein
RT-PCR	reverse transcription polymerase chain reaction
RNA	ribonucleic acid

RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
ROS	Reactive oxygen species
SNP	single nucleotide polymorphism
ssDNA	single stranded deoxyribonucleic acid
ssRNA	single stranded ribonucleic acid
SUC	sucrose symporter
t/ha	tonnes per hectare
T-DNA	transfer deoxyribonucleic acid
tRNA	transfer ribonucleic acid
TMV	Tobacco mosaic virus
TuYV	Turnip yellows virus
TuMV	Turnip mosaic virus
μg	mircogram
μl	mircolitre
μM	mircomolar
UTR	untranslated region
UV	ultra violet
V	volts
VPg	viral protein genome-linked
VSR	viral suppressor of ribonucleic acid silencing
W	watts
w/v	weight/volume

Chapter 1

General Introduction

1.1 *Brassicaceae*

The angiosperm family *Brassicaceae* are a large group of morphologically varied and economically valuable species of plants (formally known as *Cruciferae*). It contains around 340 genera and over 3700 species (Warwick *et al.*, 2006). Many members of the family are important crop species, hence the ability to increase their yield would be highly valuable. One way of increasing yield is by introducing resistance to detrimental viruses that can cause reductions in plant productivity or induce necrosis.

Due to the economic importance of the *Brassicaceae*, extensive genetic and molecular analyses has been conducted for the six cultivated *Brassica* species. Their relationship has been clarified by cytological analyses (Schmidt *et al.*, 2001). Among species of the family, chromosome numbers vary from n = 4-128 (Kubitzki *et al.*, 1990). The three diploid species, *Brassica nigra* (BB), *Brassica oleracea* (CC) and *Brassica rapa* (AA) (syn. *campestris*) have 2n = 16, 18 and 20 chromosomes, respectively. Hybrids of these diploid species have resulted in the amphidiploid species *Brassica juncea* (AABB), *Brassica napus* (AACC) and *Brassica carinata* (BBCC) (U, 1935). These species are of worldwide importance for root, leaf, stem and oil-type crops, providing a diverse range of food sources.

Also within the family *Brassicaceae* is the model plant *Arabidopsis thaliana* (L.) (thale cress) (Al-Shehbaz *et al.*, 2006). It is currently used throughout plant science due it being the first fully sequenced genome, with annotations

being widely available (Bevan and Walsh, 2005), paving the way to a better understanding of every aspect of plant biology. This makes *Arabidopsis* very useful for understanding genetic interactions which may then be transferable to the crop species within *Brassicaceae*, allowing the further understanding of gene interactions and identification of novel gene functions.

1.1.1 *Brassica napus* L.

One member of the *Brassicaceae* family of interest is oilseed rape (OSR, *Brassica napus* L.), which is an amphidiploid (AACC, n = 19) and evolved through hybridisation between wild parental progenies of *B. rapa* (syn. campestris; genome AA, n = 10) and *B. oleracea* (CC, n = 9) (See Section 1.5.2). OSR has low genetic diversity as a result of its recent origin. Extensive cultivation and breeding programmes started to occur as its international importance grew over the last 50 years (Downey, 1983). Although cultivated for around 2000 years, it is only relatively recent breeding activity has intensified (Colton and Potter, 1999). In contrast other crops such as wheat have been domesticated for more than 10,000 years. There is little genetic diversity as no naturally occurring wild *B. napus* has been discovered (Prakash *et al.*, 1980). It is thought to have originated from around the Mediterranean as *B. oleracea* was known to be confined to that region, so the only possible location for this hybridisation to occur was around the Mediterranean (Downey, 1983).

The UK is ranked ninth in global OSR production and accounts for 1.9 million metric tonnes (Bayer CropScience, 2007a). OSR is the third most widely grown crop in the UK after barley and wheat, with around 737 kHa under cultivation per annum (HGCA, 2012). The UK 2014 yield of winter OSR yield was 3.3-3.6 t/ha valued at £270 per tonne (July 2015). The total world production area of OSR in 2014 was 36,374kHa second only to soybean in oil production (111,269kHa) (Food and Agriculture Organization of the United Nations, 2015). In European and many Asian countries, OSR is used as the primary oil crop as it is better suited to the local environments. OSR is grown globally for food production and its ability to aid the modern crop rotation, improving establishment and yields of subsequent wheat crops (Angus *et al.*, 1991).

The seeds are harvested and crushed to separate the oil, which makes up

approximately 40% of the seed by weight, leaving the meal. The oil has been used as a lubricant, in the chemical industry, but now is most often used in cooking or the production of food. The meal contains approximately 40% protein by weight (Lamb, 1989). It has been used as an organic fertilizer, but most often it is blended into animal feed. Sales of this by-product contribute substantially to the value of the crop (Downey, 1983). This added value of oilseed products has caused breeding efforts to focus on changing the content of glucosinolates and erucic acid, for specific industry uses (Robbelen *et al.*, 1980; Daun, 1984; Cartea and Velasco, 2008). Low glucosinolates are desired for animal meal, as high levels have pesticidal properties causing concern over toxicity. The erucic acid is a valuable raw material for manufacture of a wide array of industrial products such as plasticizers, surfactants, detergents, coatings, polyesters (Bhardwaj and Hamama, 2003).

OSR growth and production faces many problems. There are major diseases of OSR such as phoma, stem canker, light leaf spot, sclerotinia, downy mildew, alternaria, powdery mildew, clubroot, verticillium wilt and viruses, all of which reduce OSR production. As well as animal pests such as aphids, flea beetle, pollen beetle, slugs, pigeons and deer that cause large amounts of physical damage. When crops are ready for harvest premature pod-shattering can cause yield loss of up to 25% on top of the pest and disease problems (Price *et al.*, 1996). All these reduce OSR popularity as a crop for farmers and need attention, but with the low genetic diversity of OSR it means it is one of the harder crops to breed improved elite lines for.

1.2 Plant Viruses

1.2.1 Virus Families

Plant viruses are completely reliant on their plant hosts for replication and survival, whilst causing diseases within their hosts. There are 23 confirmed families of plant viruses (Table 1.1) as well as a limited number of unassigned viruses. Classification of viruses into specific families is based on morphology, physical properties and genome sequences (King *et al.*, 2012). Genus definition is less formal, but there are over 70 genera recognised based on similar characteristics.

Viruses fall between two other forms of parasitic organisms, Mycoplasmas and transposable elements (Hull, 2002). Mycoplasmas are 150-300nm in size and contain a bilayer membrane but no cell wall. One of the major differences when compared to viruses are that they contain their own ribosomes and ATP producing enzymes along with their DNA. This allows Mycoplasmas to replicate by binary fission and thus some are able to be cultured in *vitro* which is not possible for viruses. Transposable elements differ by not possessing a particle structure that protects its genetic material. Their impact on the cell is generally positive and does not cause disease or cell death, but could cause gene knock-outs when insertion into plant genes occurs.

Table 1.1 – Plant Virus Families.

Family	Genome	Morphology	Reference
Bromoviridae	ssRNA	Icosahedral or Bacilliform	(Scott, 2006)
Bunyaviridae	ssRNA	Enveloped Spheres	(Haan *et al.*, 1991)
Caulimoviridae	dsDNA	Bacilliform or Icosahedra	(Bousalem *et al.*, 2008)
Circoviridae	ssDNA	Icosahedral	(Delwart and Li, 2012)
Closteroviridae	ssRNA	Flexuous Filaments	(Martelli *et al.*, 2002)
Comoviridae	ssRNA	Icosahedral	(Gall and Wellink, 2000)
Endornaviridae	dsRNA	No true capsid	(Roossinck, 2012)
Flexiviridae[a]	ssRNA	Flexuous Filaments	(Martelli *et al.*, 2007)
Geminiviridae	ssDNA	Twinned Icosahedral	(Stanley *et al.*, 2001)
Luteoviridae	ssRNA	Icosahedral	(Mayo and dArcy, 1999)
Metaviridae	ssRNA	Icosahedral	(Eickbush *et al.*, 2005)
Nanoviridae	ssDNA	Icosahedral	(Bressan and Watanabe, 2011)
Ophioviridae	ssRNA	Flexuous Filaments	(Milne *et al.*, 2011)
Partitiviridae	dsRNA	Icosahedral	(Nibert *et al.*, 2009)
Potyviridae	ssRNA	Flexuous Filaments	(Berger, 2001)
Pseudoviridae	ssRNA	Icosahedral	(Peterson-Burch and Voytas, 2002)
Reoviridae	dsRNA	Icosahedral	(Urbano and Urbano, 1994)
Rhabdoviridae	ssRNA	Enveloped Bacilliform	(Redinbaugh and Hogenhout, 2005)
Secoviridae	ssRNA	Icosahedral	(Sanfaçon *et al.*, 2009)
Sequiviridae	ssRNA	Icosahedral	(Reddick *et al.*, 1997)
Tombusviridae	ssRNA	Icosahedral	(Stuart *et al.*, 2004)
Tymoviridae	ssRNA	Icosahedral	(Maccheroni *et al.*, 2005)
Virgaviridae	ssRNA	Non-Enveloped Bacilliform	(Adams *et al.*, 2009)

[a]Alpha/Beta/Gamma

The *Luteoviridae* family are plant viruses that can infect a wide range of host plants, including many important crops (Brault *et al.*, 2011). The *Luteoviridae* genus *Polerovirus* is named after Potato leaf roll virus (PLRV), this genus also contains Turnip yellows virus (TuYV) (Mayo and dArcy, 1999) a virus emerging as an important OSR disease causing species. The *Luteoviridae* genus *Luteovirus*

members are restricted to a single plant family unlike *Polerovirus* (Fauquet *et al.*, 2005) and cause serious diseases. Another *Luteoviridae* genus *Enamovirus* consists of Pea enation mosaic virus 1 (PEMV-1) and an umbravirus Pea enation mosaic virus 2 (PEMV-2), these are the only species of *Luteoviridae* not limited to the phloem (Gray and Gildow, 2003).

The relationships of virus families are complex and interlinked, but also expanding. One of these interactions is between the *Tombusviridae* species Umbravirus, that relies on *Luteoviridae* viruses for successful infection of plants (Taliansky *et al.*, 2003), which facilitates better cell infection of *Luteoviridae* in return (Ryabov *et al.*, 2001), by acting as a satellite. The *Luteoviridae* family is expanding with the newly discovered *Polerovirus* Luff aphid-borne yellows virus (LABYV (Knierim *et al.*, 2015)), the author also provides a new phylogenetic analysis of the *Luteoviridae* family. The *Luteoviridae* are an interesting and important groups of viruses, hence understanding their diversity and interaction is key for gaining insight into how to control the diseases they can cause.

1.2.2 *Polerovirus*

TuYV was previously known as Beet western yellows virus (BWYV), until Schubert *et al.* (1998) proposed that European strains of BWYV should be renamed TuYV, due to previous observations by Duffus and Russel (1970) noting the differences between the European and USA strains of BWYV. Most notably that European isolates that infect OSR are unable to infect sugar beet. Other names were suggested such as Brassica yellows virus by Hauser *et al.* (2000); this has now been used for Chinese isolates of a virus closely related to TuYV found in brassicas (Xiang *et al.*, 2011; Lim *et al.*, 2014). The reclassification of European BWYV strains, splitting the virus in two: TuYV as an independent virus in the genus *Polerovirus*, *Luteoviridae* family (Mayo, 2002; Stevens *et al.*, 2008b) that infects OSR and Beet mild yellowing virus (Guilley *et al.*, 1995) as a separate species that infects sugar beet but not OSR.

1.3 Turnip yellows virus (TuYV)

1.3.1 TuYV Genome Organisation

Poleroviruses, such as Turnip yellows virus, all have the same basic genome structure and it is thought that they all share similar 5′ genome organisation and gene function (Stevens *et al.*, 2005). All Poleroviruses have isometric (icosahedral) non-enveloped virions (Figure 1.1), 24-30nm in diameter, with 32 capsomeres per particle (Miller *et al.*, 1995).

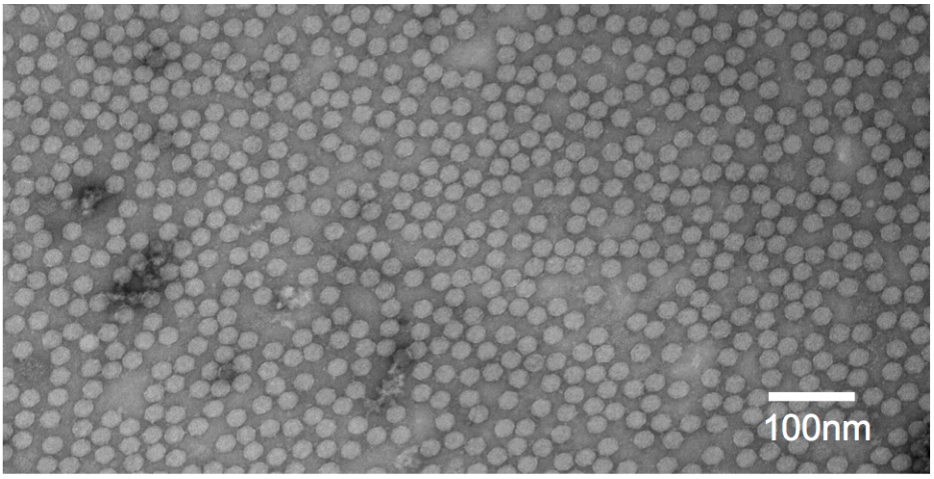

Figure 1.1 – Electron micrograph of icosahedral particles of *Turnip yellows virus* (Colin Clay)

The *Polerovirus* genome consists of a single-stranded plus sense RNA molecule 5300-6000 nucleotides long. The 5′ terminus has a viral genome-linked protein (VPg). It is known that VPg can interact with the eukaryotic translation initiation factors (eIF) of the infected cells to start gene translation (Reinbold *et al.*, 2013). TuYV does not contain an internal ribosome entry site (IRES) unlike the closely related PLRV (Jaag *et al.*, 2003). TuYV like other poleroviruses does not possess a polyA tail (Mayo and Ziegler-Graff, 1996). The genome encodes six open reading frames (ORFs) numbered from 0 to 5 (Figure 1.2) and the proteins translated from these ORFs are referred as P0 to P5. These were originally referred to as ORFs 1 to 6. The genes within the genome of TuYV are arranged in a 5′ and 3′ block separated by what was thought to be a 200 nucleotide non-coding sequence (Veidt *et al.*, 1988), but the 200 nt region is now thought to contain the gene P3a (Smirnova *et al.*, 2015).

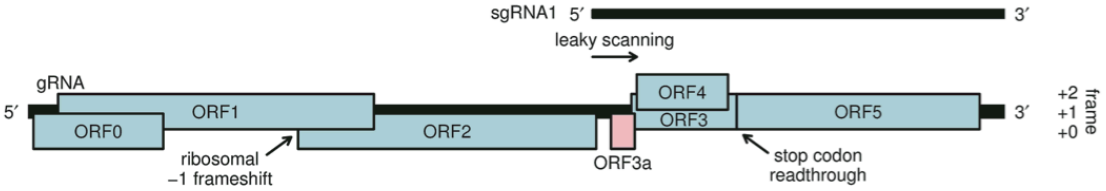

Figure 1.2 – Structure of the Turnip yellows virus genome (Smirnova *et al.*, 2015).
Functions of the Open Reading Frames (ORF): ORF0, involved in symptoms, define host range, suppressor of gene silencing; ORF1/2, a RNA dependent RNA polymerase; ORF3a, complements ORF4 aiding cellular movement; ORF3, major coat protein; ORF4, transport protein; ORF5, minor coat protein, virus accumulation and persistence within the vector. Translation frames and mechanisms also labelled.

The genome of TuYV (Figure 1.2) contains overlapping ORFs implying complex gene expression utilising different mechanisms such as subgenomic RNA, frame-shift, leaky ribosome scanning and termination suppression (Veidt *et al.*, 1988). This allows TuYV to retain a compact genome by having several mechanisms that allow overlapping genes. This understanding is based on studies on BWYV and PLRV (Mayo, 2002; Taliansky *et al.*, 2003; Beuve *et al.*, 2008).

1.3.2 P0 TuYV Gene Silencing Suppressor

P0 is a viral suppressor of RNA silencing (VSR) (Ziegler-Graff *et al.*, 1996; Dunoyer *et al.*, 2002). P0 acts as an F-box protein, recruiting the post-transcriptional modification system, stopping the degradation of translated virus messenger RNA (mRNA) (Bortolamiol *et al.*, 2007; Pazhouhandeh, 2007). P0 targets plant ARGONAUTE1 for ubiquitination by the host proteasome, thus inhibiting the restriction enzyme in the RNA induced silencing complex. It is one of the most variable components of the viral genome, which is useful in detecting new strains when used in tandem with P3 (Pazhouhandeh *et al.*, 2006), allowing serological and pathological understanding of the virus. This gene is not present in the closely related species *Luteovirus*; the 5′ end of the *Luteoviridae* is thought to be the most genetically diverse region and helps define the species (Herrbach *et al.*, 2001).

1.3.3 P1 and P2 TuYV Gene RNA-Dependent RNA Polymerase

The replication of the virus is thought to be controlled by P1 and P2 (Mayo and Ziegler-Graff, 1996). P1 is known to contain protease motifs and part of the VPg; the protease activity cleaves at the VPg site. P2 carries the viral RNA-dependent RNA-polymerase (RdRp) needed for RNA gene transcription (Mayo and Ziegler-Graff, 1996). P2 is the most conserved gene of poleroviruses, due to its intrinsic house keeping function of viral replication. The frameshift signals of the luteovirus Barley yellow dwarf virus (BYDV) include highly structured RNA and a long distance signal located in the 3′-untranslated region. This sequence has the ability to base pair to a stem loop adjacent to the frameshift site and may be part of a regulatory process to switch between virus translation and replication (Barry and Miller, 2002; Nixon *et al.*, 2002).

1.3.4 P3a TuYV Gene and Long Distance Movement

The newly discovered gene P3a (Smirnova *et al.*, 2015), has been shown not to be involved in TuYV replication, however it is necessary for the viruses systemic infection throughout a plant. Expression of P3a complements the movement of a TuYV isolate lacking ORF3a. A green fluorescent protein (GFP) localisation study showed that the P3a is targeted to the Golgi apparatus and plasmodesmata, these cell structures seem to have an essential role for P3a assisting viral movement (Smirnova *et al.*, 2015). This gene is conserved throughout the *Luteovirus* and *Polerovirus* genera with a non-AUG initiation from the sgRNA.

1.3.5 P3 TuYV Major Coat Protein

P3 is the major coat protein (CP) required for infection, efficient systemic spread and long distance movement within the phloem (Brault *et al.*, 2005). The protein is critical in the virus association with the aphid vector and may interact with cell receptors in the accessory gland of the aphid (Gray and Gildow, 2003). P3 is conserved in *Poleroviruses* (∼90% identity), as significant change would cause decapsulation that would be fatal to the virus (Mayo and Ziegler-Graff, 1996). Some regions of P3 which are in contact with viral RNA evolve near neutrality but the rest is evolutionary restricted (Torres *et al.*, 2005).

1.3.6 P4 TuYV Gene Movement Protein

P4 is necessary for movement of the virus through whole plants (Ziegler-Graff *et al.*, 1996; Stevens *et al.*, 2005) and has been thought to have a role in phloem-specific interactions as a movement protein (MP). ORF4 is present and highly conserved (∼90% identity) in all poleroviruses (Mayo and Ziegler-Graff, 1996). Poleroviruses are limited to the phloem of plants and this could be partly responsible for the muted symptoms of TuYV. It is thought the movement protein complexes with TuYV virions to allow movement and the spread of infection with the help of non-encapsidated read through proteins (RTP) (DeBlasio *et al.*, 2015).

1.3.7 P5 TuYV Minor Coat Protein

The P5 gene encodes the minor coat protein when read-through occurs; it is involved in symptoms, virus accumulation and spread. It also plays a key role in transmission efficiency and specificity, as well as virus persistence within the aphid vector. In PLRV P5 RTP has been associated with recognition with the aphid gut membrane and helps with transmission efficiency (Rouze-Jouan *et al.*, 2001). It is thought it facilitates endocytosis and exocytosis through the gut into the salivary glands of the vector (Brault *et al.*, 2005). It has also been linked to the fact that TuYV is limited to the phloem, but the mechanism is not yet understood (Peter *et al.*, 2009).

As a fusion protein resulting from a leaky stop codon, the RTP only constitutes 10% of the total capsid; this RTP has a conserved proline rich region used to incorporate it into the capsid. Non-incorporated P5 has also been shown to multimerise, cross-linking in plant cell walls following reactive oxygen species (ROS) release following the plant's immune response (DeBlasio *et al.*, 2015). This function's benefit to the virus is unknown or perhaps doesn't exist. The 3′ untranslated region (UTR) has been implicated in resistance breaking. This is related to how the viral genome interacts with the plant's eukaryotic translation initiation factors (eIF) (Nieto *et al.*, 2006).

1.3.8 P6 the Unknown *Luteoviridae* Gene

Other *Poleroviruses* have an ORF6 that is 7-9 kDa in size, which is the smallest of all predicted products. As of yet this has not been identified in TuYV. This gene's function is unknown (King *et al.*, 2012) and the closely related *Enamovirus* genus does not possess this predicted ORF, suggesting it is not essential for successfully maintaining the virus.

1.3.9 TuYV Satellite

Satellite RNAs are sub-viral RNAs which do not contain sufficient information to direct their own replication and encapsulation and do not have sequence homology with their partnered virus (Francki, 1985). TuYV has no known satellites, but it has been demonstrated that a unique strain of BWYV (BWYV ST9) has the ability to co-encapsulate a 2.8 kb RNA element (Sanger *et al.*, 1994). This ST9 element was shown to greatly increase the symptoms of infection by the same BWYV isolate, but this was not due to increased cellular movement caused by ST9. The ST9 RNA achieves this by encoding a polymerase which aids the virus' replication. The isolate BWYV-ST9 was also able to encapsulate the satRPV from the luteovirus Barley yellow dwarf virus (Rasochová *et al.*, 1997), suggesting BWYV-ST9 may not be limited to a single satellite. In keeping with satellite RNA, ST9 has no homology with BWYV, but rather with carmo-like viruses (Chin *et al.*, 1993). This suggests that other TuYV isolates have the potential to possess a satellite, or sequester one from other viruses.

1.3.10 TuYV Genetic Diversity

There is limited information available on the genetic diversity of TuYV. What little is known is based mainly on sequence analysis of P0 and P3 genes of the TuYV genomes (Miranda *et al.*, 1995; Schubert *et al.*, 1998; Hauser *et al.*, 2000; Asare-Bediako, 2011). Between TuYV and BMYV, P3 has ∼90% sequence homology, whereas P0 has as low as ∼40% sequence homology (Hauser *et al.*, 2000). As such P0 was thought to be a good candidate for genetic group classification of TuYV due to this variability and its gene function. P0 diversity has indicated three distinct genetic groups of TuYV in the UK: Common (∼80% of total isolates), Intermediate (∼18%) and Rare (∼2%) (Asare-Bediako, 2011). These groups were not shown to have differing biological activities or host origins, they were purely based on

phylogenetic analysis. There is only one full-length sequence of an European isolate of TuYV, which was from lettuce, TuYV-FL (Veidt *et al.*, 1988). This isolate has been shown to be genetically diverse from *Brassica* derived isolates of European TuYV isolates, being considered as an outlier (Asare-Bediako, 2011). The only other full length sequence published is the TuYV Australian sequence WA-1, sequenced by next generation sequencing from an orchid origin and has 89% homology to TuYV-FL (Wylie *et al.*, 2012). As the sequence homology is below 90%, this possibly indicates it is a different species to TuYV; the geographical location and host origin might support this. There is a need for full genome sequence analysis over a comprehensive geographical area to fully understand TuYV genetic diversity as at this moment there is a severe lack of genetic data on TuYV isolates.

1.3.11 TuYV Epidemiology

Unlike the closely related *Luteovirus* species, TuYV as a *Polerovirus* has had a wide host range reported including weed and crop species (Duffus and Russel, 1970; Walkey and Pink, 1990; Stevens *et al.*, 1994; Graichen *et al.*, 1996; Thurston *et al.*, 2001; Pallett *et al.*, 2002; Coutts *et al.*, 2006). These studies demonstrated that the presence of symptoms does not necessarily indicate TuYV infection, and counter to this, the lack of symptoms does not guarantee no infection (Kozłowska-Makulska *et al.*, 2007). One of the few species that demonstrates symptoms is Shepherd's Purse (*Capsella bursa-pastoris*) which undergoes leaf yellowing and curling (Sanger *et al.*, 1994; Stevens *et al.*, 2008b), and has been used in TuYV studies due to its ability to accumulate a high titre of the virus.

TuYV crop host range has been reported to include a large proportion of the *Brassicaceae* family, including *Lepidium*, *Raphanus* and *Brassica* species. Infection has also been reported in crops within the *Chenopodiaceae*, *Compositae* and *Fabaceae*. The effects of TuYV on crops outside the *Brassicaceae* family are unknown, but are obvious reservoirs of infection within agriculture in both weeds and crops.

It is necessary to note that studies that have looked into the epidemiology of TuYV over the last 30 years have used serological methods for detecting TuYV. This might be problematic as cross-reactivity of this antisera with closely rated viruses has been shown to occur (Jaegle and Van Regenmortel,

1985). Without sequencing confirmation and host range tests, it is hard to fully understand TuYV epidemiology. However, with the current reported host range of TuYV and the Home-Grown Cereals Authority (HGCA) report of 60% of OSR acreage being infected with TuYV (Nicholls, 2013), TuYV is a major threat to yields in many crop species with the possibility of high levels of inoculum within wild plant species and other crops.

1.3.12 TuYV Impact on Oilseed Rape Yields

Despite the increasing economic importance of OSR to the UK economy, yields are perceived to be low compared to other crops such as wheat 8.8 tonnes per hectare (t/ha) (for Environment Food and Affairs, 2015). The current average OSR yield is 3.5 t/ha (Department for Enviroment and Affairs, 2010), which is still far below the potential yield estimated at 6.5 t/ha (Berry and Spink, 2006). Viral diseases are a major contributing factor to low yield of OSR in the UK and elsewhere. TuYV is also a very important virus in mainland Europe, with Germany reporting incidence of upto 71.5% (Graichen *et al.*, 1997), and yield reductions upto 37% (Jay *et al.*, 1999; Graichen and Peterka, 1999).

TuYV has been overlooked for a long time due to the fact infection is largely symptomless when compared to a healthy plant (Figure 1.3 C and A), as a result it has not been seen as a problem in the agricultural industry, but visible symptoms can include discolouration (Figure 1.3 B), which may be accompanied by dwarfing (Graichen and Peterka, 1999; Bayer CropScience, 2007b; Stevens *et al.*, 2008b). Due to the the similarity of TuYV symptoms to abiotic stresses such as nutrient deficiencies (Figure 1.3 D-F) and sometimes no symptoms being present it is difficult to positively identify TuYV infection from phenotype alone. The lack of symptoms in OSR from TuYV infection has yet to be explained. TuYV can cause symptoms in other brassicas; tip burn in cabbage (Hunter *et al.*, 2002) and in Brussels sprouts by reducing their yield by upto 65% (Walsh, 2011) making both unmarketable to the public. In similar work Walsh (2012) demonstrated the dramatic effect that earlier infection has on increased yield loss. This work was conducted in cabbage but the situation is likely to be similar in OSR. As will be explained in Section 1.4 winter OSR is infected right after crop emergence, increasing the the effect of TuYV on OSR yields.

TuYV is a global threat to OSR and other *Brassica* yields. In OSR TuYV

seriously affects yield by reducing the number of pods per plant, number of seeds per pod, and the oil content per seed (Hardwick *et al.*, 1994). Some sources report that infection can actually increase individual seed weight by up to 11% but yield loss is still observed (Jay *et al.*, 1999; Jones *et al.*, 2007). The value of the yield improvement could be up to £150 per hectare resulting in a potential gain of £180 million per year (Stevens *et al.*, 2008b). Viruses are said to cause the least loss to yield in crops when compared to other pests and disease averaging 5% of total agricultural losses (Oerke, 2006). Contrary to this, TuYV causes large economic losses, reducing the yield by up to 46% (Australian Government, 2008), suggesting TuYV maybe one of the largest impacts on yield in oilseed rape. TuYV has also been reported in many mainland European countries, including the major OSR producing countries France, Germany and Poland (Kerlan, 1991; Graichen *et al.*, 2000; Schroder, 1994; Polak and Majkowa, 1992; Milosevic *et al.*, 2015). The HGCA has recently proposed the figure of 15% for yield loss for the UK with 60% of the total area of OSR affected by TuYV (392 Kha), resulting in £67 million loss per annum (Nicholls, 2013).

The other possible detrimental impact TuYV can have is on the quality of the meal and oil produced. It has been noted that TuYV-infected OSR produced oils with altered mineral content (Jones *et al.*, 2007; Coleman, 2013). As mentioned in Section 1.1.1, breeding of modern elite varieties of OSR had the aim of producing specific levels of glucosinolates and erucic acid depending on the end use desired. This ability of TuYV to affect the fatty acid composition in OSR seeds could make the oils and meals produced unmarketable as they might not meet the strict requirements needed in these products, adding to the losses of income and productivity for farmers and the industry.

1.3.13 TuYV Infection

Virus infection of plants begins with the entrance of the virus into the plant host cell. After uncoating and release of the viral genomic RNA into the host cytoplasm, the viral RNA ORF1 and ORF2 are translated to produce the RdRp, utilising plant machinery. RdRp produces negative-sense complementary ssRNA that is synthesised using the viral genomic RNA as a template, this includes a subgenomic component. The new genomic RNA is then synthesised using the negative-sense RNA as template. Translation of the 3′ subgenomic RNA component yields the capsid and movement proteins, these are then assembled

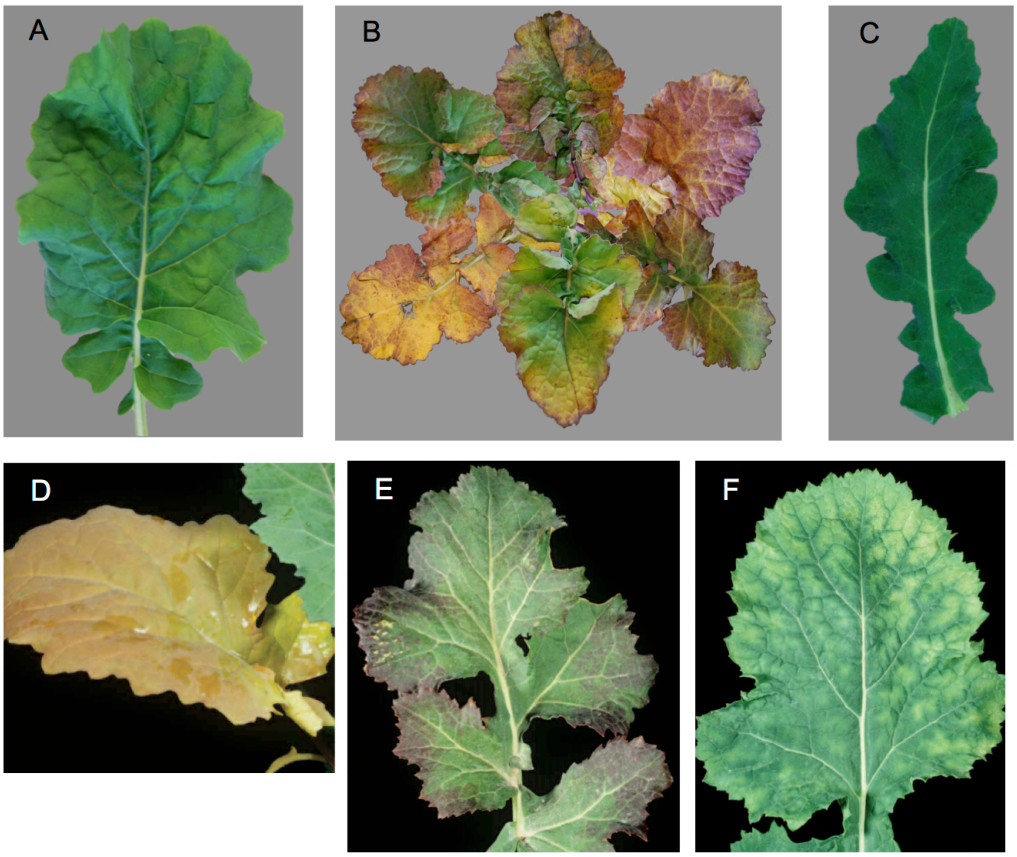

Figure 1.3 – Different oilseed rape leaf phenotypes due to TuYV infection and nutrient deficiencies.
A) Leaf from an OSR plant not infected by TuYV. B) Different phenotypes of TuYV-infected OSR leaves. C) TuYV-infected OSR leaf with no symptoms. D) E) and F) Copper, Nitrogen, Potassium deficient leaf phenotypes respectively (Nutrient images from (Billericay Fertiliser Services, 2015))

into new viral particles (Veidt *et al.*, 1988). Because poleroviruses exhibit vascular tissue tropism, TuYV replication and movement is limited to companion cells, phloem parenchyma cells, and sieve tubes (Figure 1.5) (Mutterer *et al.*, 1999). Aphid vectors transmit TuYV from plant to plant in a persistent circulative, non-propagative manner only (Gildow, 1999; Gray and Gildow, 2003). The peach potato aphid, *Myzus persicae*, is thought to be the most efficient vector for transmission of TuYV in the UK.

Aphids harbour the virus in their salivary glands (Figure 1.4), from where it is able to be transmitted to plants that the aphid feeds on (Stevens *et al.*, 2005; Bayer CropScience, 2007b). Once infected sap is ingested it is taken into the gut of the aphid, where TuYV virions may be protected from proteolytic breakdown by associating with symbionin, a chaperon protein produced by

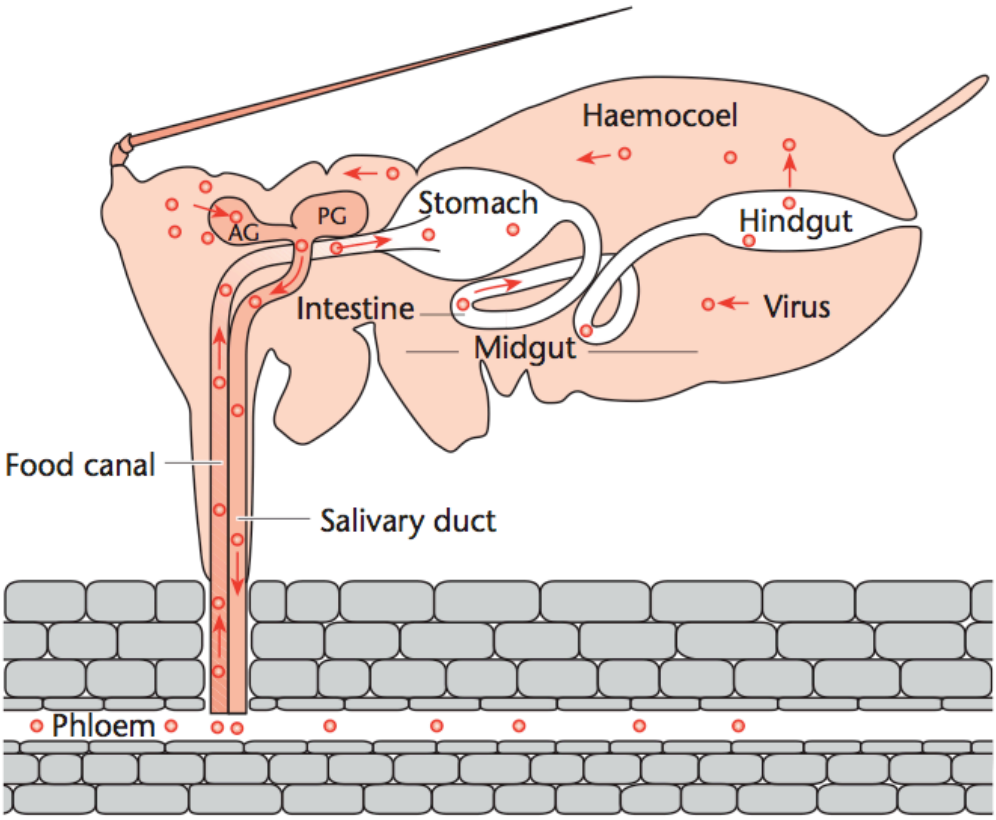

Figure 1.4 – *Myzus persicae* Acquisition of TuYV.
Diagram of the proposed route of circulative transmitted *Luteoviridae* through their aphid vector. Red Circle - TuYV Particle. AG - accessory salivary gland. PG - principal salivary gland (Brault *et al.*, 2011).

Buchnera endosymbionts (Van den Heuvel *et al.*, 1994). The virions move from the midgut lumen into the haemolymph across intestinal cells (Reinbold *et al.*, 2001). The proposed model for virus invagination through this first barrier is receptor-mediated endocytosis and exocytosis transport involving several aphid endogenous proteins (Seddas *et al.*, 2004). Once at the accessory salivary glands, virus particles cross the basal lamina and plasmalemma membrane before being released into the salivary canal, from where they can be inoculated into new plant material (Gildow, 1999). Both the CP and RTP are necessary for the movement of TuYV through the gut membrane and virion stability (Brault *et al.*, 1995; Chay *et al.*, 1996; Gildow, 1999; Brault *et al.*, 2000; Reinbold *et al.*, 2001).

From the point where the aphid acquires the virus to when it is able to transmit it back into plants can take between a few hours upto a couple of days (Hogenhout *et al.*, 2008). However, this persistent method of TuYV transmission means that aphid movements and feeding on other plants readily transmits the

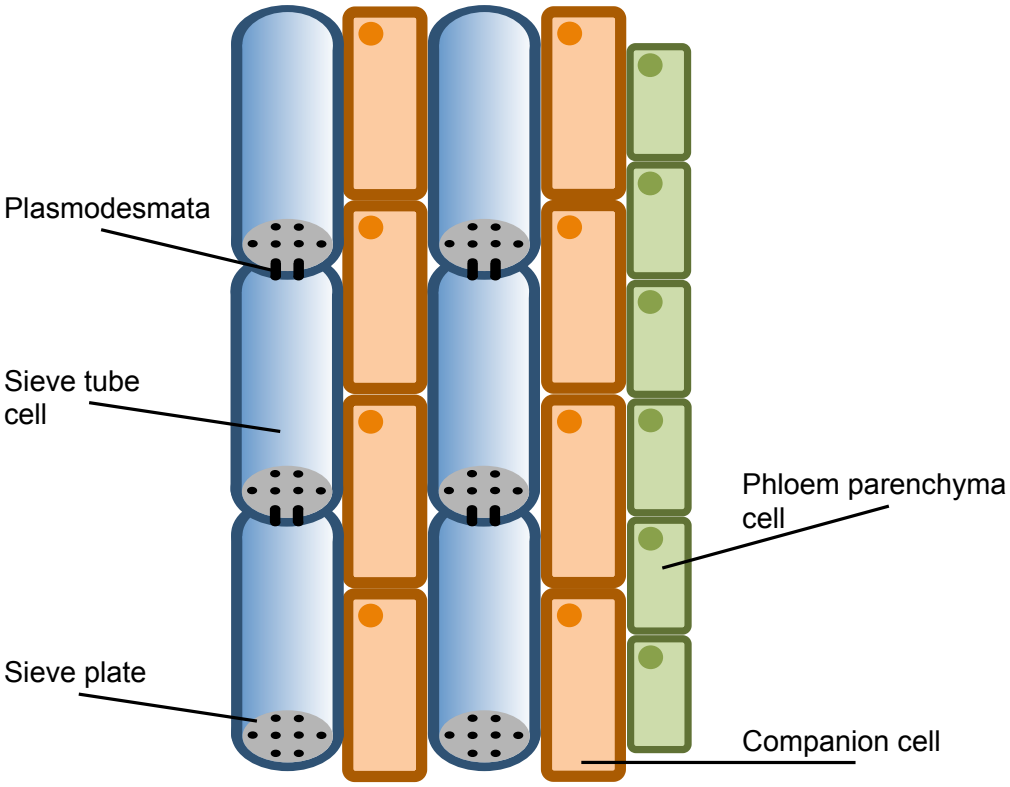

Figure 1.5 – Diagram of Cells that Constitute the Phloem.
TuYV is vascular restricted to cells surrounding the phloem, which includes: companion cells, phloem parenchyma cells, sieve tube cells and plasmodesmata.

virus, as it persists within the aphids for the remainder of their lives.

1.4 Vectors

TuYV is transmitted by aphids, in a persistent and circulative non-propagative manner. This mode of transmission needs 2-3 hours of feeding by the aphid to acquire the virus from infected plants and transmission of virus from viruliferous aphids to plants. The more transient mode of transmission of other viruses is the non-persistent, stylet-associated pattern where transmission can be achieved by aphids probing with their stylets requiring less than a minute for transmission to occur (Sylvester, 1980). Circulative viruses are usually only accessible in phloem tissues of infected plants, although inoculation may occur in non-vascular tissues. Acquisition by aphids is expected to begin with ingestion of phloem sap. This persistent manner of transmission does not pass the virus on to the aphid progeny (Schliephake *et al.*, 2000).

M. persicae is thought to be the major vector for TuYV (Reinbold *et al.*, 2001) and is present across the UK (Rothamsted Insect Survey, 2015), with the flights occurring October-November (Figure 1.6) being the major factor accounting for winter OSR infection (Asare-Bediako, 2011). The abundance of aphids during this annual flight varies year on year; 2011 and 2014 had warmer autumns comparatively (Table 1.2), which correlated with higher numbers of *M. persicae* (Figures 1.6). For winter OSR, this means warmer autumns will cause increased numbers of aphids and in turn will increase the incidence of TuYV in crops. *M. persicae* is also the most economically important aphid crop pest worldwide (Van Emden and Harrington, 2007), due its ability to spread disease so effectively. The incidence TuYV in *M. persicae* in the UK has been shown to be of up to 75% (Stevens *et al.*, 2008a), indicating the potency of this vector for the disease. Understanding the vectors of TuYV might allow better management and reduce viral spread (Bianchi *et al.*, 2008; Landis *et al.*, 2000). The spread of *M. persicae* even in years with unfavourable weather can result in infection levels sufficient to cause losses in yield, leading to the need for TuYV control.

Table 1.2 – Mean Monthly Temperatures, Rainfall, Windspeed for the Latter Months of 2011-2014 in the UK.

Year	Mean monthly measurement	August	September	October	November
2011	Rainfall (mm)	108	110	122	100
	Wind (Knots)	7.2	10.4	10.5	9.9
	Temp °C	14.1	13.9	11.2	8.9
2012	Rainfall (mm)	112	115	123	138
	Wind (Knots)	7.3	9.1	7.1	8.2
	Temp °C	15.3	12	8.2	5.7
2013	Rainfall (mm)	73	71	163	91
	Wind (Knots)	7.7	7.4	9.3	8.1
	Temp °C	15.4	12.8	11.1	5.5
2014	Rainfall (mm)	140	20	160	122
	Wind (Knots)	8.7	5.5	9.9	7.5
	Temp °C	14	14	11	7.6

Data obtained from (Met Office UK, 2011-2014)

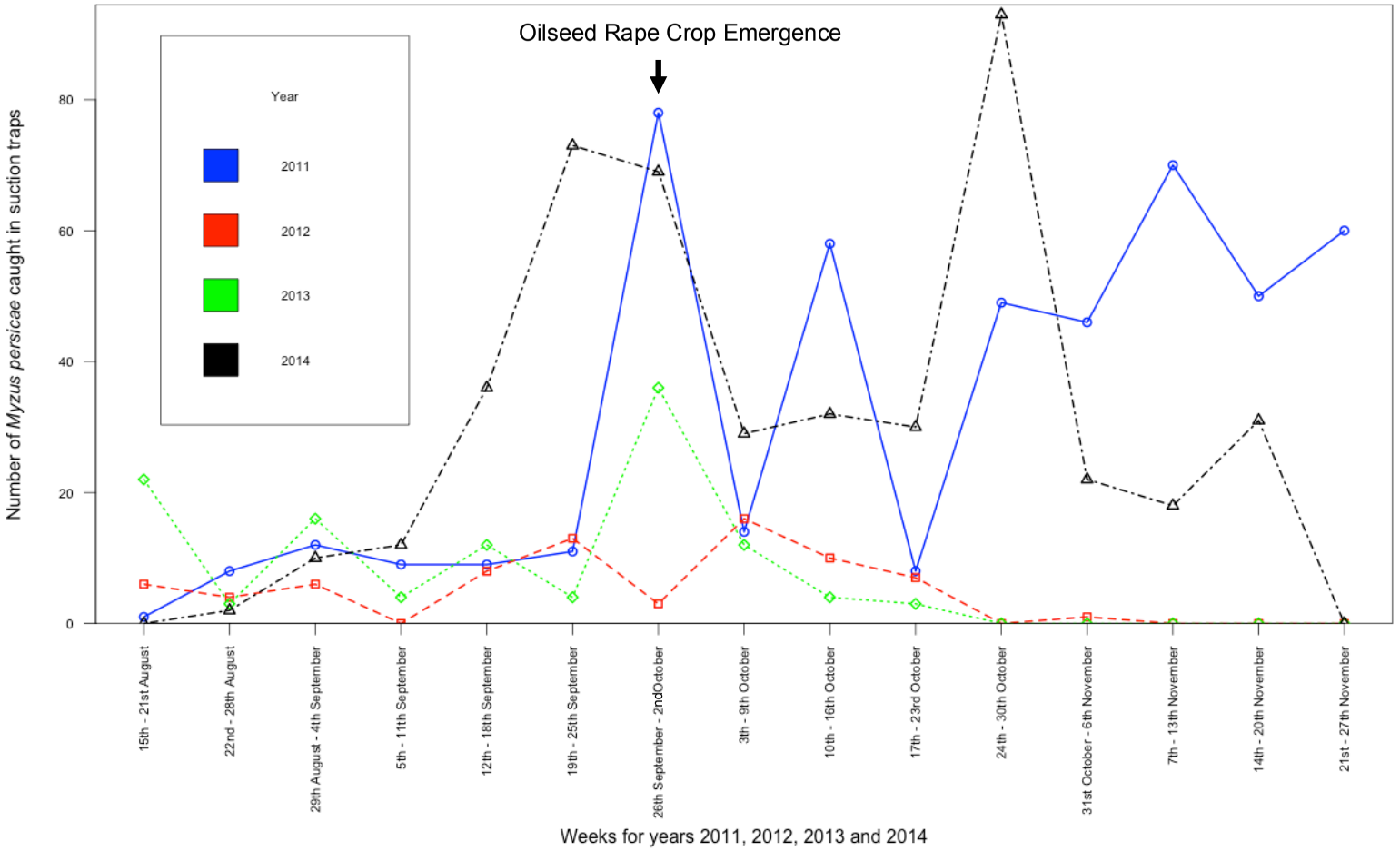

Figure 1.6 – Numbers of *Myzus persicae* caught in aphid traps around the UK between August through to November 2011-2014. *M. persicae* has two major periods of flights, usually one between June and July and the second starting late September through to November (Rothamsted Insect Survey, 2015). The second flight coincides with oilseed rape emergence (Constructed with R-Studio).

M. persicae is the main vector of not only TuYV but the closely related BMYV, though interestingly the transmission rates are vastly different between the two viruses, 96.4% for TuYV and 28.6% for BMYV (Stevens *et al.*, 1995). The transmission rates by other species of aphids are significantly lower than those of *M. persicae*. An example of a vector for both of these viruses with less efficiency is *Macrosiphum euphorbiae*, with 8.9% for TuYV and 1.8% for BMYV (Stevens *et al.*, 1995). There is a large number of shared vectors between the viruses, which indicates their close origin (Schliephake *et al.*, 2000). There is a wide variety of aphids that are vectors for TuYV, albeit with a lower efficiently than *M. persicae* (Table 1.3). Even with reduced ability to transmit TuYV, the number and increased plant host range of all vectors means they are an important consideration for understanding TuYV spread and epidemiology.

1.4.1 Vector Life Cycle

The main host crops of *M. persicae* in the UK are potato, sugar beet, lettuce, brassicas and legumes. Its behaviour in the wild is not to form dense colonies, so levels of *M. persicae* rarely reach levels that cause direct damage to plants. In crowded situations its mobility increases, walking short distances and moving to neighbouring plants, increasing it potential as a virus vector. This species overwinters on peach trees (*Prunus persica*), which are low in number in the UK but numerous in southern Europe. As such in the UK only a small number overwinter as eggs (holocyclic) on peach trees, mostly they overwinter and are mobile (anholocyclic) on herbaceous weeds and brassicas (Emden *et al.*, 1969). Aphids can reproduce more rapidly, asexually (viviparous reproduction) (Goggin, 2007). In late April to early June winged forms are produced to migrate from winter hosts to summer hosts. *M. persicae* is highly polyphagous in the summer, potentially feeding on plants in more than 40 families. From September to early November aphids migrate back to weeds and brassica hosts for overwintering. *M. persicae* has winged and wingless forms, with a range of colours, yellow, pink, red, almost black and different shades of green (Figure 1.7).

1.4.2 Food Security and Climate Change

In just one decade, the percentage of food insecure people decreased from 21.59% in 2002 to 10.98% in 2011, with more than 455 million people lifted out of food

Table 1.3 – Aphid Vectors of TuYV.

Latin name	Oilseed Rape as a Host	Common Name	Reference
Acyrthosiphon pisum	Yes	Green pea aphid	(Schliephake *et al.*, 2000)
Aphis fabae	Yes	Black Bean Aphid	(Sylvester, 1980; Schliephake *et al.*, 2000)
Aphis gossypii	Yes	Cotton Aphid	(Sylvester, 1980; Schliephake *et al.*, 2000)
Aulacorthum solani	Yes	Foxglove Aphid	(Sylvester, 1980; Schliephake *et al.*, 2000)
Brachycaudus helichrysi	Yes	Leaf-curling Plum Aphid	(Sylvester, 1980)
Brachycorynella asparagi	No	Asparagus Aphid	(Schliephake *et al.*, 2000)
Brevicoryne brassicae	Yes	Cabbage aphid	(Schliephake *et al.*, 2000)
Cavariella aegopodii	Yes	Carrot Aphid	(Schliephake *et al.*, 2000)
Lipaphis erysimi	Yes	Turnip Aphid	(Kyriakou and Close, 1983)
Macrosiphoniella sanborni	No	Chrysanthemum Aphid	(Schliephake *et al.*, 2000)
Macrosiphum albifrons	Yes	Lupin Aphid	(Schliephake *et al.*, 2000)
Macrosiphum euphorbiae	Yes	Potato Aphid	(Schliephake *et al.*, 2000)
Myzus ascalonicus	Yes	The Shallot Aphid	(Sylvester, 1980)
Myzus nicotianae	Yes	Tobacco Aphid	(Schliephake *et al.*, 2000)
Myzus persicae	Yes	Peach Potato Aphid	(Sylvester, 1980; Schliephake *et al.*, 2000)
Myzus ornatus	Yes	Violet Aphid	(Sylvester, 1980)
Nasonovia ribisnigri	No	Currant lettuce aphid	(Sylvester, 1980; Schliephake *et al.*, 2000)
Neomyzus circumflexus	Yes	Crescent-marked Lily Aphid	(Sylvester, 1980; Schliephake *et al.*, 2000)
Pentatrichopus fragaefolii	Yes	Strawberry Aphid	(Schliephake *et al.*, 2000)
Rhopalosiphoninus staphyleae tulipaellus	No	Mangold Aphid	(Sylvester, 1980)
Rhopalosiphum maidis	Yes	Corn Aphid	(Schliephake *et al.*, 2000)
Rhopalosiphum padi	No	Oat Aphid	(Schliephake *et al.*, 2000)
Sitobion avenae	No	English Grain Aphid	(Sylvester, 1980; Schliephake *et al.*, 2000)

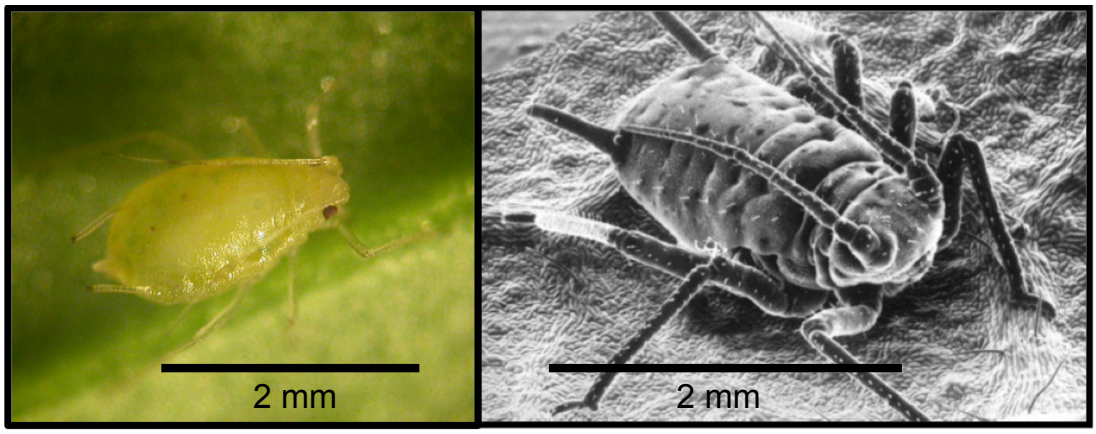

Figure 1.7 – *Myzus persicae* the main vector of TuYV in the UK
Left, Photograph of *Myzus persicae*. Right, Scanning Electron micrograph of *Myzus persicae* (Colin Clay).

insecurity. Despite such progress, 626 million people in the globe are still food insecure (Yang *et al.*, 2009; Food and Agriculture Organisation of the United Nations, 2009; FAO, 2014; Kakwani, 2015). The effects of crop diseases are reducing food availability and making food security more unpredictable. Crop losses from diseases are estimated at 16% globally, despite efforts to control them (Oerke, 2006). The demand for higher yields has led to replacement of diverse land races by genetically uniform crops that are more susceptible to diseases because the pathogens can rapidly adapt to render any host genes for resistance ineffective.

This issue is only going to be exacerbated as global mean air temperatures have increased by 0.3-0.6°C since the 19th century, and are set to increase further by 1-3.5°C by 2100 (Houghton, 1996; Johns *et al.*, 2003). With these increased temperatures and CO_2 levels, the conclusion from most studies is that the impact of insect pests will increase (Cannon, 1998; Ladányi *et al.*, 2010; Thomson *et al.*, 2010). It is thought that plants will react much slower to the increase in temperatures and CO_2 levels, thus the increased disease pressure without added benefit of more vigorous plant growth will still result in lower yields (Greogory *et al.*, 2009). As well as increased disease pressure, global food production must increase by 50% to meet the projected demand of the world's population by 2050, making the need to reduce losses in yield even greater (Bale *et al.*, 2002; Nikan *et al.*, 2013). Pest and disease management has played its role in doubling food production over the last 40 years, but pathogens still claim 10-16% of the global harvest (Chakraborty and Newton, 2011). Increased temperatures also have the potential to cause further spread of diseases such as TuYV to new regions that previously had climates that did not suit the disease vectors (Jones and Barbetti,

2012). So increased disease pressure and the possibility of extended spread of the disease due to climate change will only increase the need to be able to effectively combat yield reducing diseases such as TuYV.

1.4.3 Pesticide Resistance

Pesticides are the most common and effective way of protecting crops from many insect pests, such as aphids. For 50 years, control of *M. persicae* depended on three types of chemistry that encompass just two modes of action, OPs and carbamates acting on AChE, and pyrethroids acting on the voltage-gated sodium channel. But over time resistance can occur to commonly used pesticides. *M. persicae* clones were discovered to be resistant to a wide range of insecticides in 1990 (Moores *et al.*, 1994a), which was later confirmed in natural populations of European aphids soon after (Moores *et al.*, 1994b). The resistances were first attributed to target site resistance involving acetylcholinesterase (AChE) and then sodium channel (knock-down resistance or kdr) genes (Devonshire *et al.*, 1998). These mutations confer resistance to organophosphorus (OPs), carbamate (MACE) and pyrethroid insecticides as a consequence of both ester hydrolysis and sequestration (Devonshire and Moores, 1982). More point mutations were discovered in the sodium channels of *M. persicae*, the Super kdr (M918T) mutation (Williamson *et al.*, 1996), plus a new Super kdr (M918L) mutation (Fontaine *et al.*, 2011), which has started to become the dominant resistance over kdr in the UK (Insecticide Resistance Action Committee, 2012). MACE and pyrethroid resistance has become the major phenotype in *M. persicae* with in the UK, reducing the usefulness of a number of pesticides in agriculture for combating pests and their transmissible diseases.

The more recently introduced neonicotinoid (1990s) mode of action differed greatly from previous chemistry by targeting the central nervous system (Bass *et al.*, 2014). As a new mode of chemistry, neonicotinoids were the first line of defence as seed coatings, but *M. persicae* populations have shown differences in susceptibility to neonicotinoids up to 20 fold (Nauen and Denholm, 2005). Now over a decade later, resistance has started to occur (IRAG-UK, 2011). It was first discovered that some populations of *M. persicae* in southern France, northern Spain and northern Italy had strong resistance to neonicotinoid insecticides. So far, this resistance has only been found in *M. persicae* on peach trees, these are the first cases of resistance to neonicotinoid in any aphids, and potentially signals

the start of control failures with this class of chemical (IRAG-UK, 2011). This loss in control might already be occurring as there has been further spread of resistant aphids over a larger area of southern Europe (Slater *et al.*, 2012; IRAC-IRM, 2014). A study of six nicotinic acetylcholine receptor (nAChR) subunit genes (Mpa1 - 5 and Mpb1) from resistant and susceptible aphid clones revealed a single point mutation in the loop D region of the nAChR Mpb1 subunit of the resistant clone, causing an arginine to threonine substitution (R81T). The discovery of the mutation at this position and its association with the reduced affinity of the nAChR for imidacloprid is the first example of field-evolved target-site resistance to neonicotinoid insecticides and also provides further validation of existing models of neonicotinoid binding and selectivity for insect nAChRs (Bass *et al.*, 2011).

Currently the only effective insecticide spray against *M. persicae* as well as other pests is Plenum (Syngenta). The active ingredient is pymetrozine, that inhibits the nervous system, paralysing the cibarial pump, therefore regulating feeding behaviour that affects the food and salivary pump mechanisms of the stylet. This chemistry acts by both contact and ingestion, taking 1-2 days to have an effect, leaving time for the transmission of viruses. Only one application of pymetrozine is currently allowed for OSR in the UK, the persistence being only two weeks, leaving the crop vulnerable to pests (Syngenta, 2014). These caveats and the absence of fully effective alternatives are leaving plants open to infection even after pesticide treatment (Walsh, 2012).

The introduction of neonicotinoid insecticides expanded the modes of action for use in agriculture, but with the aphids efficiency to evolve resistance to a wide variety of pesticides, controlling TuYV by limiting the vectors will become increasingly difficult. If alternative methods are found to control spread of disease the fitness cost of insecticide resistance to the aphid, such as poor overwintering (Devonshire *et al.*, 1998) and lower reproduction (Blackman *et al.*, 1978), could reduce the high levels of insecticide-resistant aphid strains.

1.5 Major Control Options

1.5.1 Insecticide Control

Due to no TuYV-resistant OSR varieties on the oilseed rape recommended list in the UK, the only effective way to combat TuYV infection at this time in crops is with the use of pesticide. Targeted pesticide treatments during aphids flights reduce aphid numbers by up to 86%, but this method cannot be fully effective due to magnitude of the aphid flights and the ongoing development of pesticide resistance (Walsh *et al.*, 1989). Controlling TuYV is also difficult due to the ease of infection (only one aphid feeding needed to infect a plant); effective control of early infection is the best course of action. Walsh (2012) demonstrated that aphid numbers on a individual plant correlated with TuYV titre, so effective control could reduce TuYV impact, as long as it is done early during the plants development (Walsh, 2011). But no legal pesticide is fully effective against aphid colonisation.

The major issue is that the main insecticide used to reduce aphids vectors were neonicotinoids such as Crusier, as a seed coating on OSR, but these have now been banned by the EU (The European Commission, 2013). This has happened following the association of neonicotinoids with the bee population decline (Iwasa *et al.*, 2004; Girolami *et al.*, 2009; Cresswell, 2011; Blacquiere *et al.*, 2012; Whitehorn *et al.*, 2012). So alternative ways of reducing pests will need to be found to achieve this. These alternatives are needed soon as climate change is expected to increase infection levels as warmer conditions will increase the number of *M. persicae* surviving throughout the winter (Stevens *et al.*, 2008b). This led to the conclusion that the development of resistant OSR cultivars is the most viable answer for controlling the impact of the disease.

1.5.2 OSR Resistance to TuYV

There was no naturally occurring resistance TuYV in OSR known except a quantitative resistance found in the spring/winter variety Yudal by Asare-Bediako (2011). This resistance has been mapped in the *B. napus* genome but not down to the gene level, only region, thus the gene of interest is unknown. This is currently being addressed with fine mapping by Dr John Walsh's group. Due to the lack of wild *B. napus* and the low genetic diversity in existing OSR lines, alterna-

tive avenues have to be explored to obtain natural plant resistance to TuYV. It is possible to produce virus-resistant varieties by re-synthesis, a process of combining two possible sources of resistance genes from sexually compatible species *B. rapa* and *B. oleracea* (AA and CC genomes Figure 1.8) to produce *B. napus* (AACC). This has been demonstrated with the production of a TuYV resistant variety R54 (Graichen, 1994). The two *Brassica* progenitors (Figure 1.8) allow combination of genetically diverse backgrounds, which can give rise to a new resynthesised OSR expressing new phenotypes such as resistance. The mode of action of the resistance in R54 is not known.

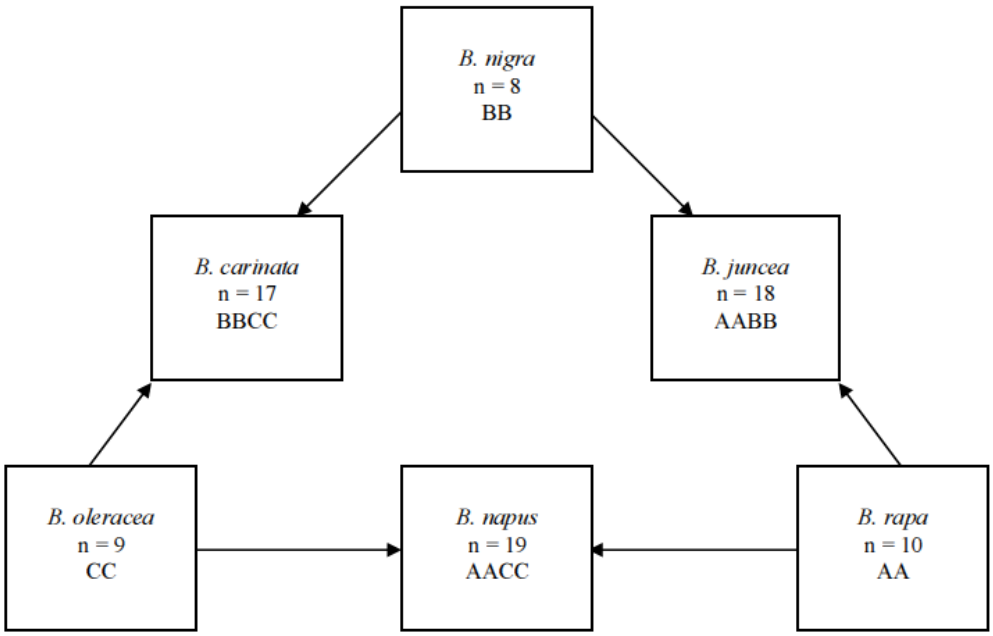

Figure 1.8 – Triangle of U showing the genetic origin of *Brassica napus* (U, 1935).
Three *Brassica* species: *Brassica nigra*, *Brassica rapa*, *Brassica oleracea* and their three hybrid species *Brassica carinata*, *Brassica juncea* and *Brassica napus*. n = chromosome count.

TuYV has very high genetic variation due to short generation times and error prone replication since no proofreading correction mechanism is associated with their RNA-dependent RNA polymerase, causing quasispecies, which are pseudospecies or sub-species (Eigen *et al.*, 1988; Andino and Domingo, 2015). This type of rapid evolution by viruses necessitates a progressive approach to breeding a resistant plant (Juergens *et al.*, 2010). The process of resynthesizing *B. napus* is slow and problematic. Another approach is genetically modifying already elite lines to produce resistance, usually with RNAi (Thomas *et al.*, 2000; Nicholls, 2013) but this will not be viable for wide-spread uptake by the industry at this point due to the restraints on genetically modified organisms (GMOs), particularly in Europe. This creates the need for natural plant resistance to be bred from existing resistant *Brassica* species using genes that can be introgressed into OSR to allow wide-scale use.

For breeding an extreme and durable form of resistance to TuYV in *B. napus*, or any species, there is a need to have a full understanding of all genetic groups of a pathogen. The limited information available on the genetic diversity within TuYV isolates is based mainly on sequence analysis of P0 and P3 genes of the TuYV genome (Miranda *et al.*, 1995; Schubert *et al.*, 1998; Hauser *et al.*, 2000). These have been of interest as P0 is thought to be the most variable genetic region and P3 is highly conserved and important for serological reasons for creation of antisera. Therefore understanding the variation and role of these genes provides the best chance of understanding how to achieve resistance, but so far full genome analysis of TuYV has not been performed on a medium, or large scale. This must be done to provide the information and virus isolates necessary to ensure no genetic groups can overcome resistance that has been, or will be introduced into crops.

1.6 Mechanisms of Natural Plant Resistance

There are five known mechanisms of resistance effective against plant viruses, these fall under two modes; passive or active immunity (also known as *R* genes) (Boualem *et al.*, 2016). Passive immunity is the first form of defence with the presence of physical barriers such as cell walls and waxy cuticles stopping virus entry to plant cells, however this can be circumvented by a virus' vector. A second form of passive resistance occurs once a virus enters a potential propagative plant cell, where absence or altered forms of plant gene products

stop viral requisite, thus systemic plant infection cannot occur as key viral processes that rely on plant machinery cannot be performed (Künstler *et al.*, 2016). Active immunity can be split in to three categories: Antiviral RNA silencing, NLR-mediated plant resistance (classic *R* genes) and Vat-mediated resistance (vector resistance). RNA silencing is initiated by the recognition of viral dsRNAs or partially double strand hairpin RNAs, which are processed to virus-derived small RNAs (vsRNA). Production of vsRNA removes viable virus genomes stopping viral protein production and replication (Hammond *et al.*, 2001). Nucleotide-binding domain and leucine-rich repeat (NLR) plant resistance is mediated through NLR genes recognising viral effectors (or avirulence factor, Avr) expressed from the virus genome. Directly or indirectly specific plant NLR genes interact triggering virus resistance (Jones and Dangl, 2006). Model for Vat-mediated resistance involving separate recognition and response phases. In the case of *A. gossypii* resistant plants, the Vat-NLR recognizes an elicitor molecule from the aphid (Chen *et al.*, 1997). This recognition phase induces local resistance mechanisms that inhibit aphid colonisation, viral replication and movement of viruses transmitted by the same aphid, while being more broad-spectrum than NLR mediate resistance (Boualem *et al.*, 2016).

One common feature of the host active immunity resistance, it is the rapid induction of programmed cell death at the site of pathogen invasion and the immediately surrounding cells. This symptomatic manifestation is called Hypersensitive Response (HR) (Künstler *et al.*, 2016). The HR is triggered by a wide variety of pathogens, as well as viruses, to prevent pathogen spread in the plant. Hypersensitive reactions are initiated by the recognition of the pathogen-encoded Avr by the plant host *R* genes, either NLR or Vat. The Avr-*R* protein interactions may trigger a mitogen-activated protein kinase (MAPK) signaling cascade and lead to a fast accumulation of reactive oxygen species (ROS) and defence hormones, salicylic acid (SA) and jasmonic acid (JA) (Hammond-Kosack and Jones, 1996).

1.6.1 Eukaryotic Translation Initiation

No active forms of plant resistance are known to be effective against *Luteoviridae* family, however passive forms of resistance might be viable as all viruses are reliant on plant machinery for propagation. Ribosomes are the driving force behind protein synthesis in eukaryotes including plants, large ribonucleoprotein

assemblies of approximately 4 MDa work in concert with a number of accessory factors to translate mRNA (Preiss and Hentze, 2003). The first step in this process is the transcription of plant DNA into mRNA, this occurs in the nucleus of the cell. The mRNA then migrates into the cytoplasm for translation into a polypeptide chain by the eIF (Browning, 1996). In the case of viral RNA such as TuYV it is already present in the cytoplasm (Duprat *et al.*, 2002). The process of mRNA translation is divided into three phases: initiation, elongation and termination (Sonenberg *et al.*, 2000). The initiation phase represents all processes required for the assembly of a ribosome with a initiator-methionyl-transfer-RNA in its peptidyl (P-) site at the start codon of the mRNA. Polypeptide synthesis is known as the elongation phase, by the process of peptide bond formation in ribosomal RNA (rRNA) catalytic centres of the large subunit (60S). Termination is controlled by stop codons within the mRNA, signalling the release of the completed polypeptide from the rRNA (Doudna and Rath, 2002).

The initiation phase of RNA translation is thought to occur by two distinct mechanisms, a cap-dependent mechanism, involving the eIFs (12 separate factors (Preiss and Hentze, 2003) this process makes up 95-97% of cellular mRNA translation, or a cap-independent mechanism, involving ribosomes which are recruited to an internal ribosome entry site (IRES) within RNA sequences. The cap-independent mechanism is between 1-25% efficient relative to the initiation of translation by a cap-dependent process (Merrick, 2004).

Cap-dependent translation requires preparation of a pool of small ribosomal subunits on which to build the initiation complex driven primarily by association of 40S subunits with eIF3 (Browning, 2004). Then binding of the ternary complex (eIF2 GTP Met-tRNA) to the 40S subunit, ATP-dependent activation of the mRNA, primarily by eIF4F (Complex of eIF4E, poly A-binding protien (PABP) and eIF4G) (Browning, 1996). Binding of the mRNA to the 40S subunit activates ATP-dependent scanning to locate the initiating start code. Elongation phases begin with the addition of the 60S subunit to form the 80S complex. In tandem with eIF2 recycling eIF2 GTD to eIF2 GTP by eIF2B (Kawaguchi and Bailey-Serres, 2002; Merrick, 2004).

1.6.2 Candidate Resistance Genes

Natural resistance sources for *Luteoviridae* are scarce in cultivated plants (Maule *et al.*, 2007). Two quantitative trait loci (QTL) conferring resistance to TuYV are known (Dreyer *et al.*, 2001; Asare-Bediako, 2011), but both their modes of action are unknown. Barley yellow dwarf virus has also had a QTL connected with resistance to it, but again the mechanism behind this is unknown (Scholz *et al.*, 2009). However, it is known that TuYV utilises its host plant's replication machinery for it own reproduction (Mayo and Ziegler-Graff, 1996). It has been shown with the TuYV-FL isolate that plants with altered production of eIF factors, or more accurately lack of them reduces viral titre in infected plants (Reinbold *et al.*, 2013). *Arabidopsis thaliana* has been the model to investigate this interaction not only with poleroviruses but others, as multiple virus species rely on plant machinery for their replication (Yoshii *et al.*, 2004). Most notably of these are eIF factors 4E and 4G (Yoshii *et al.*, 2004), but other components have been involved such as (iso)4E and 3d (Daughenbaugh *et al.*, 2003; Reinbold *et al.*, 2013; Nellist *et al.*, 2014), all conveying a passive mechanism of resistance as opposed to the *R* gene system of active recognition (Maule *et al.*, 2007).

The interaction of the capped mRNA molecule with plant eIF proteins initiates the translation process; TuYV can produce its own RNA with endogenous RdRp (Figure 1.9 A). Figure 1.9 (B) lists interactions and genomic locations of these genes in the model plant *Arabidopsis thaliana*, these genes are possible candidates for resistance knock-out studies, as they would halt virus replication and thus stop accumulation, resulting in passive resistance. TuYV lacks the usual methylguanylate cap (m^7G), but instead possesses a VPg, located within ORF1, that mimics RNA tertiary cap structure. It is known that some uncapped and non-polyadenylated viruses utilise this machinery and its removal/alteration can allow resistance to these viruses (Nieto *et al.*, 2006). All characteristics of TuYV suggesting passive resistance with the alteration or knock-out of eIF genes might convey resistance.

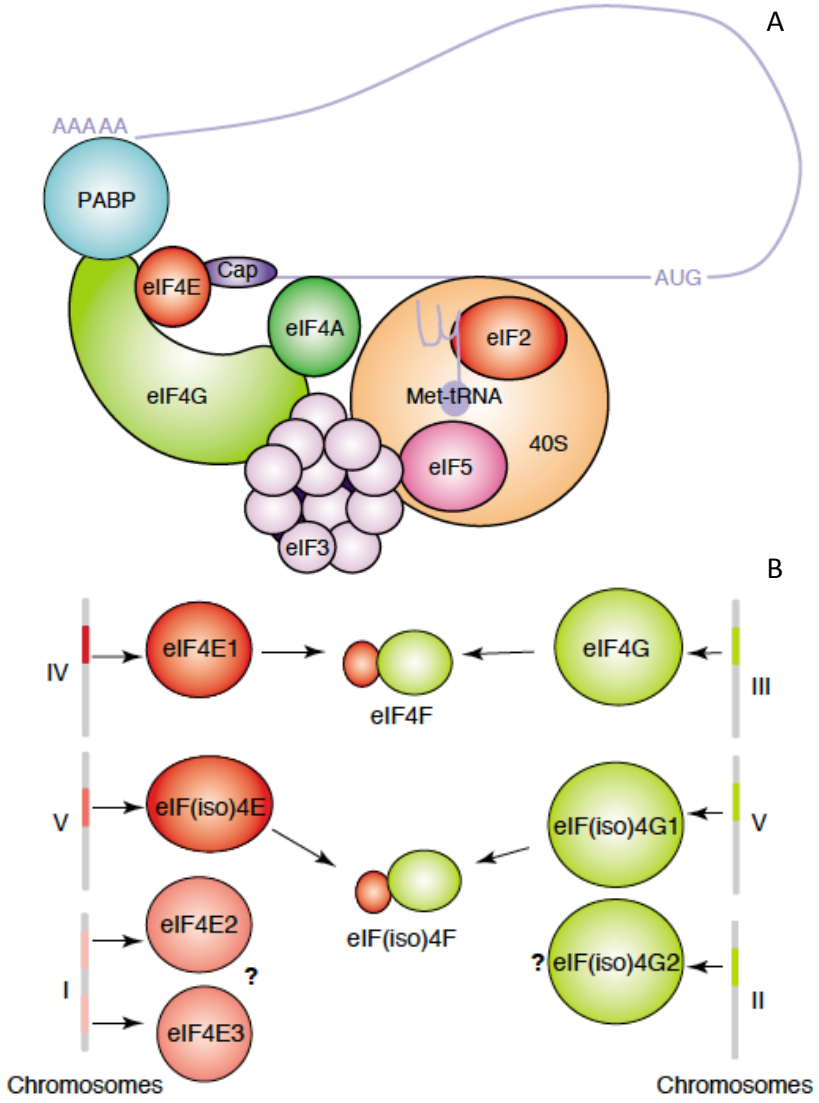

Figure 1.9 – Plant Eukaryote Initiation Factor Machinery and Chromosomal Location of Gene Copies within *Arabidopsis thaliana* (Robaglia and Caranta, 2006)
A) Plant eukaryote initiation factor (eIF) protein complex interacting with capped mRNA. B) Interaction and location of eIF components possibly involved with virus replication. TuYV is thought to interact with this pathway to replicate, thus removal or mutation of these components could convey resistance.

1.7 Aims and Objectives

The main aims of the study were to investigate the genetic diversity of TuYV in European OSR and UK weeds, with a view to evaluating plant resistance to TuYV.

The specific goals of this study were to:

- Explore whole genome diversity of TuYV infecting oilseed rape and weeds in Europe.
- Construct an infectious clone of TuYV with a UK isolate for identifying determinants of pathogenicity and host range
- Utilise *Arabidopsis thaliana* to identify new plant resistance strategies by investigating novel sources of resistances with gene knock-out lines.
- Investigate the host range of TuYV from oilseed rape and whether isolates from weeds are able to infect oilseed rape.

Chapter 2

Materials and Methods

2.1 Plant Lines

2.1.1 *Brassica napus*

Oilseed rape *Brassica napus* L. ssp. *napus*, (Roxb.) Hanelt variety Mikado, is extremely susceptible to (TuYV) (Hunter *et al.*, 2002) and was used as the propagation host for TuYV isolates, as well as the OSR variety Castelle. Other plant lines used were *Brassica oleracea* varieties Genius and Doric, as well as *Nicotiana benthamiana*. These lines were sourced from The University of Warwick's Genetic Resource Unit (GRU) and were selected on preliminary work conducted within the group on their susceptibility to TuYV.

2.2 Plant Growth Methods

2.2.1 *Brassica* Growth Methods

Brassica plants were grown in Pot and Bedding - M2 compost (Levington; medium grade sphagnum moss peat 100% (pH 5.3 6.0; N = 200, P = 150, K = 200 mg/litre)) in the glasshouse under natural light conditions. During the winter months the day length were extended using halogen lamps to ensure a 16 hour day length. Glasshouse compartment temperature were maintained at 20°C ± 2°C for growing plants. MLR-352-PE Panasonic plant growth cabinets were also used to grow plants at a temperature of 18°C ± 2°C with artificial lighting (at 18.000 lux). Seeds were sown into FP7 pots (7 cm diameter) for growing healthy plants,

the plants were grown until they were ready to be challenged with TuYV (after approximately three weeks) at which point they were moved to compartments in an Insect Rearing Unit (IRU) to be challenged at 18°C ± 2°C with artificial lighting of 18.000 lux. Plants were watered three times a week and kept inside insect proof cages (Bugdorm cage 44545, Watkins and Doncaster).

2.3 Turnip yellows virus (TuYV)

2.3.1 TuYV Isolates

TuYV can be divided into three distinct groups, common, intermediate and rare, based on the frequency of phylogenetic groups identified following sampling of oilseed rape plants in England (Asare-Bediako, 2011). The Rare group consisted of recombinant isolates between the two other genotypes. TuYV isolates maintained by the Plant-Virus Interactions Group led by Dr. John Walsh were from two sources, the LAB isolate originated from Suffolk (Patron, 1999) and was obtained from Dr. Mark Stevens of Broom's Barn and the other isolates were obtained by Dr. Elvis Asare-Bediako. The gene P0 has been used to classify the isolates into their designated genetic groups (Asare-Bediako, 2011). The intermediate phylogenetic group isolates were collected by Dr. Elvis Asare-Bediako (Asare-Bediako, 2011) from Lincolnshire (L1926 and L1937), as well as the LAB isolate. At the same time other isolates were collected (L1806, L1843, L1851 and L1906) all of which belong to the common phylogenetic group. These isolates were maintained with continuous reinfection of plants with each isolate kept separately in insect proof cages.

For inoculation tests the three isolates were chosen to be representative of the three different genetic groups: L1851 was used as the common genotype isolate and will be referred to as L1851-C, LAB for the intermediate genotype isolate and will be referred to as LAB-I, and Cau74 for rare genotype isolate and will be referred to as Cau74-R. The Cau74 isolate was obtained from a cauliflower in the UK. The three genotypes are based on phylogenetic analysis of TuYV gene P0 (Section 1.3.10) (Asare-Bediako, 2011).

2.3.2 Aphid Transmission

To facilitate the successful transfer of aphids between plants without damaging their stylets, allowing effective transmission of virus, agitation and natural aphid movement was induced. TuYV-free *Myzus persicae* (Strain *Mp1s* formally known as Genotype J (Nikan *et al.*, 2013)) were propagated on healthy brassica plants (See Section 2.1.1) within insect proof cages. Leaves infested with non-viruliferous aphids were placed under a 60 W lamp at a distance of 8 cm. This process irritated the aphids, causing them to stop feeding and to remove their stylets due to the light and temperature increase. Once aphids were mobile they were collected in petri dishes and starved in the dark for an hour to encourage subsequent feeding. The aphids were placed on TuYV-infected leaves and allowed to feed overnight to ensure uptake of virus. After feeding on infected plant material, parts of leaves with aphids on were placed on healthy plants so that as the detached leaves dried out; the aphids moved to feed on the healthy plant.

TuYV isolates maintained in brassica plants were then used to challenge plants, this was achieved by placing 1 cm^2 pieces of leaves infested with aphids onto the stem and leaves of plants to be tested. Aphids were left to move naturally over to the new plant as the original plant material dried. In some cases to ensure aphid feeding on plants to be challenged, clip cages were used for 2 days to limit movement and establish colonies. Plants maintaining TuYV were used for inoculum after 4 weeks of feeding by aphids with TuYV, 10-20 aphids per 1 cm^2 were present on each portion of leaf. These two techniques of aphid agitation and leaf placement were used for infecting of plants depending on whether aphids were killed by insecticide sprays.

Following 1-2 weeks of aphid feeding plants had two insecticide sprays applied, Hallmark Zeon (0.4 ml/L) and Plenum W.G. (0.75 g/L) (both from Syngenta). The active ingredients of these insecticides were Lambda-cyhalothrin and Pymetrozine respectively. Plants were then left in locked glasshouse compartments for 24 hours before being moved or inspected again.

2.4 TuYV Detection

2.4.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to detect plant samples infected with TuYV utilising antibodies specific for the major coat protein (P3). *B. napus* had one leaf ground into sap using a mechanical macerator (Leaf Juice Press, Meku-Pollaehne). For *Arabidopsis* plants, whole plants were harvested and plant sap was extracted using Bioreba extraction bags (Bioreba AG) with a hand-held homogeniser (Bioreba AG). Samples were randomised on 96-well plates. The outer most wells were not used to avoid any edge effect caused by temperature. Each sample was put into two wells of a microtitre plate (96-well Nunc Maxisorp) based on a method described by Walsh *et al.* (1999), to allow an average absorbance to be calculated. One positive control and three negative controls were located in outside wells of each plate.

The primary polyclonal antibody (rabbit IgG, AS-0049, DSMZ), which was specific to TuYV, was diluted 1:1000 in coating buffer, 200μl was pipetted in each well of the ELISA plate and incubated for 4 hours at 37°C. After each antibody incubation plates were washed three times with Phosphate Buffered Saline with Tween (PBST) (9809S, New England Biolabs (UK) Ltd). Plant samples were added to wells (150 μl) and incubated overnight at 4°C. After washing the secondary mouse monoclonal antibody (AS-0049/1, DSMZ) was added 1:1000 in PBST plus 0.25g/100 μl Bovine Serum Albumin (BSA)(B2064, Sigma), 150 μl was pipetted in each well and incubated for 3 hours at 37°C. After washing the tertiary goat anti-mouse antibody IgG (A4416, Sigma) was applied 1:4000 in PBST plus BSA; 150 μl was pipetted in each well and incubated for 3 hours at 37°C. Following a final wash, the substrate solution was prepared. One alkaline phosphatase tablet added per 5 ml (Sigma S0942) to a solution of 8 parts dH_2O to 1 part diethanolamine titrated to 9.8 pH with 10 M HCl. This was then added to each well (150μl). Absorbances were then detected using a Biochem Anthos 2010 ELISA reader at 405 nm with reference measurement at 620 nm (Biochem Ltd.).

ELISA data was analysed in Excel. TuYV infection was determined by positive A_{405} values after subtracting the value of two healthy (TuYV negative, grown in controlled environment) control wells and from the average of the two values for each test sample. Samples with negative corrected values were replaced with 0. Negative samples had ELISA results within two standard deviations of

the negative controls (Table 2.1). If the a sample's values were between (+) two standard deviations and 1.5x the ELISA values of the negative controls were labelled as a marginal with further molecular testing of candidate samples were conducted to detect TuYV (Table 2.1). If the sample's ELISA values were 1.5x above the negative controls, the sample is labelled as positive (Table 2.1).

Table 2.1 – Methodology of ELISA Result Interpretation.

Sample	ELISA A_{405} Result [a]
Positive Control Mean	1.845
Negative Control Mean	0.163
Negative Control + 2 * Standard Deviations	0.188
Negative Control + 50%	0.245
Negative Result	0.162
Marginal Result	0.208
Positive Result	1.272

[a]ELISA data from Yorkshire site 1 (See Table 3.1).

2.4.2 Visual Assessments of Plant Symptoms

Visual assessments of the plants were performed 1, 2 and 3 weeks post-transfer of viruliferous aphids which had spent 4 weeks feeding on TuYV infected plants. Plant symptoms and the severity were noted each week. Symptoms recorded were: yellowing or purpling of the leaves, stunted growth, curling of the leaves, or any other unusual symptoms relative to the healthy unchallenged control plant phenotypes.

2.5 Nucleic Acid Techniques

2.5.1 Nucleic Acid Extraction

Genomic DNA Extraction

Leaf samples were collected and stored in 2ml Eppendorf tubes (Eppendorf®) at -20°C prior to DNA extraction. Frozen leaves were crushed to a fine powder using a pre-cooled modified drill-piece, that fits flush with the Eppendorf tubes. The Qiagen DNasey Kit (Qiagen Cat No.74904) was used to extract DNA

using silica-membrane purification. The kit was used according to the manufacturer's guidelines. The concentration and contamination of the DNA was assessed using a NanoDrop® ND-100 spectrophotometer (Thermo Scientific). DNA was stored at -20°C.

Total RNA Extraction

Leaf samples were collected in 2ml Eppendorf tubes (Eppendorf®), flash frozen at -170°C in liquid nitrogen and stored at -70°C prior to RNA extraction. Frozen leaves were crushed into a fine powder using a pre-cooled modified drill-piece, that fitted flush with the Eppendorf tubes. The Qiagen Plant RNeasy kit was used to extract total RNA. The kit was used according to the manufacturer's guidelines. Total RNA was also extracted by TRIzol extraction (Gehrig *et al.*, 2000). The concentration and contamination of the RNA was assessed using a NanoDrop® ND-100 spectrophotometer (Thermo Scientific). RNA was stored at -70°C.

Plasmid DNA Extraction

Qiagen Plasmid Mini Kit (Cat No.12125). The kit was used according to the manufacturer's guidelines, on both *Escherichia coli* and *Agrobacterium* cells.

DNA Gel Extraction

For PCRs containing multiple products when visualised using Agarose Gel Electrophoresis (Section 2.5.8) a clean scalpel was used to cut out bands to be extracted while visualising under UV light. Bands were purified using the QIAquick Gel Extraction Kit Protocol (Qiagen) and a microcentrifuge. 30μlof EB elution buffer (10 mM Tris Cl, pH 8.5) was used to elute the DNA, which was then stored at -20°C.

2.5.2 DNase Treatment

DNA was removed from purified RNA samples using the DNase I, RNase-free protocol (0.002 U/μl)(Fermentas cat No.EN0525) for the treatment of RNA following the manufacturer's protocol. The RNA was then used as a template for reverse transcription.

2.5.3 RNase Treatment

RNA was removed from DNA samples with RNase A, following the manufacturer's protocol (DNase and protease-free 10 mg/ml) (Thermo Scientific cat No.EN0531). RNase treated samples were then used in PCR reactions.

2.5.4 Digestion Enzyme

SalI (New England BioLabs cat No.R0138S) was used to cut specific locations in TuYV PCR products, using the manufactures protocol.

2.5.5 Ligation of Poly A Tails

The enzyme *E. coli* Poly (A) Polymerase (M0276S New England Biolabs (UK) LTD) was used to ligate addition of AMP from ATP to the $3'$ end of 4 μg RNA. The protocol was followed using the manufactures protocol, then the prepared RNA was used as a template for the reverse transcription reaction using the $3'$ RACE primers as described in subsection 2.5.7.

2.5.6 Reverse Transcriptase Reaction (RT-)

For RT- the standard protocol was a two step protocol, which was carried out on total RNA to synthesise complementary DNA (cDNA). The first step consisted of 1μg RNA, 2 μl reverse primer (5 μM, see tables 2.2-2.3), made up to 20 μl with double distilled water. This was then subjected to denaturing conditions (70°C for 10 minutes, 25°C for 10 minutes), then placed on ice. After this step, the enzymes and other reagents were added (8 μl 5x first strand buffer, 4 μl DTT, 1 μl dNTPs (Invitrogen), 0.5 μl Superscript^{TM} II (Invitrogen), 1 μl RNaseOUT^{TM} (Invitrogen), 5.5 μl double distilled water). The following conditions: 20°C for 10 minutes, 37°C for 45 minutes, 42°C for 45 minutes, 70°C for 10 minutes, 12°C ∞, reverse transcribing viral RNA. The final volume of 40 μl was stored at -20°C after completion.

2.5.7 Polymerase Chain Reaction (PCR)

PCR was performed on cDNA and gDNA. 4 μl of cDNA from the RT-reaction was added to a 46 μl PCR reaction and gDNA (5 μg) was used at various

volumes depending on concentration (total reaction volume of 50μl used). The PCR reaction mixture comprised of 5 μl 10x PCR Buffer (Invitrogen), 1.5 mM magnesium chloride (up to 3 mM was also used), 0.5 μl 0.25 mM dNTPs (Invitrogen), 2 Units Taq-DNA Polymerase (Invitrogen) and 2 μl of 5 μM forward and reverse primers (see tables 2.2-2.3). The total volume was made up to 50 μl with double distilled water. The PCR reaction mixture was amplified under the following conditions: one cycle at 95°C for 5 mins, 35 cycles at 95°C for 30 secs, between 50° and 66°C for 30 secs depending on the specific primer pair and 72°C for 1 min per kb and one cycle at 72°C for 10 mins and 12°C ∞. Reactions were performing in a thermocycler (Bio-Rad, MyCycler^{TM}). PCR products were stored at -20°C.

PCR reaction used Phusion® High-Fidelity DNA Polymerase (M0530S, New England BioLabs). The PCR reaction mixture comprised of 10 μl 5x GC Buffer (M0530S, New Englands Biolabs), 1.5 mM magnesium chloride, 0.5 μl 0.25 mM dNTPs (Invitrogen), 1 Unit Phusion® and 2 μl of 5 μM forward and reverse primers (see tables 2.2-2.3). The total volume was made up to 50 μl with double distilled water. The PCR reaction mixture was amplified under the following conditions: one cycle at 98°C for 30 secs, 35 cycles at 98°C for 15 secs, between 54° C and 66°C for 30 secs depending on the specific primer pair and 72°C for 30 secs per kb and one cycle at 72°C for 10 mins and 12°C ∞.

Primers were designed in PimerSelect and SeqBuilder (DNASTAR, Lasergene v10.0; Misener and Krawetz, 1999). The ABI trace files of forward and reverse sequences were aligned and edited in SeqMan (DNASTAR Lasergene v10.0) and the consensus was exported in fasta format.

Table 2.2 – Details of Primers Designed for Whole TuYV Genome Amplificaton.

Primer Name	Sequence (5′-3′)	Orientation	Target Region
MN17[a]	CAGGYGCTGCCTGGGCTA	Forward	2937-2954nt
MN22[b]	AACRYTCGTTGCCTATCC	Reverse	5520-5537nt
MN25	CACCGAAGTGCCGTAGGGAT	Reverse	5621-5642nt
MN27[c]	ACACCGAAGTGCCGTGGGGATTTCTC	Reverse	56415666nt
MN30	CGGAKTCGTTCCAGTTTR	Reverse	5630-5648nt
MN45	ACAAAAGAAACCAGGAGGGAATC	Forward	1-23nt
MN46	ACAAAAGAAACCAGGWGGGAATC	Forward	1-23nt
MN37	GGACAACTGGAATTCTGCTCTC	Forward	3040-3062nt
MN42b	GRACCAGCTATCGATGAAGAACC	Reverse	4027-4049nt
MN48	GTTTAATGTCTCTGGCTTGACTTTAT	Reverse	5569-5595nt
MN49	GGGCACTCCATGGCAGTC	Forward	4815-4844nt
MN57	GACCACAACCACTGGCTGAG	Reverse	3690-3709nt
MN59	GGCAGTCTGATAGACTCGGC	Reverse	3726-3746nt
MN64	TGTAGCCATAGATCAGTTTGTAGAT	Reverse	3100-3124nt
MN66	ATCGTTTTATCGTCTATACTGGAGTC	Forward	4200-4225nt
MN67	CCATCTTGTTTGTCCTTATTAG	Reverse	4400-4421nt
MN68	CCACAACCACTGGCTGAGAG	Reverse	3500-3520nt
MN69	CGTCGTCGTCTWGGTCGGCGTT	Reverse	3600-3622nt
MN72	CACTCCATGGCAGTCTCRACC	Forward	4800-4821nt
MN73	GTCGATGGRAAAGAGATGATGG	Forward	1630-1652nt
MN74	CTGTTTCTTTGGGGCTCTTCTG	Reverse	1745-1767nt
MN75	GCTGTACACTGTTACAAGATTTATGG	Forward	640-665nt
MN77	TTCCGCTTGCGTGGGTGAT	Reverse	800-819nt
MN78	ACACCGAAGTGCCGTAGG	Reverse	5624-5642nt
MN90	CCACGAGTAAAGAAGYTCAACGG	Reverse	3201-3223nt

[a]AB9 (Asare-Bediako, 2011)
[b]CAB5608R (Zhao *et al.*, 2003)
[c]TYR3 (Xiang *et al.*, 2011)

RACE PCR Reaction

Rapid amplification of cDNA ends (RACE PCR) was performed on cDNA. 4 μl (minimum concentration 1 μg) of cDNA from the RT-reaction was added to a 46 μl RACE PCR reaction used at various volumes depending on the concentration (total reaction volume of 50 μl used). The kit and protocol of the 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen) was used. Primers listed in Table 2.3 were used with previous conditions outlined

in sections 2.5.6 and 2.5.7.

For 3′ RACE was carried out after polyA tail addition to the total RNA (see section 2.5.5). Primers listed in Table 2.3 were used with previous conditions outlined in sections 2.5.6 and 2.5.7.

Table 2.3 – Details of Primers Used for RACE Amplificaton.

Primer Name	Sequence (5′-3′)	Orientation	Target Region
MN36[a]	CAGCAAGGCGAGACAGAAGAC	Reverse	620-641nt
MN32[a]	TCATACAAACATTTCGGTGTAGAC	Reverse	760-785nt
MN49[b]	GGGCACTCCATGGCAGTC	Forward	4815-4833nt
Vial 8[a]	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV	Forward	N/A
Vial 9[a]	GACCACGCGTATCGATGTCGAC	Forward	N/A
PolyT Adapter[b]	GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTTTVN	Reverse	N/A
TAG Primer[b]	GCGAGCACAGAATTAATACGACTCACTATAGG	Reverse	N/A

[a]5′ RACE primer, Vial 8 and 9 from Rapid Amplification of cDNA Ends, version 2.0 Invitrogen kit
[b]3′ RACE primer

Bacterial PCR Reaction

Individual bacterial colonies (*Escherichia coli*) were stabbed with a 10 μl pipette tip and the cells were transferred into a 0.2 ml PCR tube with 10 μl dH_2O. The pipette tip was agitated to dislodge the cells and the 10 μl of re-suspended cells was used in standard PCR.

2.5.8 Agarose Gel Electrophoresis of DNA

DNA PCR products and gDNA were visualised by separating and quantifying their size using electrophoresis and UV imaging. UltraPure^{TM} Agarose (Invitrogen) powder was dissolved by heating in 1x Tris-Borate-EDTA (TBE) buffer (9 parts dH_2O and 1 part 10x TBE; National Diagnostics). Gels for general use were 1%, for PCR products that were hard to separate 1.5% and 2% agarose gels were made. The UV sensitive nucleic acid stain GelRed (Biotium Inc.) was added at 2 μg/50 ml. Gels with a thickness of 8-10 mm were prepared in gel casts; gel tanks contained 1% TBE buffer.

Loading buffer was added to samples (1/5 volume of 5x DNA Loading Buffer; 0.25 % Bromophenol blue, 40 % (w/v) sucrose in dH_2O) for electrophoresis. The 1 Kb Plus DNA Ladder (Cat no. 10787, Invitrogen) was used at a concentration of 0.5 μg to estimate molecular mass of single and double-stranded DNA bands and give an indication of concentration (Figure 2.1). Gels were run at room temperature between 40-120 V for anything between 60-240 mins for diagnostic tests and 30-70 V for isolation of products of similar size. After electrophoresis, separated DNA fragments were viewed on a Syngene G Box transilluminator, using GeneSnap 7.07 software (Syngene, a division of Synoptics Ltd.).

2.5.9 Sequencing

Three Sanger methods were used for sequencing, the first was performed in-house using BigDye (Applied Biosystems) and the second and third by an external companies, GATC Biotech and BioScience Source.

For in-house sequencing PCR products had a final volume of 10 μl (2 μl BigDye, 2 μl sequencing buffer, 1 μl of 5 μM appropriate primer) (Tables 2.2

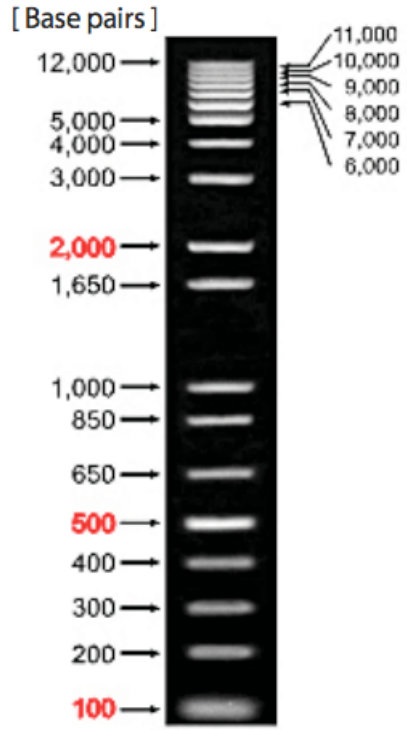

Figure 2.1 – 1 kb Plus DNA Ladder
1 % agarose gel. Size range 0.1 to 12 kb. Concentration 270 ng per lane (Invitrogen, 2008).

- 2.3), up to 5 μl of 10-50 ng template DNA and the remaining volume of dH_2O. Reactions were performed in a thermal-cycler (Bio-Rad MyCycler^{TM}), using the temperature profile published by Applied Biosystems (96°C for 1 min, 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min). The products were then sequenced using an Applied Biosystems 3130xl Genetic Analyser (School of Life Sciences Genomics Centre).

For GATC Biotech and BioScience Source sequencing, a total volume of 10 μl was prepared (5 μl template DNA (80-100 ng/μl purified plasmid DNA or 20-80 ng/μl purified PCR product) and 5 μl of 5 μM appropriate primer) (Table 2.2). BioScience Source samples DNA and Primers were sent in 5 μl volumes in 1.5ml micro-centrifuge tubes. External Sanger sequencing was carried out on Sanger ABI 3730xl machines with reads between 800-1100 bases.

2.6 Cloning and Transformation of Plasmids into *Escherichia coli*

2.6.1 Media

LB-medium (1 L) contained: 10 g tryptone, 5 g yeast extract and 10 g NaCl in 950 ml dH_2O. Adjusted pH to 7.0 using 1 M NaOH and volume made up up to 1 L with dH_2O, then autoclave. The solution was cooled to 55°C then antibiotics were added (100 μg/mL / 50 μg/ml of Ampicillin or Kanamycin). Media was then stored at room temperature.

For LB agar-plates, LB media was prepared as above, but 15 g/L agar was added before autoclaving (1.5%). After autoclaving, the media was cooled to approx. 55°C, appropriate antibiotics were added, and 20 ml poured into petri dishes, stored at room temperature.

Cloning

TA cloning was used to separate mixed PCR products readying them for sequencing. TA cloning relies on the ability of thymine (T) overhangs left after polymerase extension to ligate to open vectors with and adenine (A) overhangs. This was performed following the manufacturer's instructions (TOPO TA Cloning® Kit for Sequencing; Invitrogen) for transforming chemically competent One Shot® (Invitrogen) *E. coli*. For blunt end products PJET1.2 vector from CloneJET PCR Cloning Kit (Fermentas, K1231) was used following the manufacturer's instructions.

Chapter 3

Genetic Diversity of TuYV

3.1 Introduction

Across Europe OSR production is increasing, with Europe being the world's number one producer (Ufop and Unilever, 2007; Fediol and EUROSTAT, 2014; United States Department of Agriculture, 2014). OSR is valued for its oil, as it is used in food production and the protein rich meal for animal feeds. OSR also has the ability to increase farming intensification as a rotation crop as cultivation methods enrich soil with nitrogen, as low uptake of by OSR reduces nitrogen depletion in the soil (Angus *et al.*, 1991; Rathke *et al.*, 2006) adding value and making it essential in modern agriculture. However, OSR does not reach it potential yields of 6.5 t/ha (Berry and Spink, 2006) in field conditions in the UK, instead OSR yield are around 3.5 t/ha (Department for Enviroment and Affairs, 2010), which is due to external factors causing losses (Alford *et al.*, 2003). One of these potential sources of yield loss is TuYV, as it is known to reduce yields by up to 26% in Europe (Jay *et al.*, 1999) and up to 46% in Australia (Australian Government, 2008). The potential to mitigate these losses would be highly desirable to the industry. With aphid vectors of TuYV becoming more and more difficult to control due to EU insecticide regulations (The European Commission, 2013) and pesticide resistance (IRAG-UK, 2011; IRAC-IRM, 2014), natural plant resistance to TuYV is a more desirable, sustainable and effective approach. For plant resistance to be an effective method of controlling disease and thereby reducing yield loss, knowledge of the genetic diversity of pathogens is a necessity in order to produce resistant plant lines that are not overcome by resistance breaking isolates (García-Arenal and McDonald, 2003).

TuYV is a member of the genus *Polerovirus* of the family *Luteoviridae* (D'Arcy and Domier, 2005), with little knowledge of its genetic diversity compared to other members of the *Luteoviridae*. TuYV has a single-stranded plus sense RNA genome of approximately 5.6-5.7 kb, which consists of seven ORFs numbered from 0 to 5 (Miller *et al.*, 1995; Smirnova *et al.*, 2015). The 5′-proximal half of the genome (ORF0, ORF1, ORF2) is expressed from the genomic RNA and encodes viral proteins (P0, P1, P2, respectively) necessary for infection (Reutenauer *et al.*, 1993). The 3′-terminal ORFs (ORF3a, ORF3, ORF4, ORF5) are translated from sub-genomic RNA and encode polypeptides responsible for the formation of viral particles (P3, major coat protein), transmission by aphids (P5, RTD minor coat protein) and cell-to-cell movement (P3a, aids plant long distance movement and P4, transport protein) (Reinbold *et al.*, 2001; Brault *et al.*, 2005; Smirnova *et al.*, 2015).

The 5′-proximal half of the genome ORFs start with ORF 0 beginning at 32nt (AUG start) and ending at 779nt (UGA stop); previous to this ORF is the 5′ UTR (Veidt *et al.*, 1988). The largest single gene ORF 1 begins at 174nt (AUG start) and ends at 1995nt (UGA stop). ORF 2 begins at 1520nt with the start codon at 2168nt (AUG start) up to 3281nt (UGA stop). It was thought that there was a non-coding region of 202nt between ORF2-ORF3 separating the 5′ and 3′ genes clusters, however ORF3a is now known to be located within a portion of this central "non-coding region". ORF3a is a small ORF starting at 3365nt with an ACG start codon (alternative start codons are AUU AGA CUG) and the stop codon at 3502nt (UAG) (Smirnova *et al.*, 2015). ORF 3 begins at 3483nt (AUG start) continuing until 4089nt (UAG stop). ORF4 begins at 3514 (AUG start) and continues until 4041 (UGA stop) (Veidt *et al.*, 1988). ORF 5 RTD begins directly after P3 stop codon, but also contains a start codon at 4200nt (AUG start) both the RTD and ORF ending at 5493nt (UGA stop). After ORF5 up to the end of the TuYV-FL genome (5643nt) there is a 150nt 3′ UTR (Figure 1.2).

So far TuYV genetic diversity is poorly characterised, only P0 and P3 genes have substantial sequence information (Hauser *et al.*, 2000; Asare-Bediako, 2011). There was only one full genome sequence published at the start of this project, TuYV-FL, which is the original sequence the TuYV species is based on (Mayo, 1999), it was isolated from a lettuce host in France (Veidt *et al.*, 1988). Over the course of this project more information has been published, including an Australian TuYV isolate with 90% whole genome nucleotide identity to TuYV-FL (Wylie *et al.*, 2012). Also several closely related virus species

(BrYV) full genome sequences have been released with <90% amino acid identity to TuYV-FL except in P3 which has above 90% identity (Xiang *et al.*, 2011). Due to low homology to the European isolate of TuYV-FL use of the BrYV sequences for the design of primers for brassica isolates of European TuYV could be questionable.

3.1.1 Viral RNA Recombination and Variation

Recombination in plant viruses was first observed in 1986 (Bujarski and Kaesberg, 1986) in Brome mosaic virus repairing a 3′ deletion in its genome; previous to this it was thought that plants did not support viral recombination. There are two kinds of recombination, self recombination and recombination with host or other organisms. This has been put forward as one of the strongest forces shaping plant RNA viruses. There are three forms of self recombination, firstly it can occur when two viral genomes recombine by homologous crossing over (Sztuba-Solinska *et al.*, 2011), secondly self recombination can occur in non-homologous regions of the viral genome (Lai, 1992). Both of these occur commonly within the family *Luteoviridae* even between poleroviruses and luteoviruses (Moonan *et al.*, 2000; Lim *et al.*, 2014). The third type of self recombination occurs if the virus contains a segmented genome, this does not require proximity during replication, only during packaging within the coat protein (reassortment) (Pérez-Losada *et al.*, 2015). Recently a very closely related virus BrYV has been shown to have two areas where self-homologous recombination occurred, in the 3′ proximal half of the genome located at positions 3531nt and 4819nt in P3 and P5 respectively (Lim *et al.*, 2014).

Recombination can also occur with the host or other organisms' genetic material. This has been shown to have occurred with a virus closely related to TuYV, PLRV (Mayo and Jolly, 1991), where an isolate was found to contain sequences homologous to an exon of tobacco chloroplast RNA. Natural selection can result in retention of the acquired sequence if it gives an evolutionary advantage to the virus and mutations can modify its original functions. These recombination events can be of evolutionary advantage for the virus if they help to evade host immune defences, for example by changing surface protein antigenicity (mammalian infecting viruses) or repairing deleterious deletions and mutations (Worobey and Holmes, 1999).

Recombination is one of the major forces driving virus variation and evolution, the other source is via mutations either by introduction of SNPs or additions/deletions by slippage (Roossinck, 1997). These are essentially the same processes that are driving all evolution, virus forces are analogous to; recombination in meiosis, error prone replication. Mutation rates of ssRNA viruses utilising RdRp have error rates ranging between 10^{-3} - 10^{-5} (Andino and Domingo, 2015) per nucleotide copied, almost a million-fold higher than the mutation rates of cellular DNA. Variation can also be introduced by RdRp slippage introducing deletions or additions of nucleotides into the sequence of certain repetitive motifs, up to ∼2% of transcripts (Olspert *et al.*, 2015). These processes can overwrite ORFs and due to selection and genetic drift can fix new genes which are unrecognisable to their original sequences.

3.1.2 Taxonomy

Understanding the genetic diversity of a virus is only one form of information used for the classification of viruses. Viruses are classified firstly by genetic material be it single or double stranded RNA or DNA. Then virus particle size and morphology, all the way down to chemical stability (Van Emden and Harrington, 2007). Classification at a species level is done on host range, sequence similarity plus genome organisation (King *et al.*, 2012). The aim of taxonomy is the dissemination of a group of organisms that share common characteristics to allow, in the case of viruses, better understanding of a pathogen and its effects. Virus species to become accepted by the virologic community, in 1991, the ICTV endorsed the following definition: A virus species is a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche (Mayo and Jolly, 1991). In the context of human development and the understanding of yield reducing pathogens the ecological niches that are most important are crops. BWYV was split into Beet chlorosis virus (BChV), BMYV and TuYV in Europe due to host range differences as well as low genetic similarity between the sequences (Hauser *et al.*, 2002; Mayo, 2002).

This chapter discusses whole genome phylogenetic analysis of TuYV isolates sequenced during this study along with sequences available in the databases. In this work *B. napus* and multiple weed species from around the UK and Europe were sampled and tested for TuYV. Field sampling across the UK was planned for sites that cover the breath of mainland UK with even coverage. Collaborations

were established across mainland Europe to source samples from the major OSR growing countries. Full genome phylogenetic analysis to ascertain the genetic diversity of TuYV will be important for research aimed at identifying and maintaining plant resistance to TuYV. Potential hot spots of recombination within the genome of TuYV were also investigated to better understand the relationship of any genetic groups found. Identifying distinct genetic groups is a first step towards identifying pathogenic determinates.

3.2 Materials and Methods

3.2.1 Sampling

The highlighted areas in Figure 3.1 are the counties within the quadrats that were selected for OSR and weeds sampling. This selection allowed an even spread of samples across the UK to represent TuYV UK genetic diversity. Northern regions had less sampling locations due to lack of OSR agriculture available for sampling.

In each location (county) three fields were sampled with 50 evenly distributed samples taken per field, this was done by splitting fields into seven transects then using the five internal transects for sampling and ignoring the edges to try and avoid differences in the environment surrounding each sample (Figure 3.2). Samples from each of the five transects sampled were taken with even spacing. To avoid damage to the OSR crop, each transect run along the tramlines of the field. To aid the investigation into the TuYV host range (See Chapter 4) within and around each field 20 weed plants were collected, both from species that had previously been reported as hosts and those that had not. Weeds sampled were any dicots that had enough foliage for ELISA, aphid transmission tests and RNA exactions. A GPS location was taken as a reference point at the centre of each field.

Once the samples had been tested for TuYV by ELISA (Section 2.4) three infected samples were selected, these were located within the field to form a "V" (Figure 3.2), and were used for RNA extractions, RT-PCR and sequencing. If this could not be achieved due to non-uniform field incidence, samples were taken as close as possible to these areas (Figure 3.2). Samples received from overseas, were sampled in a single transect across fields, each collaborator was sent a standard operating procedure for sample collection to maintain a standard protocol were possible. Samples from overseas varied from 30-90 plant samples per site.

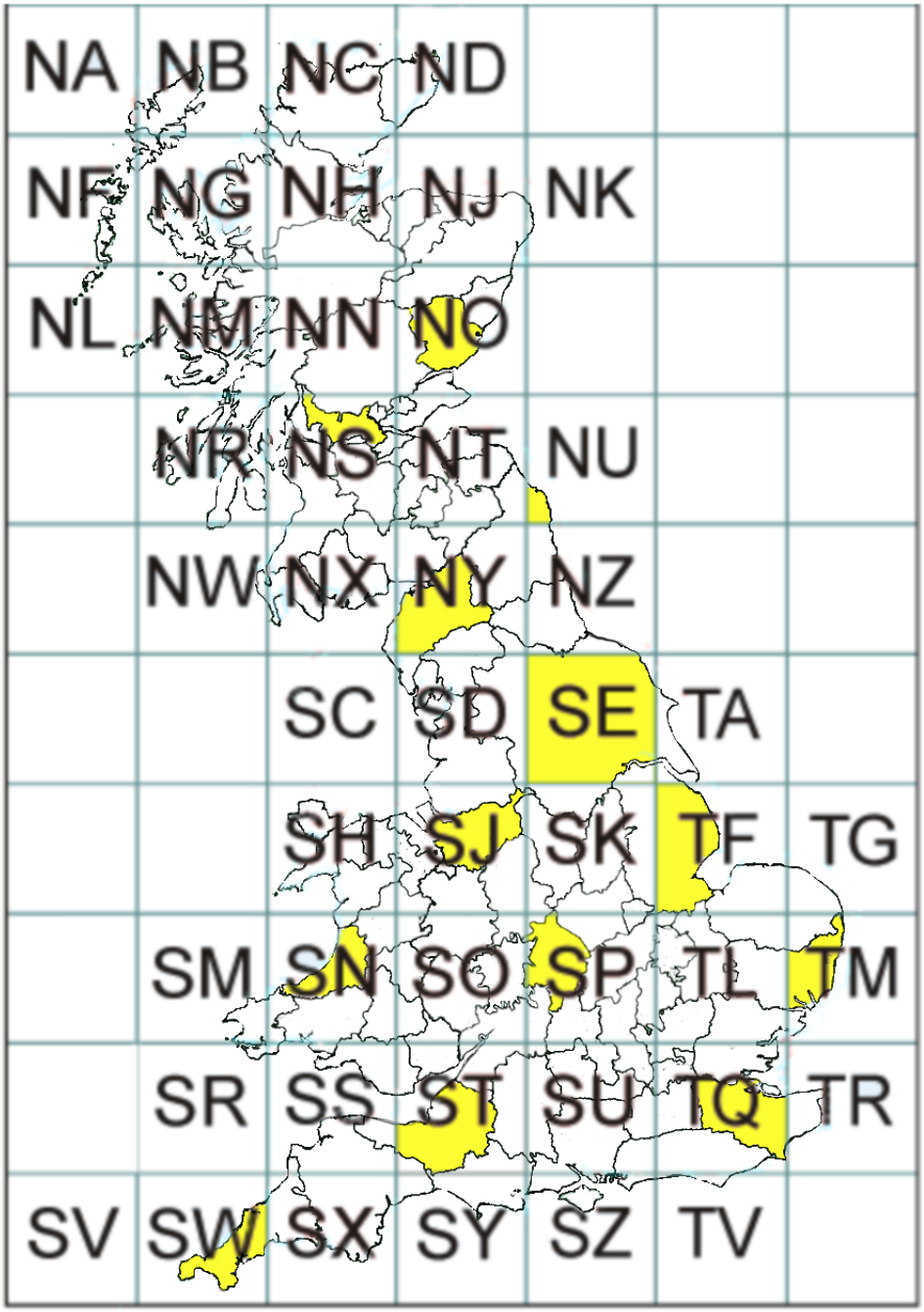

Figure 3.1 – Sampling Grid for the UK
Sampling sites were spread out evenly across the UK in an attempt to obtain a representative sample to study the genetic diversity of TuYV, SW-Cornwall ST-Somerset TQ-Kent SN-Cardiganshire SP-Warwickshire TM-Suffolk SJ-Cheshire TF-Lincolnshire SE-Yorkshire NY-Westmorland NU-Northumbria NS-Stirlingshire NO-Angus

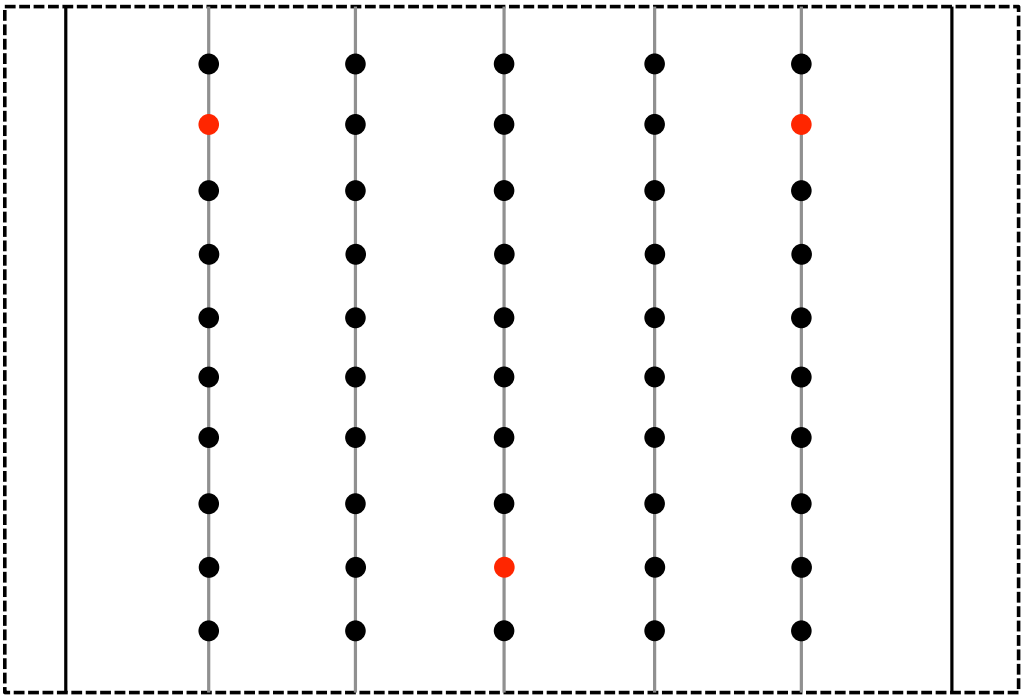

Figure 3.2 – Field Sampling Plan
Field outline indicated by the dotted lines. Transects indicated by solid lines, black lines are transects not used, grey are the used transects. Dots are sampling points, red dots are samples taken forward for sequencing if infection was uniform across the field.

3.2.2 TuYV Isolate Amplification and Sequencing

TuYV isolates were reverse transcribed with primer MN48; this provided the cDNA template for the subsequent 5′ and 3′ PCRs, which amplified the genome in two halves using the primer pairs MN46-MN59 and MN37-MN48, respectively (See Table 2.2). This allowed efficient amplification of the TuYV genome as a single PCR targeting the whole genome was not repeatable and reliable. Primers that had full sequencing coverage across the genome were designed (Figure 3.3), primers used were, MN77, MN75, MN74, MN73, MN90 and MN57 for the 5′ TuYV PCR amplicon (See Table 2.2). For 3′ TuYV PCR amplicon sequencing primers MN42b, MN66, MN67 and MN72 were used (See Table 2.2). These primers allowed full coverage and overlapping sequences (800-1100 bp potential amplification per primer) for alignment and confirmation of single isolates with no mixed genotypes detected.

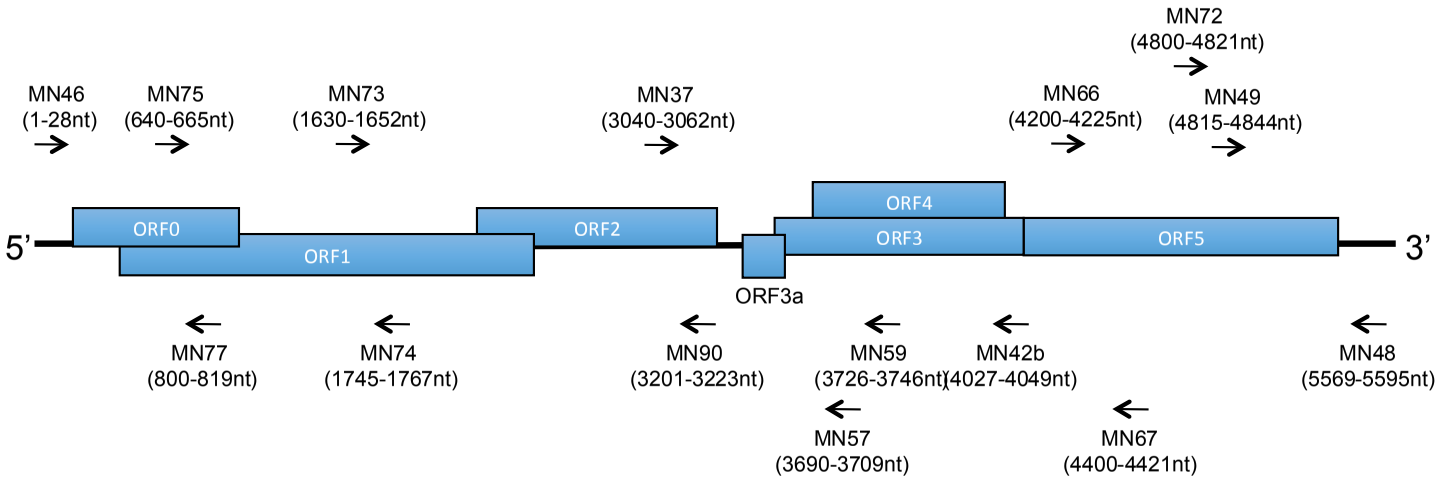

Figure 3.3 – Diagram Showing Designed Primers and their Positions within the TuYV Genome (not to scale).
The arrows show the orientation of the primers: ⇀ = forward primers, ↼ = reverse primers.

3.2.3 Phylogenetic Analysis

Sequences were compared to previously published results using the Basic Local Alignment Search Tool (BLAST; Johnson *et al.* (2008)) on the National Centre for Biotechnology Information (NCBI, GenBank, http://www.ncbi.nlm.nih.gov/;Benson *et al.* (2012)). MegAlign (DNASTAR Lasergene v10.0) was used to infer amino acid sequences from DNA sequences and to compare amino acid sequences using the ClustalW algorithm (Chenna *et al.*, 2003; Li, 2003). MEGA 6.0 (Tamura *et al.*, 2011) was also used to compare amino acid sequences using the MUSCLE algorithm (Edgar, 2004). Alignments were also performed with T-Coffee (Notredame *et al.*, 2000).

Both nucleotide and amino acid substitution model testing for maximium likelihood and Bayesian phylogenetic analysis were conducted with JModeltest2 (Darriba *et al.*, 2012) to find the optimum model based on the Akaike information criterion (AIC) score (Akaike, 1987).

The program Structure (Pritchard *et al.*, 2000; Hubisz *et al.*, 2009) and Structure Harvester (Earl *et al.*, 2012) were utilised to help understand distinct populations, hybrid zones and understand admixture within the population. Sequencing data was transformed in Microsoft Excel to be used in Structure. Batch runs estimating K between 1 and 10 were used to detect which value of K represented the data best, where each K is an individual populations within the data.

Bayesian approaches were undertaken as well as maximum likelihood, with the program Beast (Drummond *et al.*, 2012). Constant size population and relaxed lognormal clock were implemented. Gene concatination was carried out with 30 million MCMC steps sampling every 30,000 steps to produce 1,000 logs. Two runs were carried out and TreeAnnotator was used to compile the tree output file. LogCombiner was used to combine multiple runs of BEAST analysis, then the data was assessed in Tracer to ensure good effective sample size (ESS) coverage. FigTree 1.4.2 (Rambaut, 2009) was used to visualise the resultant trees from the phylogenetic analyses.

3.2.4 Recombination Analysis

For analysis of recombinant areas, the Recombination Detection Programme v.4.46 was used utilising: RDP, Chimaera, SiScan, GENECONV (Sawyer, 1989),

Maxchi, BOOTSCAN and 3SEQ (Boni *et al.*, 2007). Employing the default parameters (Martin *et al.*, 2015), except disentangle overlapping signals was not used as this resulted in an infinite loop in the analysis. For more reliable results, only recombination signals detected by five or more of the methods used, with significant support to known sequence (unknown homology was excluded as unique sequences rather than recombination), with Bonferroni-corrected P-value cut off at 0.05 were considered as recombinant (Pagán and Holmes, 2010).

3.2.5 Genetic Diversity

The programme DnaSP V.5.10.1 (Librado and Rozas, 2009) was used to investigate the genetic diversity of each TuYV gene: nucleotide diversity (π), number of segregating sites (S), haplotype (h) and total number of mutations (Eta) for all European isolates. Haplotype in the context of viruses is the number of unique sequences indicating how many genetically different viruses are within the sequenced population.

3.2.6 Determination of Genetic Distance and Selection Pressure

The overall genetic distance (the number of base substitutions per site from averaging over all sequence pairs in a population) within genes' nucleotide and amino acid sequence datasets were estimated using maximum likelihood model (Tamura *et al.*, 2011) with gamma rate of variation among sites (shape parameter r = 4.0). Standard error estimates were obtained by bootstrap procedure (1000 replicates). The pairwise deletion method was used to account for gaps. The analyses were conducted in MEGA 6.

The Maximum Likelihood analysis of natural selection codon-by-codon method via the programme HyPhy (Pond and Muse, 2005) was used to estimate the numbers of inferred synonymous substitutions per synonymous site (d_S) and the numbers of non-synonymous substitutions per nonsynonymous site (d_N). These estimates were produced using the joint Maximum Likelihood reconstructions of ancestral states under the default of FEL (Pond and Frost, 2005) utilising the General Time Reversible model (REV) (Nei and Kumar, 2000) with MG94 (Muse and Gaut, 1994). The test statistic d_N-d_S was used for detecting codons that had undergone positive selection and negative selection. A positive value for the

test statistic indicates an over-abundance of nonsynonymous substitutions. If P values were <0.05 they were considered significant at the 5% level. The overall ratio dN/dS was also calculated from the mean values of dN and dS to compare the selection pressures acting on the genes of TuYV. Genes under positive (diversifying) selection had a d_N/d_S ratio >1, genes under negative (purifying) selection d_N/d_S ratio <1, and neutral selection when d_N/d_S ratio = 1.

3.2.7 Neutrality Test

Tajima's *D* (Tajima, 1989) and Fu and Li's *D* and *F* statistics (Fu and Li, 1993) were used to test the hypothesis that patterns of diversity in TuYV are consistent with the neutral theory of molecular evolution (Kimura, 1984). This was conducted within DnaSP V.5.10.1. The neutral theory of molecular evolution states that the vast majority of evolutionary changes at the molecular level are caused by random shift of selectively neutral mutants. The significance of each test statistic was estimated by 10,000 permutations; statistical support indicates that the gene is neutrally evolving and not under functional constants.

3.3 Results

3.3.1 TuYV Incidence UK

OSR can be as profitable as other arable crops but is favoured as a break crop and is used in modern day crop rotation in-between wheat, barley, potato or sugar beet to enrich the soil with nitrogen and reduce disease build up. Winter OSR is normally sown in England between late August and early September, germinating late September to early October, overwintering, flowering in the spring (April-May) and is harvested in July/August. Sampling for this study was carried out mostly in the spring following the autumn planting, late January - May. Where available, information on OSR cultivars, location and incidence for the UK are listed in Table 3.1.

Incidence of TuYV in UK weeds (0-45%) was always lower than that of OSR in the proximal fields, between two to six fold; for more information on weed hosts see Chapter 4. TuYV was found in every county sampled during the course of this study. There were only two fields with 0% incidence, one in Cornwall and one site in Stirlingshire. However, this was not due to general cultivar resistance to TuYV as the variety in which no TuYV was detected (Compass) was susceptible, as other fields planted with Compass were found to have TuYV incidence of up to 20%. The only cultivar sampled that has a reported quantitative resistance (Limagrain, 2016) to TuYV was Amalie (samples ALi1-30 Table 3.1.4), which had a low incidence of 6.6% compared to the adjacent field of Incentive (62%), however TuYV was still detected in some of the Amalie plants.

The mean incidence in OSR for each year in this study was, 59.3% (2012), 34.8% (2013), 18.3% (2014), 40.3% (2015) with the years with higher average incidence also having higher numbers of aphids flying during the OSR germination period (Figure 1.6) as warmer temperatures (Table 1.2) allowed aphid numbers to increase during the later autumn months (Asare-Bediako, 2011). TuYV was present across the UK in both OSR and weed species, at high enough incidences (2012-2015) to cause economically important losses in yields of affected crops. However due to this study being a spacial study to accumulate varied samples of TuYV, further statistical analysis of the incidence data would be confounded by lack of repeated sample points.

Table 3.1 – The Incidence of TuYV in Oilseed Rape (*B. napus*) and Weed Species within the United Kingdom.

Location	GPS co-ordinates	Sample type	OSR Variety	Sample ID	Date of Sampling	Incidence
Suffolk	52°07'21.57" N 000°48'08.23" E	OSR	Camelot	S1-50	04/05/12	68%
Suffolk	52°07'21.57" N 000°48'08.23" E	Weeds	N/A	W-S51-70	04/05/12	20%
Suffolk	52°07'33.55" N 000°47'02.76" E	OSR	Cabernet	S71-120	04/05/12	66%
Suffolk	52°07'33.55" N 000°47'02.76" E	Weeds	N/A	W-S121-140	04/05/12	10%
Suffolk	52°03'46.50" N 000°45'30.57" E	OSR	Excalibur	S141-190	04/05/12	70%
Suffolk	52°03'46.50" N 000°45'30.57" E	Weeds	N/A	W-S191-210	04/05/12	0%
Cheshire	53°13'08.97" N 002°12'12.07" W	OSR	Osprey	C211-260	09/05/12	76%
Cheshire	53°13'08.97" N 002°12'12.07" W	Weeds	N/A	W-C261-280	09/05/12	5%
Cheshire	53°18'42.96" N 002°21'41.97" W	OSR	W21	C281-330	09/05/12	30%
Cheshire	53°18'42.96" N 002°21'41.97" W	Weeds	N/A	W-C331-380	09/05/12	0%
Cheshire	53°14'54.23" N 002°25'57.23" W	OSR	DK Cabernet	C381-400	09/05/12	46%
Cheshire	53°14'54.23" N 002°25'57.23" W	Weeds	N/A	W-C401-420	09/05/12	10%
Kent	51°15'22.56" N 001°02'03.69" E	OSR	W21	K421-470	11/05/13	70%
Kent	51°15'22.56" N 001°02'03.69" E	Weeds	N/A	W-K471-490	11/05/13	0%
Kent	51°10'13.99" N 001°15'52.11" E	OSR	W21	K491-540	11/05/13	22%
Kent	51°10'13.99" N 001°15'52.11" E	Weeds	N/A	W-K541-560	11/05/13	25%
Kent	51°06'03.50" N 001°11'22.94" E	OSR	DK Excellium	K561-610	11/05/13	92%
Kent	51°06'03.50" N 001°11'22.94" E	Weeds	N/A	W-K611-630	11/05/13	10%

Location	GPS co-ordinates	Sample type	OSR Variety	Sample ID	Date of Sampling	Incidence
Cornwall	50°30'54.32" N 004°44'37.40" W	OSR	Compass	Cw631-680	20/05/13	0%
Cornwall	50°30'54.32" N 004°44'37.40" W	Weeds	N/A	W-Cw681-700	20/05/13	20%
Cornwall	50°20'19.50" N 004°33'06.69" W	OSR	W21	Cw701-750	20/05/13	12%
Cornwall	50°20'19.50" N 004°33'06.69" W	Weeds	N/A	W-Cw751-770	20/05/13	0%
Cornwall	50°16'45.31" N 003°35'53.82" W	OSR	Cabernet	Cw771-820	20/05/13	14%
Cornwall	50°16'45.31" N 003°35'53.82" W	Weeds	N/A	W-Cw821-840	20/05/13	0%
Warwickshire	52°16'02.62" N 001°36'45.31" W	OSR	W21	Wa841-890	16/07/13	38%
Warwickshire	52°16'02.62" N 001°36'45.31" W	Weeds	N/A	W-Wa891-910	16/07/13	0%
Warwickshire	52°10'54.09" N 001°26'54.35" W	OSR	W21	Wa911-960	16/07/13	20%
Warwickshire	52°10'54.09" N 001°26'54.35" W	Weeds	N/A	W-Wa961-980	16/07/13	0%
Warwickshire	52°10'33.16" N 001°27'16.98" W	OSR	W21	Wa981-1030	16/07/13	46%
Warwickshire	52°10'33.16" N 001°27'16.98" W	Weeds	N/A	W-Wa1031-1050	16/07/13	10%
Angus	56°30'57.18" N 002°59'38.28" W	OSR	Artoga	A1051-1100	18/03/14	32%
Angus	56°30'57.18" N 002°59'38.28" W	Weeds	N/A	W-A1101-1120	18/03/14	25%
Angus	56°30'46.23" N 002°59'29.48" W	OSR	Artoga	A1121-1170	18/03/14	26%
Angus	56°30'46.23" N 002°59'29.48" W	Weeds	N/A	W-A1171-1190	18/03/14	35%
Angus	56°30'58.97" N 002°59'20.13" W	OSR	Artoga	A1191-1240	18/03/14	24%
Angus	56°30'58.97" N 002°59'20.13" W	Weeds	N/A	W-A1241-1260	18/03/14	45%

Location	GPS co-ordinates	Sample type	OSR Variety	Sample ID	Date of Sampling	Incidence
Stirlingshire	56°08'31.96" N 004°02'35.31" W	OSR	Compass	St1261-1310	19/03/14	0%
Stirlingshire	56°08'31.96" N 004°02'35.31" W	Weeds	N/A	W-St1311-1330	19/03/14	25%
Stirlingshire	56°08'31.96" N 004°03'05.15" W	OSR	Compass	St1331-1380	19/03/14	6%
Stirlingshire	56°08'31.96" N 004°03'05.15" W	Weeds	N/A	W-St1381-1400	19/03/14	20%
Stirlingshire	56°08'31.93" N 004°03'33.70" W	OSR	Compass	St1401-1450	19/03/14	20%
Stirlingshire	56°08'31.93" N 004°03'33.70" W	Weeds	N/A	W-St1451-1470	19/03/14	30%
Warwickshire	52°11'14.12" N 001°27'41.33" W	OSR	Expower	Wa1471-1520	05/04/14	10%
Warwickshire	52°11'14.12" N 001°27'41.33" W	Weeds	N/A	W-Wa1521-1540	05/04/14	35%
Warwickshire	52°11'23.59" N 001°27'24.16" W	OSR	Expower	Wa1541-1590	05/04/14	22%
Warwickshire	52°11'23.59" N 001°27'24.16" W	Weeds	N/A	W-Wa1591-1610	05/04/14	15%
Warwickshire	52°11'32.42" N 001°27'09.51" W	OSR	Expower	Wa1611-1660	05/04/14	10%
Warwickshire	52°11'32.42" N 001°27'09.51" W	Weeds	N/A	W-Wa1661-1680	05/04/14	15%
Wigtownshire	55°29'36.02" N 001°54'11.54" W	OSR	Crackers	Wi1681-1730	18/04/14	16%
Wigtownshire	55°29'36.02" N 001°54'11.54" W	Weeds	N/A	W-Wi1731-1750	18/04/14	25%
Wigtownshire	55°29'22.17" N 001°54'02.97" W	OSR	Crackers	Wi1751-1800	18/04/14	30%
Wigtownshire	55°29'22.17" N 001°54'02.97" W	Weeds	N/A	W-Wi1801-1820	18/04/14	15%
Wigtownshire	55°29'26.68" N 001°53'42.85" W	OSR	Crackers	Wi1821-1870	18/04/14	18%
Wigtownshire	55°29'26.68" N 001°53'42.85" W	Weeds	N/A	W-Wi1871-1890	18/04/14	15%

Location	GPS co-ordinates	Sample type	OSR Variety	Sample ID	Date of Sampling	Incidence
Northumberland	55°29'36.02" N 001°54'11.54" W	OSR	Expower	N1891-1940	19/04/14	18%
Northumberland	55°29'36.02" N 001°54'11.54" W	Weeds	N/A	W-N1941-1960	19/04/14	20%
Northumberland	55°29'22.17" N 001°54'02.97" W	OSR	Exhilarate	N1961-2010	19/04/14	16%
Northumberland	55°29'22.17" N 001°54'02.97" W	Weeds	N/A	W-N2011-2030	19/04/14	25%
Northumberland	55°29'26.68" N 001°53'42.85" W	OSR	Explicit	N2031-2080	19/04/14	26%
Northumberland	55°29'26.68" N 001°53'42.85" W	Weeds	N/A	W-N2081-2100	19/04/14	20%
Lincolnshire	52°47'53.92" N 000°13'44.63" E	OSR	W21	Li2101-2150	22/01/15	46%
Lincolnshire	52°47'53.92" N 000°13'44.63" E	Weeds	N/A	W-Li2151-2170	22/01/15	15%
Lincolnshire	52°48'29.08" N 000°13'44.35" E	OSR	W21	Li2171-2220	22/01/15	36%
Lincolnshire	52°48'29.08" N 000°13'44.35" E	Weeds	N/A	W-Li2221-2240	22/01/15	5%
Lincolnshire	52°46'17.65" N 000°06'01.96" E	OSR	Incentive	Li2241-2290	22/01/15	62%
Lincolnshire	52°46'17.65" N 000°06'01.96" E	Weeds	N/A	W-Li2291-2310	22/01/15	20%
Lincolnshire	52°46'17.65" N 000°06'01.96" E	OSR	Amalie	A1-30	22/01/15	6.6%
Cardiganshire	52°05'21.42" N 004°35'55.03" W	OSR	PT229CL	Ca2311-2360	28/01/15	46%
Cardiganshire	52°05'21.42" N 004°35'55.03" W	Weeds	N/A	W-Ca2361-2380	28/01/15	10%
Cardiganshire	52°05'26.34" N 004°36'09.51" W	OSR	PT229CL	Ca2381-2430	28/01/15	44%
Cardiganshire	52°05'26.34" N 004°36'09.51" W	Weeds	N/A	W-Ca2431-2450	28/01/15	20%
Cardiganshire	52°05'20.62" N 004°36'13.69" W	OSR	PT229CL	Ca2451-2500	28/01/15	38%
Cardiganshire	52°05'20.62" N 004°36'13.69" W	Weeds	N/A	W-Ca2501-2520	28/01/15	20%

Location	GPS co-ordinates	Sample type	OSR Variety	Sample ID	Date of Sampling	Incidence
Somerset	51°04'30.90" N 003°03'25.61" W	OSR	W21	So2521-2570	03/02/15	42%
Somerset	51°04'30.90" N 003°03'25.61" W	Weeds	N/A	W-So2571-2590	03/02/15	10%
Somerset	51°05'07.24" N 003°02'17.87" W	OSR	W21	So2591-2640	03/02/15	72%
Somerset	51°05'07.24" N 003°02'17.87" W	Weeds	N/A	W-So2641-2660	03/02/15	20%
Somerset	51°04'58.83" N 003°02'10.23" W	OSR	W21	So2661-2710	03/02/15	74%
Somerset	51°04'58.83" N 003°02'10.23" W	Weeds	N/A	W-So2711-2730	03/02/15	15%
Yorkshire	53°55'22.89" N 001°09'11.76" W	OSR	Picto	Y2731-2780	03/02/15	52%
Yorkshire	53°55'22.89" N 001°09'11.76" W	Weeds	N/A	W-Y2781-2800	03/02/15	20%
Yorkshire	53°56'23.54" N 001°21'36.64" W	OSR	Charger	Y2801-2850	03/02/15	72%
Yorkshire	53°56'23.54" N 001°21'36.64" W	Weeds	N/A	W-Y2851-2870	03/02/15	25%
Yorkshire	54°02'52.33" N 001°18'12.68" W	OSR	Incentive	Y2871-2920	03/02/15	62%
Yorkshire	54°02'52.33" N 001°18'12.68" W	Weeds	N/A	W-Y2921-2940	03/02/15	30%
Cornwall	50°29'57.53" N 004°43'34.81" W	OSR	Unknown	S-Cw301-350[a]	12/05/15	28%
Cornwall	50°29'49.84" N 004°43'35.43" W	OSR	Unknown	S-Cw351-400[a]	12/05/15	32%
Cornwall	50°30'16.86" N 004°43'37.75" W	OSR	Unknown	S-Cw401-450[a]	12/05/15	38%

[a]Samples taken and ELISA results produced by an undergraduate student working in the Laboratory

3.3.2 Mainland Europe TuYV Incidence

The incidence of TuYV in mainland European was determined from samples provided by collaborators (Table 3.2). TuYV was detected in every country that was sampled (Table 3.2) and on average at higher incidences than those found in the UK (Table 3.1). The wide range of TuYV-infected samples collected from across Europe (Figure 3.4) allowed for an in-depth study of the genetic diversity of TuYV throughout Europe. In the major OSR growing countries (France, Germany and Poland) there were TuYV incidences of $\geqslant 90\%$ in all countries. These high incidences were consistent from year to year, revealing the chronic nature of TuYV within OSR crops of these countries. Areas of Europe with lower incidences, down to 0% in some areas, were in regions with more extreme temperatures. Lower incidences were seen in the Ukraine and Denmark. Extreme low temperatures particularly in colder climates are likely to affect the vectors, halting the spread of TuYV in years with harsher weather; Holeby in Denmark had incidences of 77% and 0% in different years. A small collection of plants samples were also obtained from China, these samples also had a high incidence of 70% however this could be the recently described BrYV which is endogenous to China (Xiang *et al.*, 2011). However, due to the close relationship, especially in the gene P3 of TuYV and BrYV, the antisera was able to detect the virus.

Figure 3.4 – European Oilseed Rape Sampling Sites.

Table 3.2 – The Incidence of TuYV in Oilseed Rape (*B. napus)* Mainland Europe and China.

Country	Location	OSR Variety	Sample ID	Sample Date	Incidence
France	Chartes	Variable	FRA1-100	23/04/12	92%
France	Aire	DK Exstorm	FRA101-130	22/06/12	70%
France	Aire	Sensation	FRA131-160	22/06/12	67%
France	Aire	Aviator	FRA161-190	22/06/12	87%
France	Courcelles	Palmedor	FRA191-220	22/06/12	73%
France	Bulquoy	DK exstorm	FRA221-250	22/06/12	66%
France	Aire	Aviator	FRA251-280	22/06/12	73%
France	Aire	Aviator	FRA281-310	22/06/12	97%
France	Liverdy en Brie	Variable	FRA311-40	27/06/13	93.3%
France	Buzet	Variable	FRA341-F430	03/06/13	96.7%
Germany	Peine	Amillia	DEU1-50	23/04/12	96%[a]
Germany	Sulbeck	Remy	DEU51-100	02/05/12	100%[a]
Germany	Bergtheim	Variable	DEU101-130	14/05/12	90%[a]
Germany	Marburg	Visby	DEU131-180	06/06/12	98%[a]
Germany	Einbeck	Remy	DEU181-240	12/05/13	97%
Ukraine	Kiev	Variable	UKR1-38	07/06/12	0%[a]
Ukraine	Lviv	Variable	UKR39-68	07/06/12	30%[a]
Denmark	Abildgard	Variable	DNK1-30	14/06/12	0%[a]
Denmark	Holeby	Variable	DNK31-60	14/06/12	77%[a]
Denmark	Dyngby	Variable	DNK61-90	14/06/12	10%[a]
Denmark	Dyngby	Unknown	DNK91-110	25/06/14	20%
Denmark	Fehmarn	Unknown	DNK111-130	25/06/14	30%
Denmark	Holeby	Unknown	DNK131-150	25/06/14	0%
Poland	Kondratowice	Hibrirock	POL1-90	24/05/13	99%
Poland	Kondratowice	Hibrirock F1	POL91-180	24/04/14	100%
Poland	Dabrowka	Unknown	POL181-240	28/04/15	85%
Poland	Kondratowice	KWS Frodo	POL241-330	02/05/15	100%
China	Wuhan	Variable	CHN1-28	08/04/14	70%[b]

[a]Incidence from a bag of unmarked leaves, unable to find true incidence of the field
[b]Incidence from samples selected with virus-like symptoms

3.3.3 Identifying Conserved Genome Sites of TuYV

The first step in generating sequence information for TuYV was to successfully amplify a wide range of isolates. After first efforts to amplify the whole genome of TuYV were unsuccessful, further work was done to understand TuYV genetic information at the extremities of the viral genome in order to allow successful amplification. Initial and subsequent whole genome PCR attempts cause very little or no target amplicons with multiple miss amplified products, which were small portions of the TuYV genome due to its repetitive nature. Previously collected UK *Brassica* TuYV isolates: L1843, L1851, L1906, L1926, L1937 ((Asare-Bediako, 2011), See Section 2.3.1) were used to gain information on sequence of the 3′ untranslated region (UTR) by amplifying the TuYV genome between 2900-5643nt with primers: MN17, MN22, MN25, MN27 and MN30 (Figure 3.5). This was essential as only one sequence was available for primer design in this location (TuYV-FL), causing primer design for RT-PCR amplification of European isolates difficult. Primer for this experiment were either designed using the TuYV-FL sequence or utilising primers designed by other research groups (See Table 2.2). The aim was to amplify and sequence the 3′ UTR to find a conserved region for RT-PCR primer design. Different primer combinations were used for RT-PCR (Figure 3.5), allowing the successful amplification of all laboratory isolates. However, lack of sequence information lead to multiple and smeared products, but target size amplicons were extracted and sequenced to gain information on conserved regions for further primer design used throughout this project (Figure 3.6).

After sequencing, isolate nucleotide sequence data were BLASTed and aligned to produce a consensus of the region for primer design. Blasting the sequences resulted in high E-scoring hits from recently released genome sequences of Brassica yellows viruses (BrYV) (Xiang *et al.*, 2011). This extra information was used for alignment and primer design (Figure 3.6). The primers designed successfully amplified laboratory isolates with specificity; the reverse primer was located within the 3′ UTR, allowing P5 to be fully sequenced following RT-PCR. This primer allowed RT amplification of all isolates in this study.

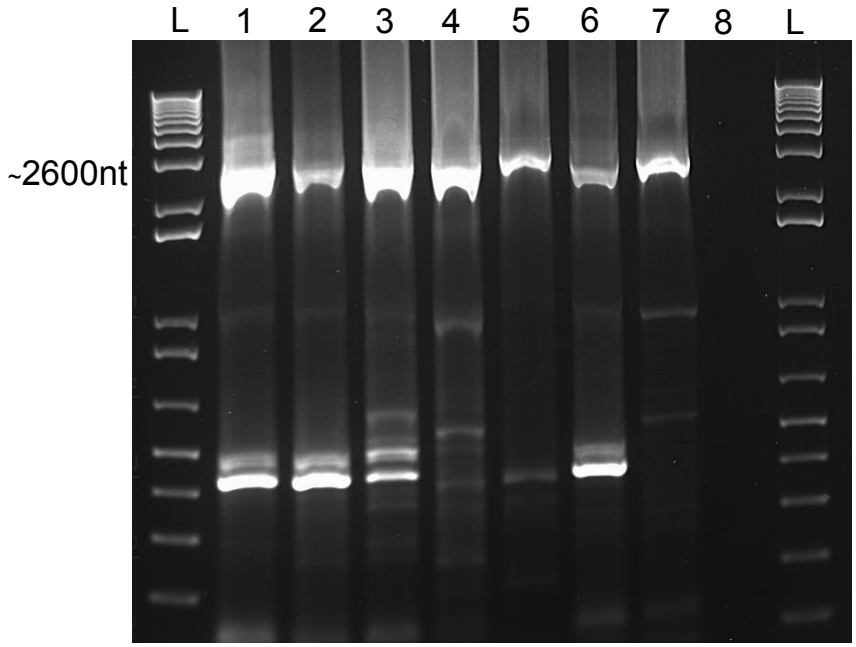

Figure 3.5 – RT-PCR of 3′ UTR Fragments from UK Isolates Maintained at Warwick.
L) 1Kb+ ladder. 1) L1808 amplified with the MN17+MN27 primer pair. 2) L1843 amplified with the MN17+MN30 primer pair. 3) L1851 amplified with the MN17+MN22 primer pair. 4) L1906 amplified with the MN17+MN27 primer pair. 5) L1926 amplified with the MN17+MN25 primer pair. 6) L1937 amplified with the MN17+MN22 primer pair. 7) LAB amplified with the MN17+MN25 primer pair. 8) Uninfected OSR RNA -ve control. L) 1Kb+ ladder.

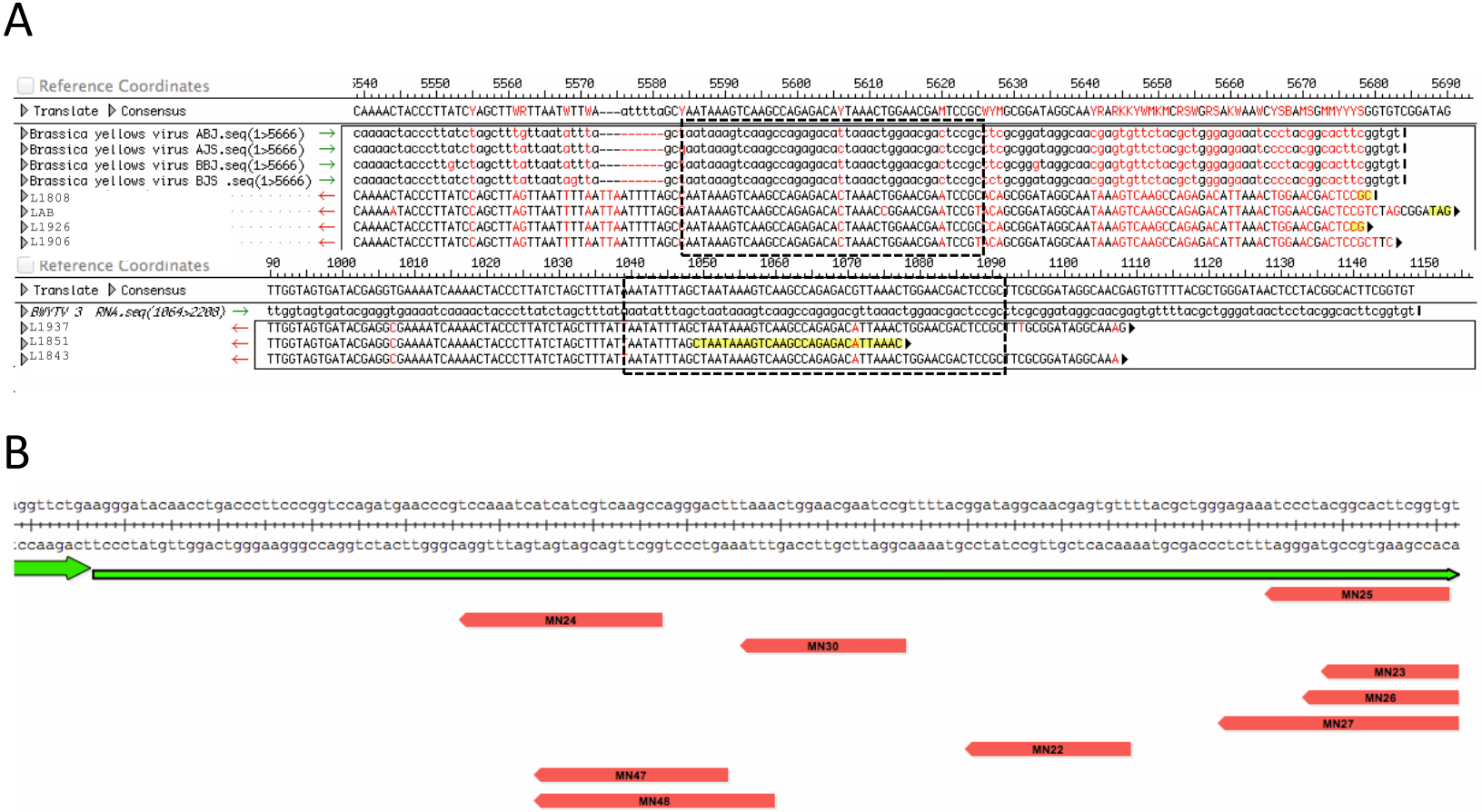

Figure 3.6 – 3′ UTR Sequences of UK TuYV Isolates Maintained at Warwick.
A) Sequences of laboratory isolates aligned with the published sequence, the boxes indicate regions conserved between the sequenced isolates, BrYV isolates and BWYV 3′ sequence (Accession numbers: HQ388348, HQ388349, HQ388350, HQ388351, X13062) (Veidt *et al.*, 1988; Xiang *et al.*, 2011). B) 3′ untranslated region terminus of TuYV genome (thin green arrow), with new MN47+MN48 Primer positions in the 3′ untranslated region, compared to previously used primers.

3.3.3.1 RACE PCR

Similar to the 3′ UTR, a greater understanding of the 5′ UTR was needed to allow a specific primer to be designed within this region. RACE PCR results were used to help shed light on the sequence upstream of the start codon of P0, allowing better primer design. Again the only sequence of the TuYV 5′ UTR (1-32nt) was from TuYV-FL (Veidt *et al.*, 1988) as other published sequences only included the P0 and no sequence information in the 5′ UTR. Two TuYV isolates maintained at Warwick were amplified and sequenced (Figure 3.7 and Section 2.5.7), one was a common group isolate L1851-C and the other was the LAB-I isolate belonging to the intermediate phylogenetic group. This revealed a conserved region which was used to design the 5′ primers necessary for this study (Figure 3.8).

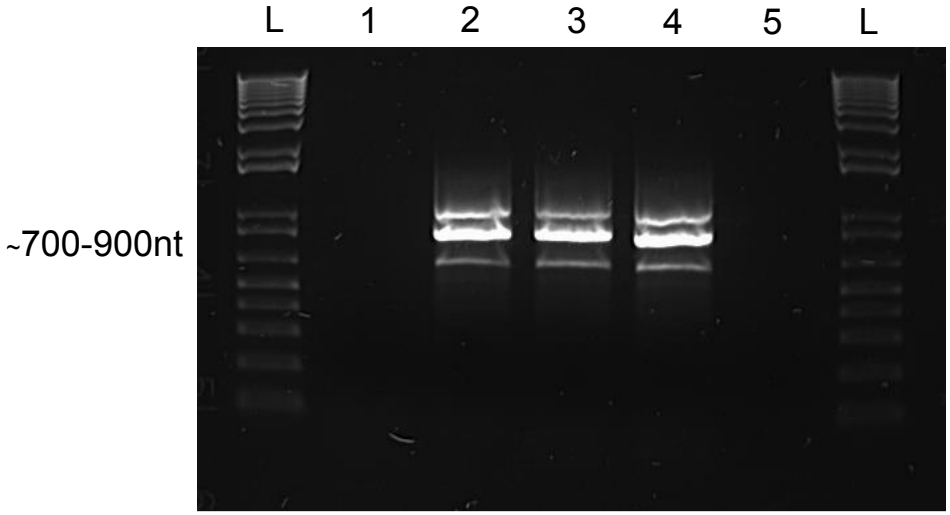

Figure 3.7 – 5′ RACE PCR of TuYV UK Isolates Maintained at Warwick. L) Ladder 1Kb+. 1) Uninfected OSR RNA -ve control. 2) LAB-I amplified with MN36 and vial 9 primers (Table 2.3). 3) L1851-C amplified with MN36 and vial 9 primers. 4) L1851-C (duplicate) amplified with MN36 and vial 9 primers. 5) Water -ve control. L) Ladder 1Kb+. Sequencing revealed the middle amplicon was the correct product.

The sequences obtained from the RACE PCR approach again showed similarity to those of both TuYV-FL and the BrYV 5′ UTR. Sequence alignments were carried out incorporating the related sequences (Figure 3.8). This data was then used to design new primers which incorporated the new information so as to avoid the region of the indel in L1851 and the two SNPs in subsequent primers. This allowed the development of primers capable of amplifying the genome. Sequencing primers were designed by using primers amplifying out from regions with significant sequence data (P0 and P3), in a primer walking approach (Figure 3.8).

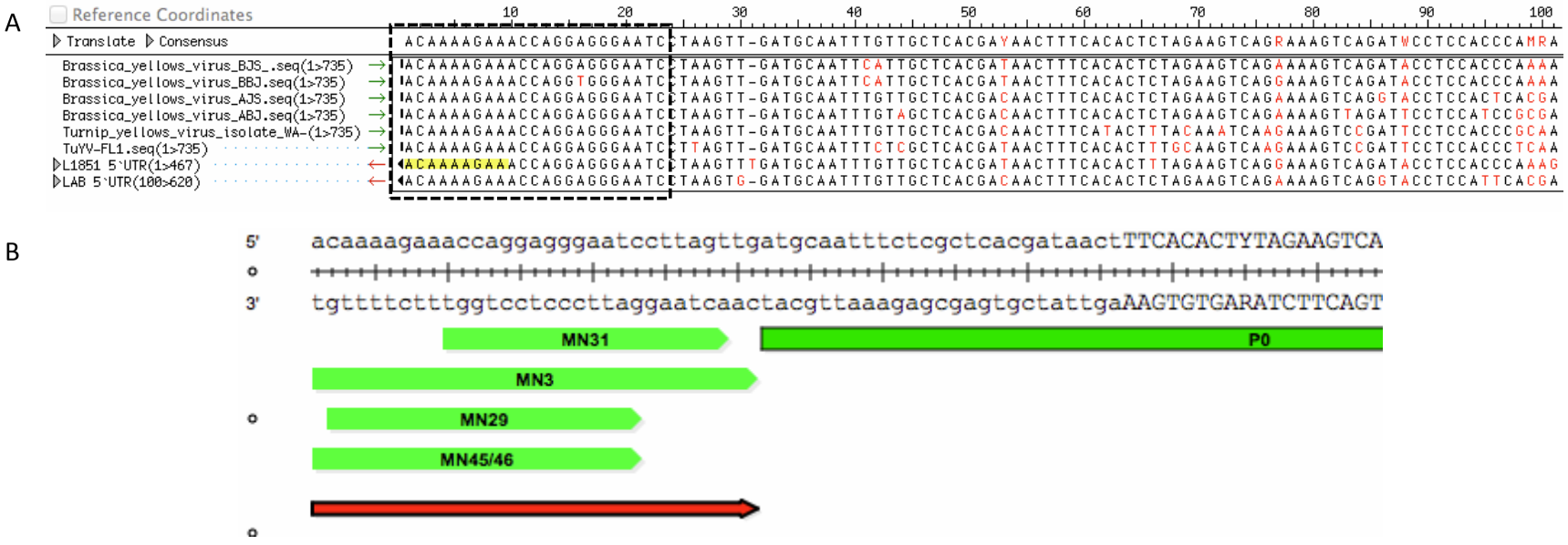

Figure 3.8 – 5′ RACE UTR Sequences of UK Isolates LAB-I+L1851-C.
The start of the TuYV genome with the 5′ untranslated region indicated by the red arrow in B. A) Laboratory isolates L1851 and LAB 5′ untranslated region sequences aligned with BrYV and TuYV-FL, target site for primer design indicated by box. B) From the sequencing information MN45/46 were designed within the 5′ untranslated region and to avoid the tyrosine indel (position 31bp).

3.3.4 Investigation of the Genetic Diversity of TuYV

In this study 179 full TuYV genome sequences were obtained along with 7 partial genomes (Table 3.3) Two locations from which TuYV could not be amplified and sequenced were Angus and Stirlingshire within the UK. The close geographical location of these sites could indicate that there was a divergent TuYV or different polerovirus isolate detected by ELISA. The successful amplification of the TuYV genome was accomplished with primers within the UTR of both ends of the TuYV genome. This allowed the sequencing of all gene coding regions in efforts to estimate the genetic variability of TuYV isolates. This included P0, which encodes a protein that is involved in post transcriptional gene silencing, host range specificity and symptom expression. P1 and P2 are form a fusion protein that is TuYV's RNA dependent RNA polymerase (RdRp). P3a aids the long distance movement of TuYV within plants. P3 encodes the major coat protein (CP) and within P3's ORF is P4, which encodes the movement protein (MP). After P3 stop codon is the gene P5 the RT minor coat protein, which has been linked to host range specificity, aphid uptake of the virus and encapsidation. The nucleotide length of each gene in this study are as follows, P0 gene comprises 750 nt. Sequence of RdRp (P1) gene was 1824nt, the RdRp (P2) gene was 1113nt. The recently discovered gene P3a is 138nt (Smirnova *et al.*, 2015). The CP (P3) gene was 699nt in length and internal to this is the MP (P4) gene, which is smaller at 588nt. Sequence of RTD (P5) gene was large at 1294nt. The sequences obtained were analysed together with those retrieved from GenBank.

European TuYV isolates analysed shared nucleotide identities ranging from 90.6 to 100% for P3, this being the most conserved gene and from 47.4 to 100% for P5, making it the most diverse between European isolates. The deduced amino acid sequences of the sequenced isolates ranged from 90.6 to 100% for the P3 and 58 to 100% for the P5 (Table 3.4), this demonstrated that there is higher variability within P5 than any other gene, even more so than P0 which was previously said to be the most variable gene (Stevens *et al.*, 2005).

The P5 nucleotide sequences obtained in this study had very low homology with those of published related polerovirus, 45.6 to 90.8%, the highest was to TuYV-FL. Deduced amino acid identities were 53.1 to 91.7% with those published in GenBank (Table 3.4). P3 nucleotide sequence identities between the isolates obtained in this study and the published isolates ranged between

Table 3.3 – Field Isolates of TuYV Genomes Sucessfully Sequenced.

Isolates Designation[a]
ALi1, ALi27, C212, C244, C251, C295, C301, C328, C356, C365, C392, Ca2312
Ca2312(b), Ca2327, Ca2359, Ca2382, Ca2401, Ca2425, Ca2462, Ca2475, Ca2499, Cau74
CHN15, CHN15(b), CHN22, CHN24, Cw706, Cw713, Cw728, Cw748, Cw789, Cw789(b)
Cw814, DNK31, DNK39, DNK45, DNK53, DNK64, DNK70, DNK85, DNK123, FRA28
FRA58, FRA72, FRA98, FRA117, FRA120, FRA193[b], FRA197, FRA229, FRA234[c]
FRA242, FRA250, FRA310, FRA319, FRA337, FRA341, FRA341(b), FRA381
FRA381(b), FRA430, DEU5, DEU13, DEU38, DEU50, DEU65, DEU101, DEU123[d]
DEU131[e], DEU157, DEU170, DEU201, DEU209, DEU213, DEU228, DEU253, K436
K436(b), K447, K465, K505, K505(b), K508, K516, K550[f], K574, K580, K596
L1851, L1926, L1937, LAB, Li2105, Li2126, Li2145, Li2171, Li2188, Li2198
Li2214, Li2246, Li2273, N1891, N1894, N1930, N1999[g], N2076, POL1, POL1(b)
POL60 , POL90, POL91, POL160, POL180[h], POL182, POL218, POL230, POL270
POL310, S14, S26, S50, S83, S108, S118, S142, S142(b), S188, S189, S-Cw330
S-Cw398, S-Cw449, So2551, So2570, So2593, So2622, So2640, So2667, So2689, So2708
So2713, UKR41, UKR44, UKR59, UKR67, Wa1484, Wa1518, Wa1543, Wa1584, Wa1627
Wa1643, Wa1645, Wa1682,W-C261, W-C412, W-Ca2369, W-Ca2441, W-Ca2505, W-Li2306
W-S51, W-S52, W-S53, W-S121, W-So2573, W-So2645, W-So2719, W-Y2782, W-Y2856
Wi1702, Wi1715, Wi1752, Wi1764, Wi1764(b), Wi1835, Wi1835(b), Wi1854, Wi1863
Y2742, W2769, Y2769(b), Y2780, Y2802, Y2825, Y2845, Y2871, Y2882, Y2913

[a]For information on the origin of each isolate see Tables 3.1 and 3.2
[b]Partial sequence (2237-5450nt)
[c]Partial sequence (2374-5450nt)
[d]Partial sequence (3181-5450nt)
[e]Partial sequence (1-4874nt)
[f]Partial sequence (1-4872nt)
[g]Partial sequence (1-3958nt)
[h]Partial sequence (1-4695nt)

91.4% and 97.6%, and the identities of the corresponding predicted amino acid sequences ranged from 89.6 to 97% (Table 3.4). However, between the different polerovirus species, P0 still seems the most variable gene with nucleotide sequence identity variation between 43-96.4% and 24.7-95.6% of the corresponding amino acids. Sequenced isolates had higher homology to BWYV than TuYV-FL in this gene, but not to any other genes. These results indicate that not only are the sequenced isolates a distinct species from BWYV, BMYV and BrYV but also possibly TuYV-FL with 5 of the 7 genes having lower than 90% homology to TuYV-FL genes which has classically been used to distinguished this virus species (Mayo, 1999).

Table 3.4 – Genetic Similarity between Genes of TuYV Obtained During this Study and other Poleroviruses.

TuYV Gene	Genetic Comparison	% Identity between European Isolates	% Identity between European Isolates and TuYV-FL[a]	% Identity between European Isolates and BrYV[b]	% Identity between European Isolates and BWYV[c]	% Identity between European Isolates and BMYV[d]
P0	AA	81.1-100	79.9-89.6	81.9-95.6	28.4-31.9	24.7-28.1
	nt	85.6-100	85.2-91.4	86.9-96.4	46.2-48.4	43-45.7
P1	AA	78.1-100	82.6-91.9	79.6-96.5	32.3-38.9	30.8-36.1
	nt	79.1-100	84.1-92	80.6-96.5	47.9-51.8	47.2-50.8
P2	AA	89.7-100	91.6-99	91.1-98.6	72.8-76.6	71.1-76.6
	nt	90.3-100	89.4-99.2	90.3-97.3	51.7-55.6	49.1-54.6
P3a	AA	71.1-100	68.9-95.6	66.7-95.6	61.4-72.7	61.4-70.5
	nt	64.3-100	63.2-94.7	57.2-95.4	61.7-67.8	49.1-65.6
P3	AA	90.6-100	94.1-97	92.1-95.5	90.1-94.6	89.6-94.6
	nt	93.7-100	95.5-97.1	92.2-95	92.7-96.1	91.4-94.6
P4	AA	88.6-100	92.6-96	84.6-90.3	88-95.4	86.3-91.4
	nt	93.2-100	95.8-97.6	92-94.8	93-96.5	91.7-94.8
P5	AA	58-100	64.8-91.7	53.1-80.9	64.4-84.5	65.1-86.6
	nt	47.4-100	48.3-90.8	45.6-76.9	47.5-81.7	48.9-84.4

[a]NCBI Accession: X13063
[b]NCBI Accession: HQ388348
[c]NCBI Accession: NC004756
[d]NCBI Accession: NC003491

Variation across the genome revealed hotspots of divergence in the P1 and P5 genes as well as the non-coding region (Figure 3.9), these areas had sequences that required the introduction of gaps into the alignment to successfully align all sequenced isolates. This highlights the need for full genome sequencing; P1 exemplified this as it was expected to be very conserved as part of the viral RdRp, therefore functionally constrained.

Analysis of the genetic diversity within each gene of TuYV showed that all genes were variable with high numbers of polymorphisms, high numbers of polymorphic sites and very high haplotype diversity, but low nucleotide diversity (Table 3.5). Apart from P3 (S = 97, Eta = 105, $\pi = 0.0136 \pm 0.0023$, h = 0.886 $\pm$ 0.0020) and P4 (S = 82, Eta = 85, $\pi = 0.0131 \pm 0.0017$, h = 0.886 $\pm$ 0.0021) each gene showed very high diversity with a high proportion of haplotypes. The most genetically diverse gene was P5 with Eta = 1107 and $\pi = 0.0654 \pm 0.0017$, which is five times more diverse than that of the conserved P3/P4 section of the 3′ proximal half of the TuYV genome (Table 3.5). P1 also had very high diversity which could be connected too the genetically diverse area within P1 (See Figure 3.9). P1 (S = 808, Eta = 1061, $\pi = 0.0451 \pm 0.0031$, h = 0.886 $\pm$ 0.001), this level of diversity is higher than the previously thought most variable gene P0 (S = 266, Eta = 321, $\pi = 0.0323 \pm 0.0021$, h = 0.998 $\pm$ 0.001). This is discrepancy between P1 and P0 is due to length of P1 being larger (750nt vs 1785nt) and the distinct genotypes having an area of divergence introducing gaps into the alignments. TuYV although polymophic sites vary between 16 - 59 % for each gene's sequence, haplotype diversity is high ranging between 82 - 99 %, indicating the amount of unique sequences that have a different combinations of these polymorphic sites is high, leading to greater diversity within the TuYV population.

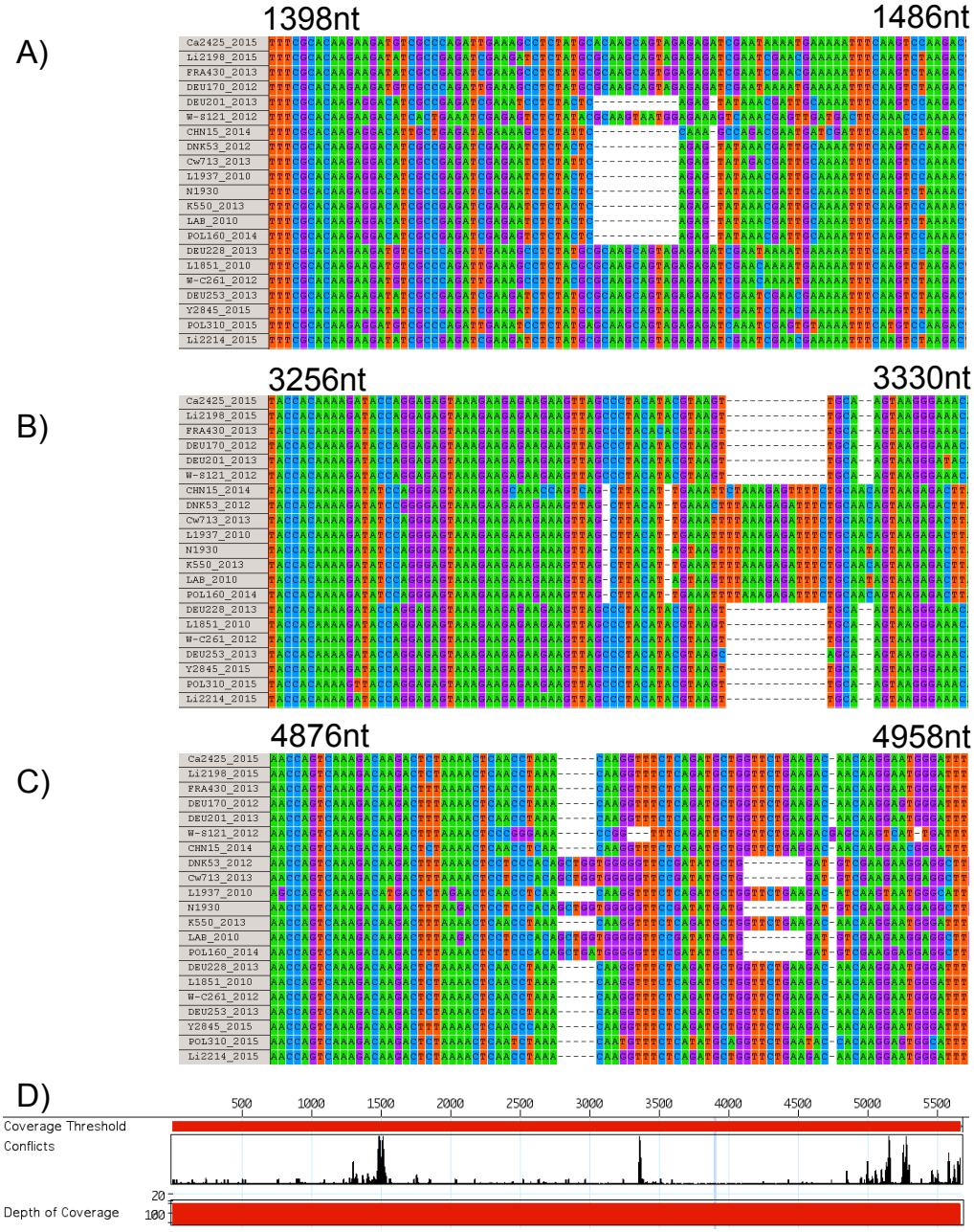

Figure 3.9 – Conflicts Across the TuYV Genome and Areas of Divergent Sequence.
A) Area of significant sequence divergence within P1 gene (1398-1486nt). B) Area of significant sequence divergence within the central non-coding region (3256-3330nt). C) Area of significant sequence divergence within P5 gene (4876-4958nt). D) Areas of sequence conflict across sequenced TuYV isolate whole genomes (n=177).

HyPhy analysis detection of synonymous and non-synonymous nucleotide changes, indicated that all genes were undergoing negative (purifying) selection as the values of dN/dS ratio were low, i.e. dN/dS <1 (Table 3.6). This provided strong evidence of heterogeneous selection pressures among codon sites as sites had both positive and negative selection pressures. This was demonstrated when the overall selection intensity of each gene was compared, the results showed that the selection intensity was highest in the P2 (d_N/d_S 0.188) with P4 displaying the least selection pressure (d_N/d_S 0.740) (Table 3.6). P4 had no positively selected codons and least negatively selected codons of any gene. The gene P1 had the most positively selected codons followed by P0 with P5 being under considerable negative selection. As P3 has such a crucial role and is well known as the most conserved region of the genome, variation is limited, as most mutations would be deleterious to the isolates' viability, and thus has limited variation and codon selection.

TuYV genes P3a, P3 and P4 were under considerable selection pressure and as such were not undergoing neutral evolution as all neutrality tests had P values > 0.05 (Tamjima's D, Fu and Li's D^* and Fu and Li's P^*). For all other TuYV genes results demonstrated that they were undergoing neutral mutation rates, detected by significant neutrality from a range of tests ($P < 0.01$ - 0.05) in the European TuYV population. However, P5 under two models (Li's D^* and Fu and Li's P^*) was also shown to not be under neutral evolution with P values of > 0.10 (Table 3.7).

Table 3.5 – Genetic Variability within TuYV Genes Nucleotide Sequences.

TuYV Gene	Number of Sequences	Total Number of Sites	Number of Polymorphic sites (S)	Total Number of Mutations (Eta)	Nucleotide Diversity (π)[a]	Haplotype Diversity (h)[a]
P0	186	750	266	321	0.0323 ± 0.0021	0.998 ± 0.001
P1	186	1785	808	1061	0.0451 ± 0.0031	0.999 ± 0.001
P2	186	1113	450	557	0.0291 ± 0.0019	0.999 ± 0.001
P3a	186	138	47	59	0.0595 ± 0.0082	0.818 ± 0.020
P3	186	609	97	105	0.0136 ± 0.0023	0.886 ± 0.020
P4	186	528	82	85	0.0131 ± 0.0017	0.886 ± 0.021
P5	186	1269	745	1107	0.0654 ± 0.0017	0.998 ± 0.009

[a]± Mean standard deviation

Table 3.6 – Mean Pairwise Genetic Distances and the Selective Pressures within TuYV Genes.

Gene	d_N	d_S	d_N/d_S	Total Number of Codons	No. Codon Under Positive Selection[a]	No. Codons Under Negative Selection [a]
P0	0.909	1.889	0.481	249	8	37
P1	1.161	4.029	0.288	595	17	157
P2	0.626	3.327	0.188	371	2	88
P3a	0.393	1.777	0.221	46	1	10
P3	0.254	0.728	0.349	203	0	14
P4	0.315	0.426	0.740	176	0	8
P5	1.0278	3.186	0.323	423	1	119

[a]P Value > 0.05

Table 3.7 – Neutrality Testing within TuYV Genes.

TuYV Gene	Tamjima's D	P Value	Fu and Li's D^*	P Value	Fu and Li's F^*	P Value
P0	-1.81314	< 0.05	-4.88413	< 0.02	-4.04698	< 0.02
P1	-1.82301	< 0.05	-3.58582	< 0.02	-3.22619	< 0.02
P2	-2.14713	< 0.01	-6.23807	< 0.02	-5.03507	< 0.02
P3a	-0.58241	> 0.10	0.75901	> 0.10	0.19841	> 0.10
P3	-1.70133	> 0.05	-1.58593	> 0.10	-1.96440	> 0.05
P4	-1.63989	> 0.05	-1.62123	> 0.10	-1.95511	> 0.05
P5	-1.83896	< 0.05	-0.66643	> 0.10	-1.48223	> 0.10

3.3.5 Recombination of TuYV Isolates

Since recombination between genomes confounds attempts to estimate evolutionary rates and phylogenetic trees (Gibbs *et al.*, 2010), it was important to omit any recombinants from subsequent evolutionary analysis. Two likely hotspots of recombination were found with breakpoints at 3488nt, which is at very the beginning of P3 and at 4823nt which is located in the middle of P5 (Table 3.8). These sites were supported with 5 or more tests with P values of 1.03^{-7} - 2.04^{-17} and 3.02^{-23} - 1.99^{-47} respectively. There were 27 isolates with potential recombination within P3 and 62 with recombination P5 (Table 3.8). These locations are consistent with BrYV recombination points (Lim *et al.*, 2014), however no double recombinants were detected, each isolate had only a single point identified as recombinant between isolates of the common group and uncommon phylogenetic groups (See Section 3.3.6). No other recombination locations were detected with strong statistical support (P value >95%). Geographical bias is apparent as a several locations have a high number of recombinant isolates: Cardiganshire, two locations in France (Buzets and Liverdy en Brie), and Wigtownshire hand 4 or more of the recombinant isolates at any one location. However the majority of locations have much lower levels of recombinant isolates.

Table 3.8 – Recombination Sites within the TuYV Isolates of this Study.

TuYV Gene	Location (nt)	Major Parent Representative	Minor Parent Representative	Recombinant Isolates	P Value Range
P3	3488		UKR44	ALi-27, C365, Ca2327, Ca2401, Ca2499, Cw728, DNK70 Cw789, Cw789(b), FRA250, FRA381, DEU38, DEU123, DEU228 K465, K508, K516, Li2273, POL182, POL60, S189, S-Cw398 So2413, Wa1543, Wi1702, Wi1715, Wi1854, Wi1863	1.03^{-7} - 2.04^{-17}
P5	4823	LAB	L1851	DEU201, C212, C251, C295, C301, C328, C356 C392, Ca2359, Ca2425, Ca2462, Cau74, Cw748, DNK39 DNK85, FRA117, FRA197, FRA242, FRA310, FRA337, FRA341 FRA341(b), FRA381(b), FRA430, FRA58, FRA98, DEU170, DEU50 DEU65, K447, K574, Li2105, Li2126, Li2145, Li2171 Li2188, Li2214, N2076, POL90, S108, S118, S14 S142, S142(b), S83, So2570, So2689, So2708, Wa1484 Wa1518, Wa1584, Wa1627, Wa1645, W-Ca2369, W-Ca2441, W-Ca2505 Wi1752, Wi1835, Wi1835(b), W-S51, Y2742, Y2780, Y2825	3.02^{-23} - 1.99^{-47}

3.3.6 Phylogenetic Analysis of TuYV

The maximum likelihood tree for P0 amino acid sequence data revealed that the 183 TuYV isolates sequenced in this study (including partial sequenced isolates) from OSR formed two main genetic groups, corresponding to the two clades supported by bootstrap values greater than 85% for all isolates (Figures 3.10). This differs from earlier work indicating three groups based on P0 (Asare-Bediako, 2011). Clade 1 contained the majority of the sequenced isolates (140 isolates); clade 2 contained 40 isolates. These clades were consistent between amino acid based trees of genes P0, P1, P2, P3a and P3 (Figures 3.10, 3.12, 3.14, 3.16, 3.18). TuYV-FL (accession number X13063) and WA-1 (accession number JQ862472) did not cluster with any of the isolates sequenced from OSR along with DEU5 in P0 phylogenetic analysis. This indicates a (relatively) long evolutionary P0 distance separating them from the other isolates, however they cluster with Clade 1 and are lost as an out-group in amino acid analysis of all the other genes except P5. TuYV isolates collected from all of the European geographical regions and across different years were spread throughout both clades (i.e. the isolates did not cluster according to geographical regions, host or years of collection), except for amino acid trees based on P4, P5, whole genome nucleotide tree and the concatenated tree. These trees were able to distinguish Chinese isolates with P5 distinguished another UK based group as well. Due to the very conserved nature of P4 the amino acid phylogenetic tree could not distinguish the common and uncommon clade of the other trees, but instead could define groups between European isolates (Clade 1) and Chinese isolates (Clade 2) (Figure 3.20). The BrYV reference isolate (ABJ) and the isolates I sequenced from China generally fell in Clade 2 (uncommon clade) unless otherwise stated.

Nucleotide based maximum-likelihood tree of P0 was able to separate the Chinese isolates away from the European isolates of Clade 1 and 2, as well as put TuYV-FL and WA-1 into an out-group (Figure 3.11). The nucleotide P1 tree again had two clades of European isolates, but was able to put TuYV-FL and WA-1 into an out-group as well as defining another clade containing three weeds and one OSR isolate (Figure 3.13). P2 nucleotide tree had the same two clades of European isolates as the amino acid tree, but could also define Chinese isolates into a separate clade (Figure 3.15). This is similar for P3a nucleotide tree however the out-group also contains TuYV-FL and WA-1 (Figure 3.17). The P3 and P4 nucleotide tree show the same clades unlike the corresponding amino acid trees, Clade 2 included, again, all Chinese isolates, TuYV-FL and WA-1, but also

included the same three weed and single OSR isolate that were genetically distinct in the P1 nucleotide tree (Figures 3.19 and 3.21). The whole genome nucleotide tree and concatenated gene trees could split the two clades of European isolates, as well as Chinese isolates into separate clades (Figures 3.25 - 3.26).

The most variable gene P5 (Tables 3.4 and 3.5) maximum likelihood trees for both nucleotide and amino acid sequences defined 5 genetics groups (including the out-group), three distinct European clades; Clades 1 and 2 were in keeping with the other amino acid phylogenetic trees (except P4), however three weed and one OSR isolate fell into Clade 3 (consistent with the P1, P3 and P4 nucleotide trees); Chinese isolates were also separated into a defined group (Figures 3.22 and 3.23), with TuYV-FL and WA-1 as out an out-group. These 5 distinct populations were supported by population genetic analysis (Figure 3.24). This demonstrates that P5 is a good candidate for genotyping European isolates as it can not only separate European isolates from closely related species but also distinguishing between the three potential European species of TuYV. The nucleotide based analyses were able to distinguish the groups in many instances better than the amino acid based phylogenetic trees, but both produced valuable information between the genotypes.

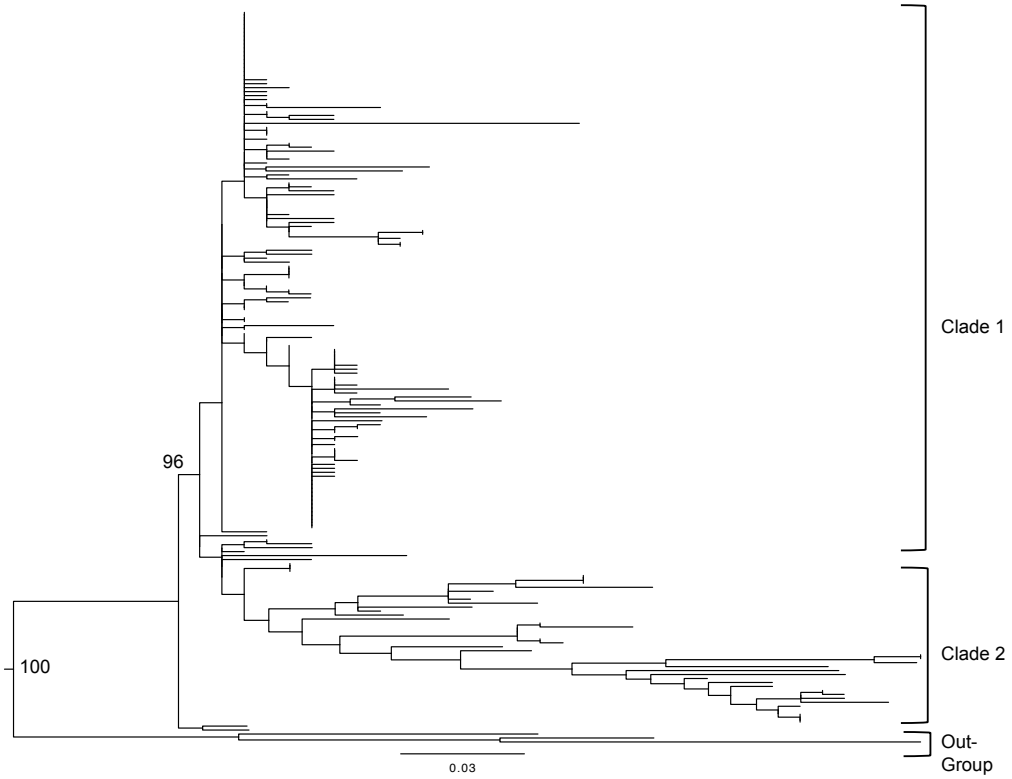

Figure 3.10 – Maximum Likelihood Phylogenetic Tree of Amino Acid Sequences of the TuYV P0 Gene (n=186).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade one contains European TuYV isolates and Chinese isolate CHN24. Clade 2 contains all other Chinese isolates plus BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) as well as a smaller proportion of European isolates. The out-group contains TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012). The scale bar signifies a genetic distance of 0.03 amino acid substitutions per site.

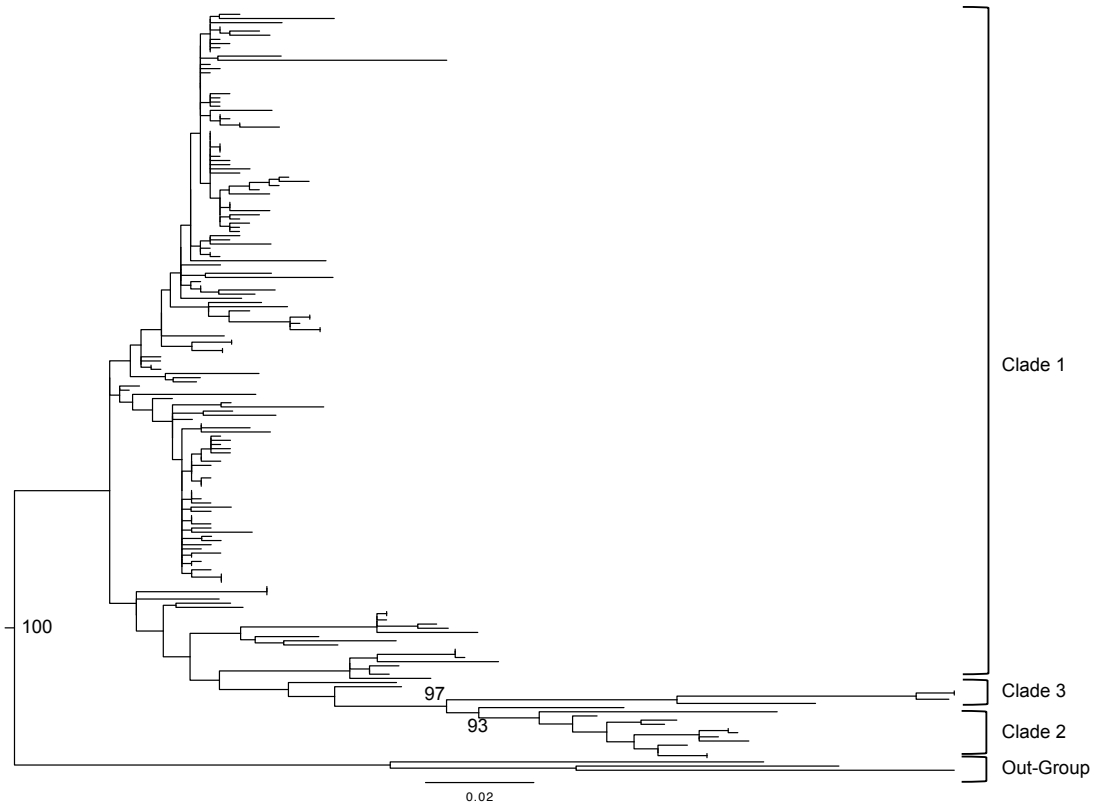

Figure 3.11 – Maximum Likelihood Phylogenetic Tree of Nucleotide Sequences of the TuYV P0 Gene (n=186).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade one contains European TuYV isolates. Clade 2 contains a smaller proportion of European isolates. Clade 3 contains all Chinese isolates plus BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) is located between Clade 1 and 2. The out-group contains TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012). The scale bar signifies a genetic distance of 0.02 nucleotide substitutions per site.

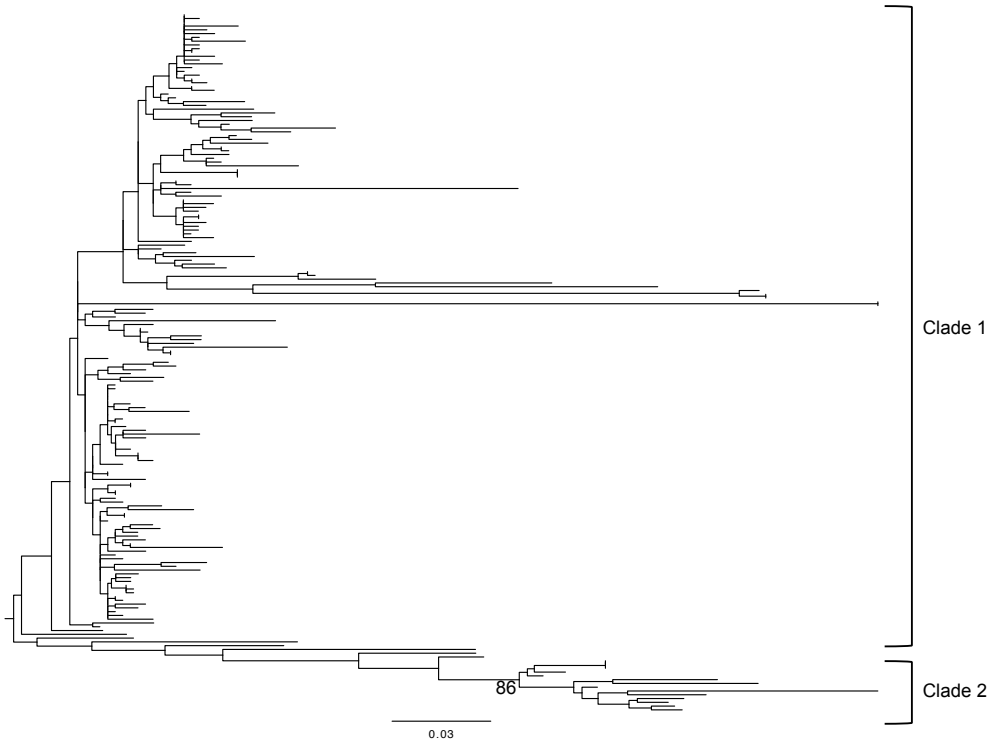

Figure 3.12 – Maximum Likelihood Phylogenetic Tree of Amino Acid Sequences of the TuYV P1 Gene (n=186).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade one contains European TuYV isolates and Chinese Isolates as well as TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012). Clade 2 contains Chinese isolate CHN24, BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) as well as a smaller proportion of European isolates. The scale bar signifies a genetic distance of 0.03 amino acid substitutions per site.

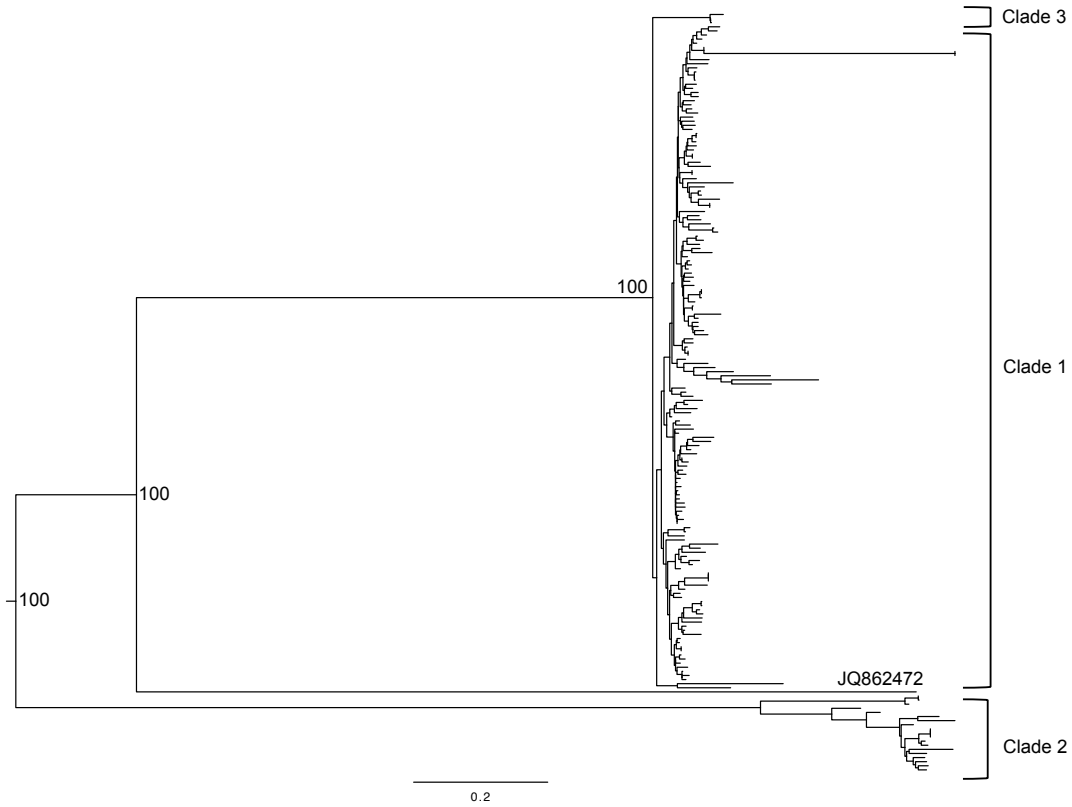

Figure 3.13 – Maximum Likelihood Phylogenetic Tree of Nucleotide Sequences of the TuYV P1 Gene (n=186).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade 1 contains European TuYV isolates and TuYV-FL (accession number X13063) (Veidt *et al.*, 1988). The TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) is an outgroup. Clade 2 contains all Chinese isolates including BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) as well as a smaller proportion of European isolates. Clade 3 consists of three weed isolates and one OSR isolate (W-S52, W-S53, W121, S-Cw330, all originating from the UK). The scale bar signifies a genetic distance of 0.2 nucleotide substitutions per site.

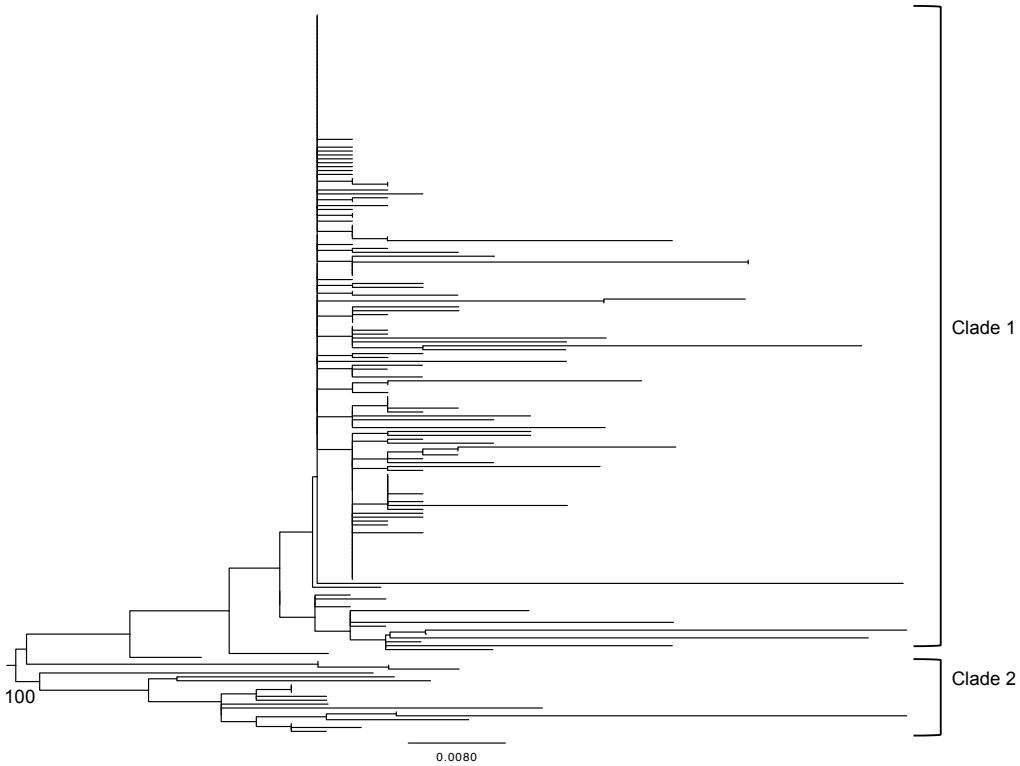

Figure 3.14 – Maximum Likelihood Phylogenetic Tree of Amino Acid Sequences of P2 (n=186).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade one contains European TuYV isolates. Clade 2 contains all Chinese isolates including BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) as well as TuYV-FL (accession number X13063) (Veidt *et al.*, 1988), TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) as well as a smaller proportion of European isolates. The scale bar signifies a genetic distance of 0.0080 amino acid substitutions per site.

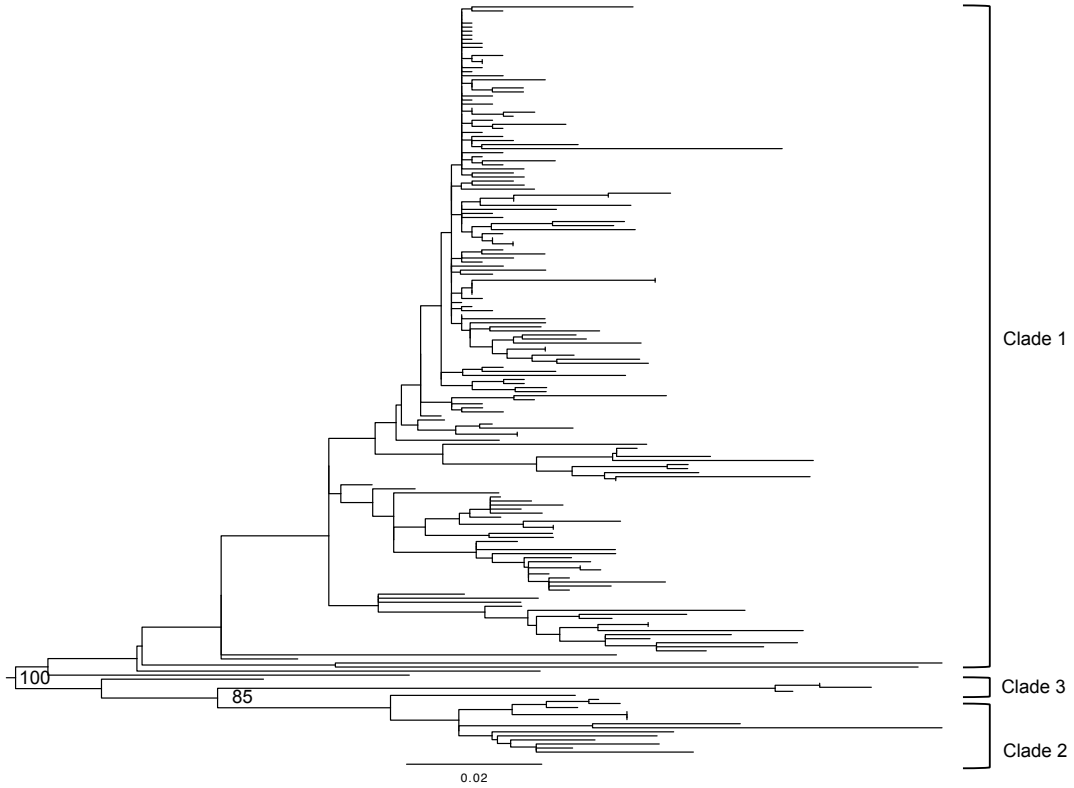

Figure 3.15 – Maximum Likelihood Phylogenetic Tree of Nucleotide Sequences of P2 Gene (n=186).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade one contains European TuYV isolates as well as TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012). Clade 2 contains a smaller proportion of European isolates. Clade 3 contains all Chinese isolates including BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348). The scale bar signifies a genetic distance of 0.02 nucleotide substitutions per site.

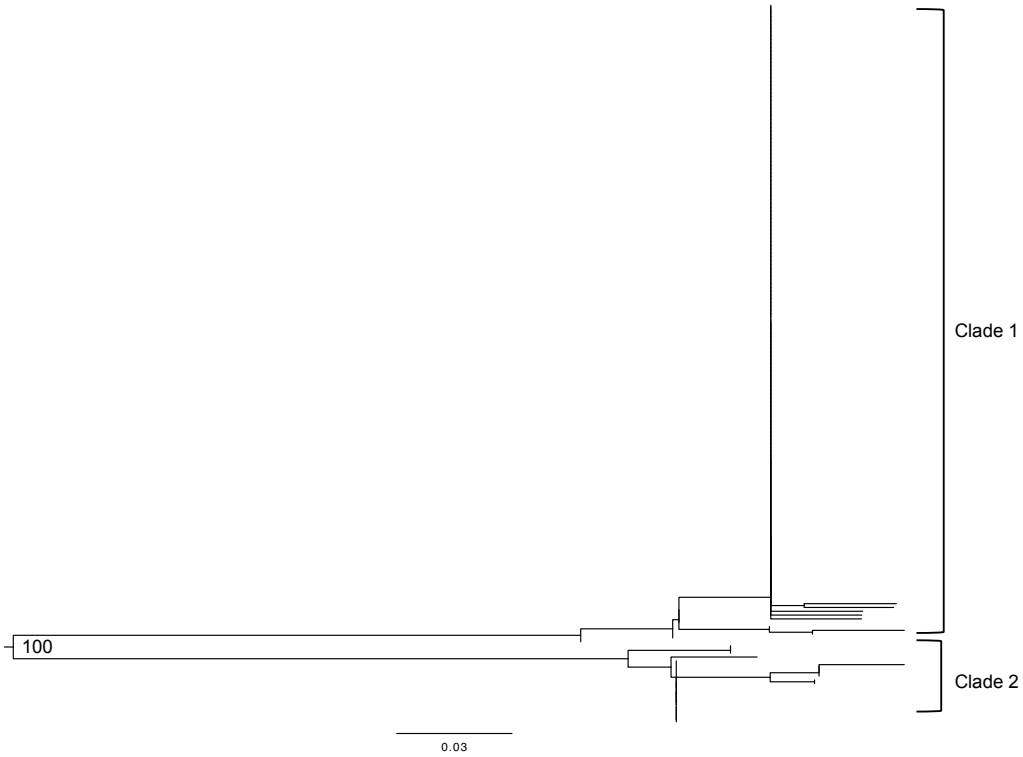

Figure 3.16 – Maximum Likelihood Phylogenetic Tree of Amino Acid Sequences of the TuYV P3a Gene (n=186).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade 1 contains European TuYV isolates. Clade 2 contains all Chinese isolates including BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) as well as TuYV-FL (accession number X13063) (Veidt *et al.*, 1988), TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) as well as a smaller proportion of European isolates. The scale bar signifies a genetic distance of 0.03 amino acid substitutions per site.

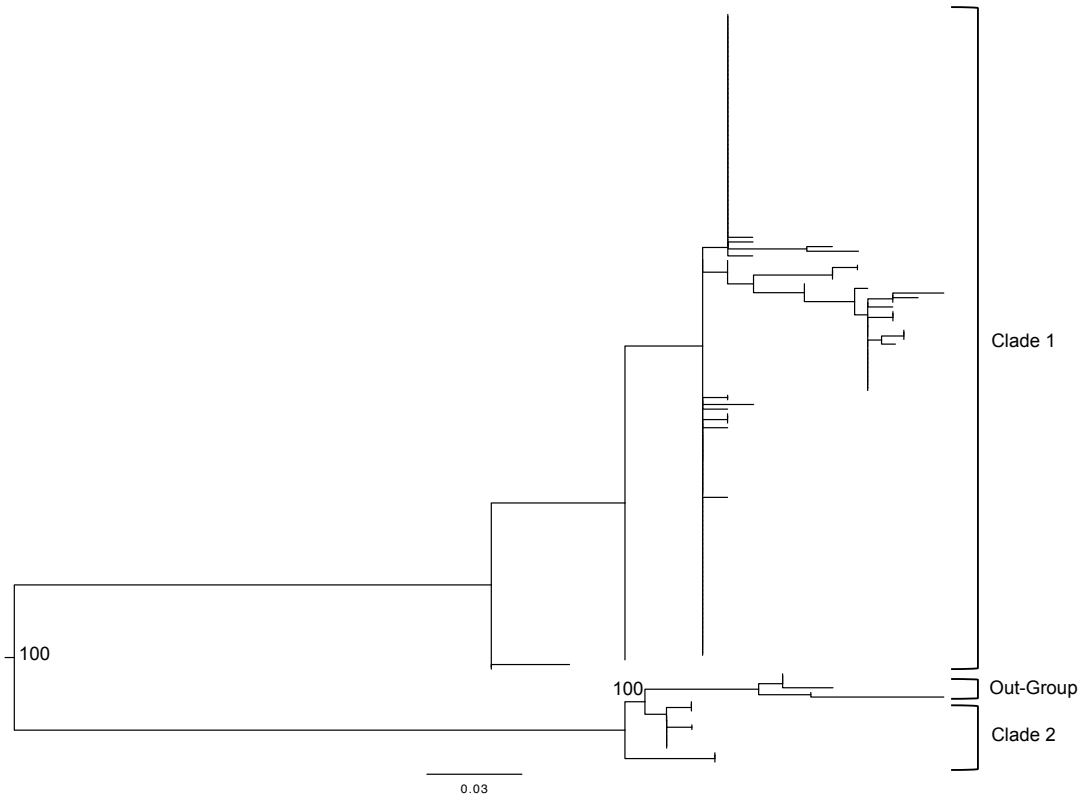

Figure 3.17 – Maximum Likelihood Phylogenetic Tree of Nucleotide Sequences of the TuYV P3a Gene (n=186).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade 1 contains European TuYV isolates. Clade 2 contains a smaller proportion of European isolates. The out-group contains all Chinese isolates including BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) as well as TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012). The scale bar signifies a genetic distance of 0.03 nucleotide substitutions per site.

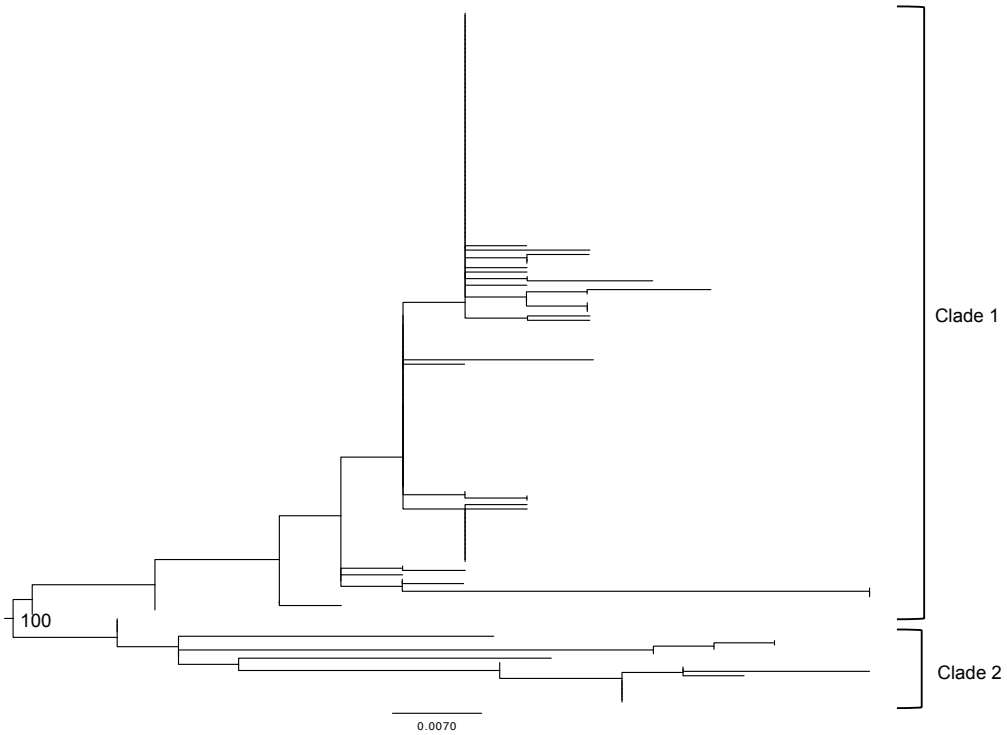

Figure 3.18 – Maximum Likelihood Phylogenetic Tree of Amino Acid Sequences of the TuYV P3 gene (n=134).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade 1 contains European TuYV isolates. Clade 2 contains all Chinese isolates including BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) as well as TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) as well as a smaller proportion of European isolates. The scale bar signifies a genetic distance of 0.0070 amino acid substitutions per site.

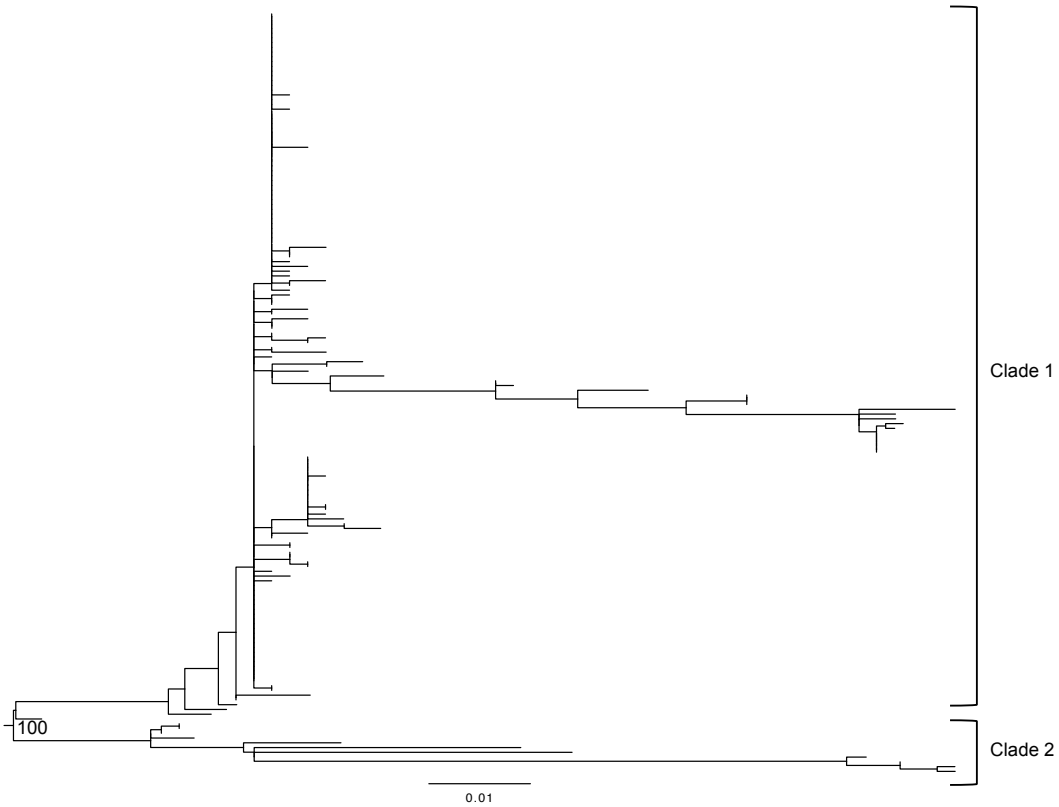

Figure 3.19 – Maximum Likelihood Phylogenetic Tree of Nucleotide Sequences of the TuYV P3 gene (n=134).
Clades are based on significant nodes of $\geqslant$ 85% indicating genetic groups after 1000 bootstrap replicates. Clade 1 contains all European TuYV isolates. Clade 2 contains all Chinese isolates including BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348), it also contains TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) as well as three weed and one OSR isolate (W-S52, W-S53, W121, S-Cw330, all originating from the UK). The scale bar signifies a genetic distance of 0.01 nucleotide substitutions per site.

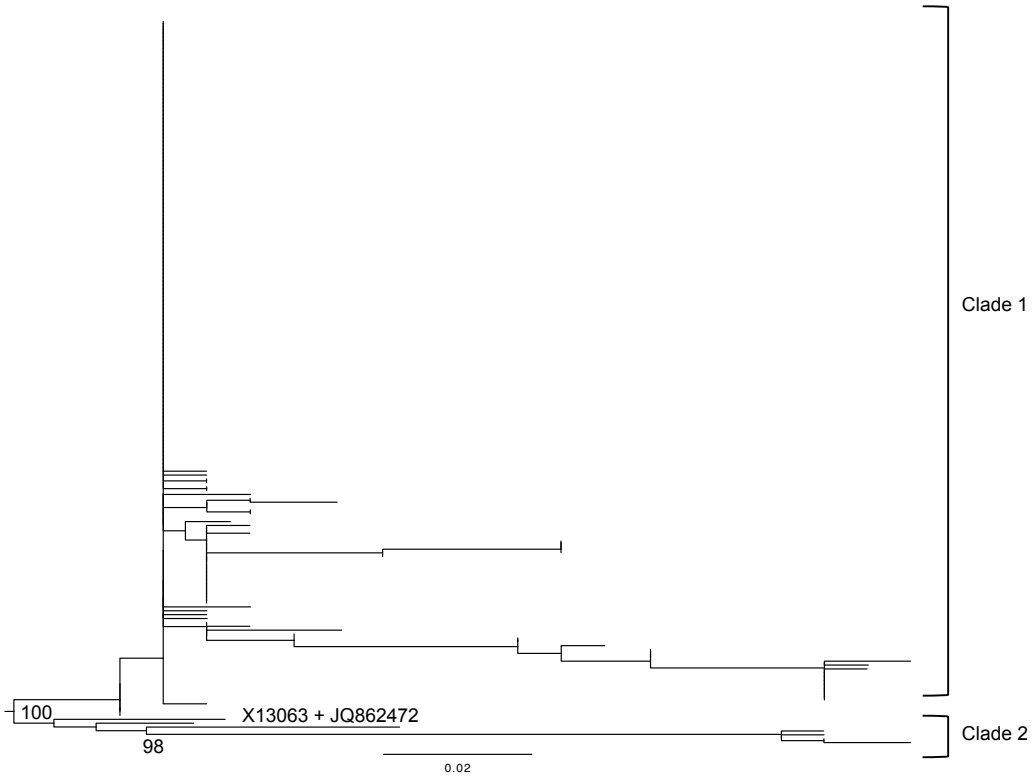

Figure 3.20 – Maximum Likelihood Phylogenetic Tree of Amino Acid Sequences of the TuYV P4 Gene (n=187).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade 1 contains all European TuYV isolates Clade 2 contains all Chinese isolates including BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348). Isolate TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) are located as an outgroup between Clade 1 and Clade 2. The scale bar signifies a genetic distance of 0.02 amino acid substitutions per site.

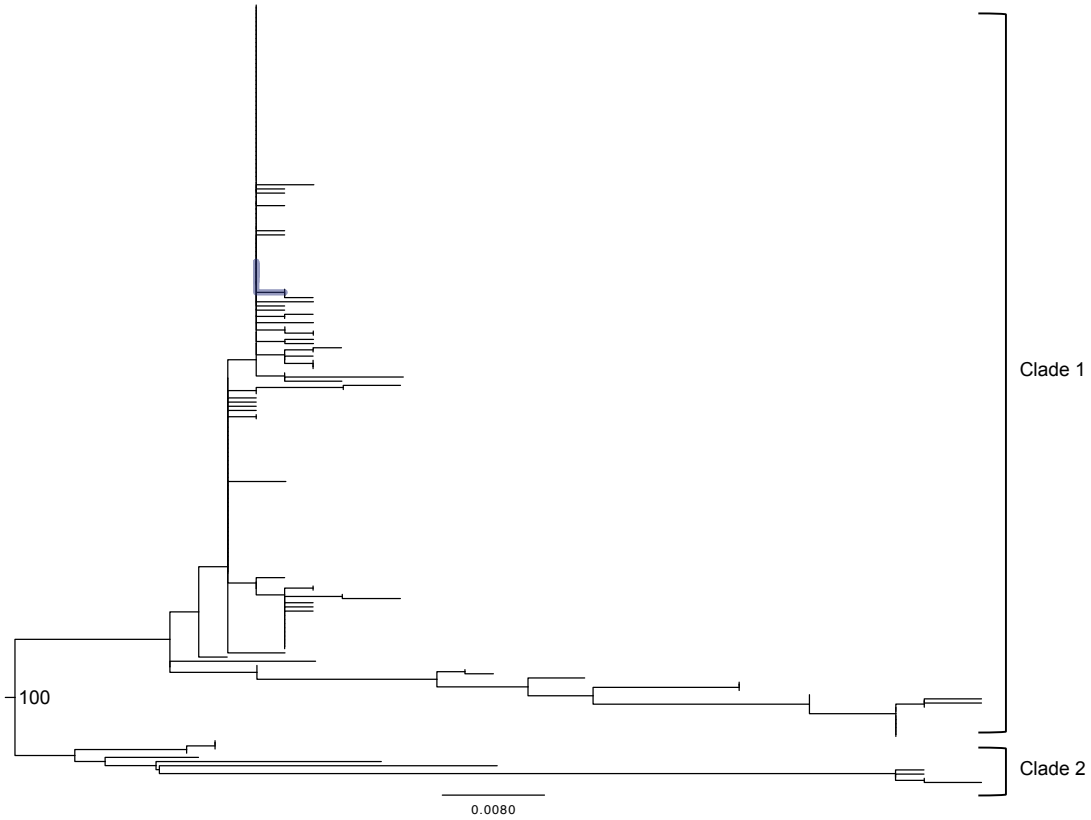

Figure 3.21 – Maximum Likelihood Phylogenetic Tree of Nucleotide Sequences of the TuYV P4 Gene (n=187).
Clades are based on significant nodes of $\geqslant$ 85% indicating genetic groups after 1000 bootstrap replicates. Clade 1 contains all European TuYV isolates. Clade 2 contains all Chinese isolates including BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348), it also contains TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) as well as three weed and one OSR isolate (W-S52, W-S53, W121, S-Cw330, all originating from the UK). The scale bar signifies a genetic distance of 0.008 nucleotide substitutions per site.

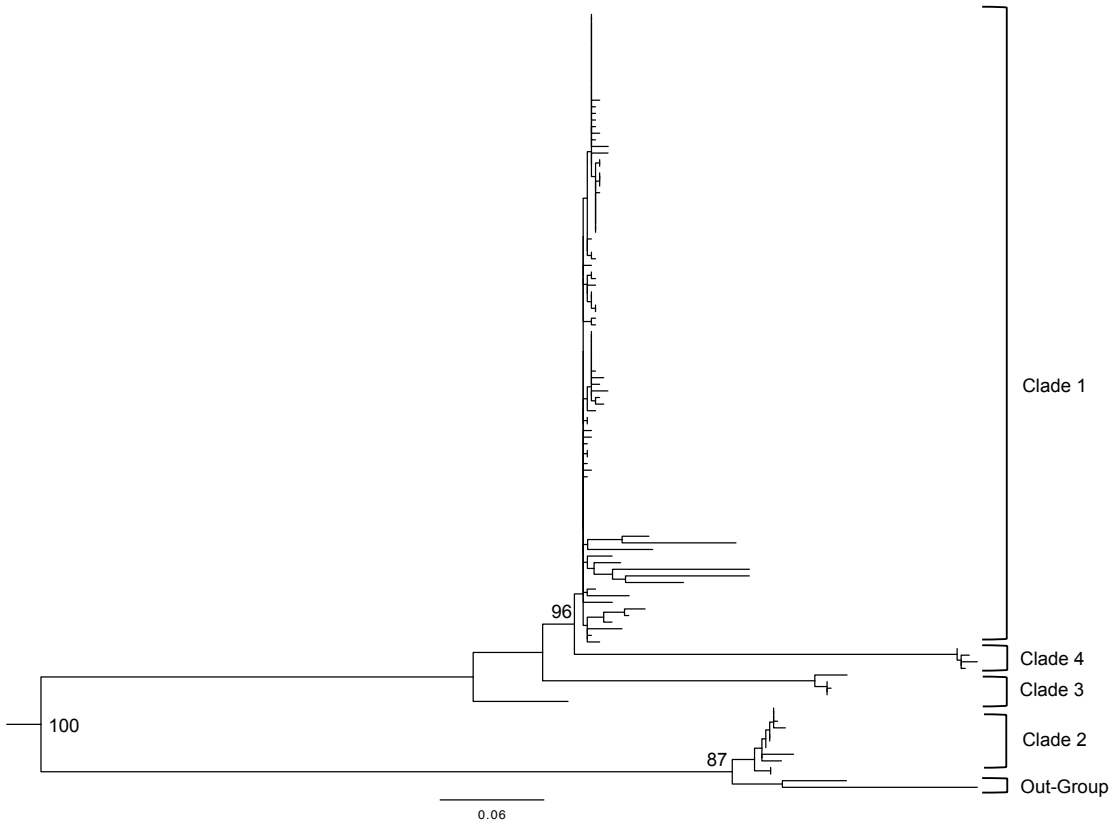

Figure 3.22 – Maximum Likelihood Phylogenetic Tree of Amino Acid Sequences of the TuYV P5 Gene (n=118).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clades 1 and 2 are European OSR and weed isolates. Clade 3 consists of three weed isolates and one OSR isolate (W-S52, W-S53, W121, S-Cw330, all originating from the UK). Clade 4 contains BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) and all Chinese isolates. Isolate TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) are an out-group. The scale bar signifies a genetic distance of 0.06 amino acid substitutions per site.

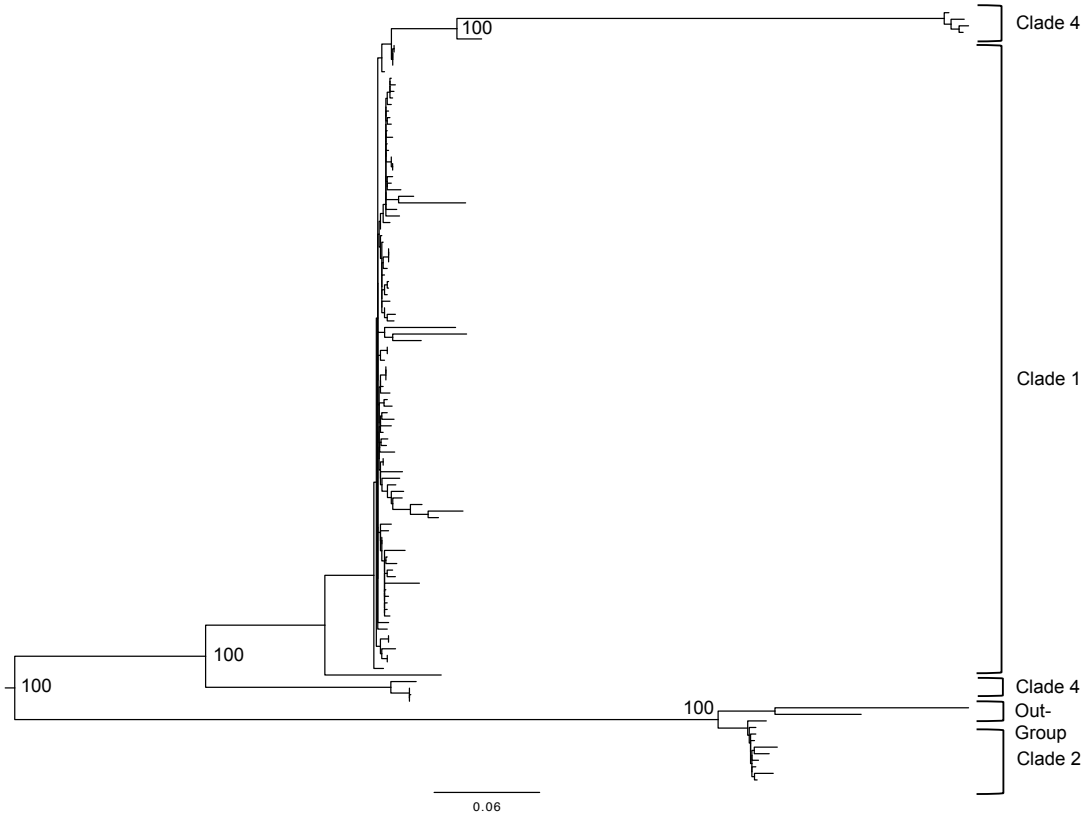

Figure 3.23 – Maximum Likelihood Phylogenetic Tree of Nucleotide Sequences of the TuYV P5 Gene (n=118).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clades 1 and 2 are European OSR and weed isolates. Clade 3 consists of three weed isolates and one OSR isolate (W-S52, W-S53, W121, S-Cw330, all originating from the UK). Clade 4 contains BrYV isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) and all Chinese isolates. Isolate TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) are an out-group. The scale bar signifies a genetic distance of 0.06 nucleotide substitutions per site.

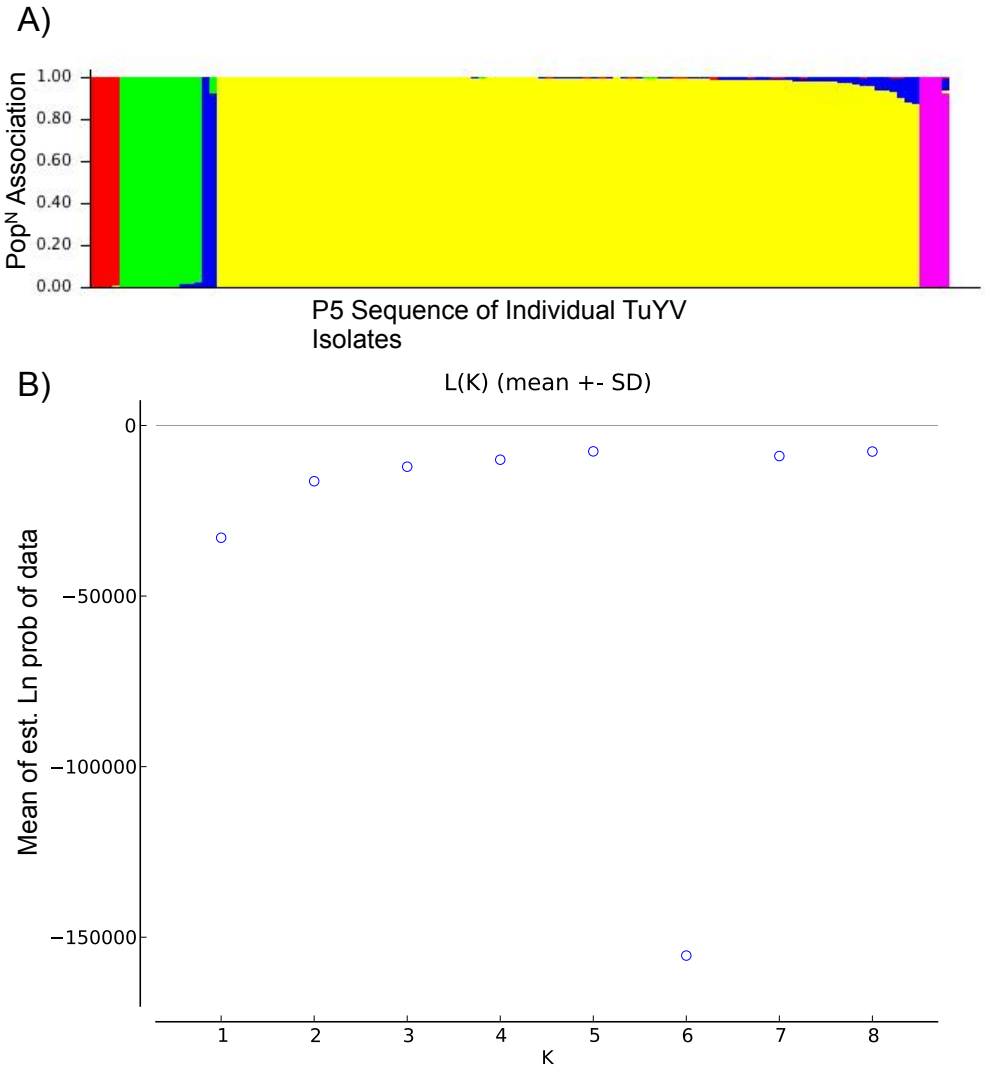

Figure 3.24 – Population Genetics Support of TuYV P5 Gene's Phylogenetic Groups.
Each individual TuYV isolate is represented by a single vertical line broken into coloured segments, with lengths proportional to each of the clades or populations (K), outlined in Figure 3.21 and 3.22. A) Population association of P5 genetic groups (n=115) in to 5 distinct populations, Clade 1 (Common) yellow, Clade 2 (Uncommon) in Green, Clade 3 in Purple ("Weed-Like"), Clade 4 (BrYV) in red and the out-group outlined in blue (TuYV-FL). B) Likelihood that K supports a population of 5 individual groups.

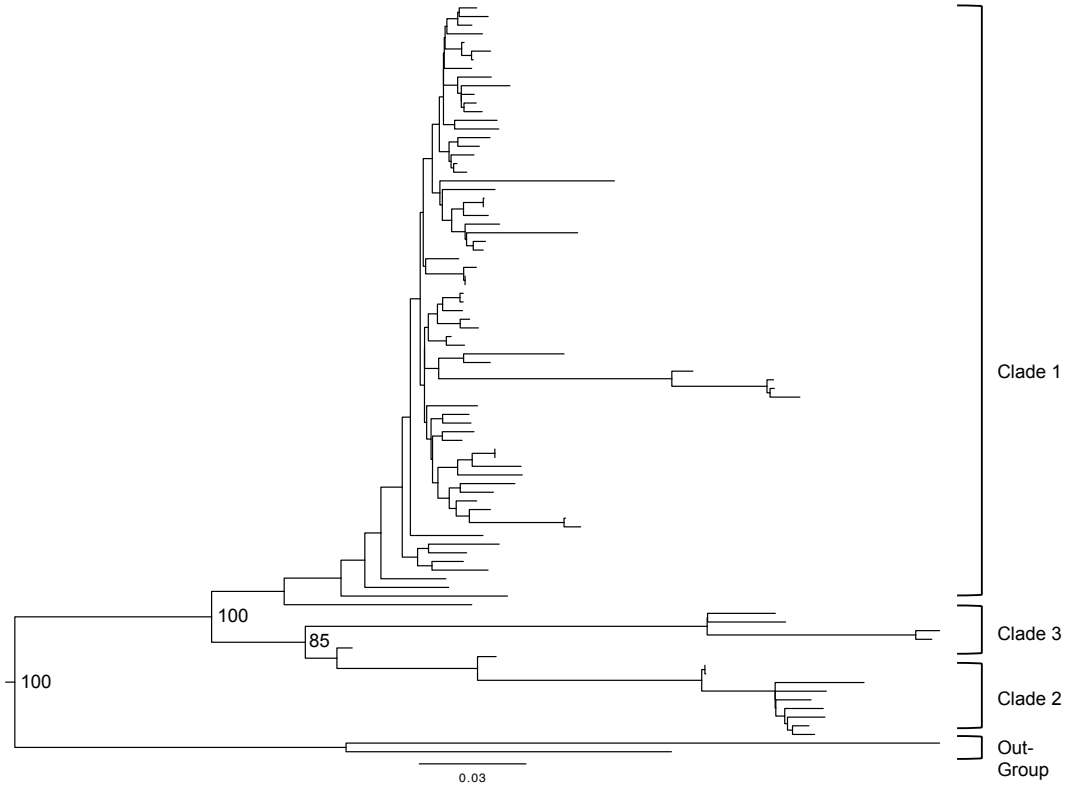

Figure 3.25 – Maximum Likelihood Phylogenetic Tree of Whole Genome Nucleotide Sequences of P5 (n=87).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clade 1 and 2 contain European isolates. BrYV Isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) and all Chinese isolates are located in Clade 3. Isolate TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) are the out-group. The scale bar signifies a genetic distance of 0.06 nucleotide substitutions per site.

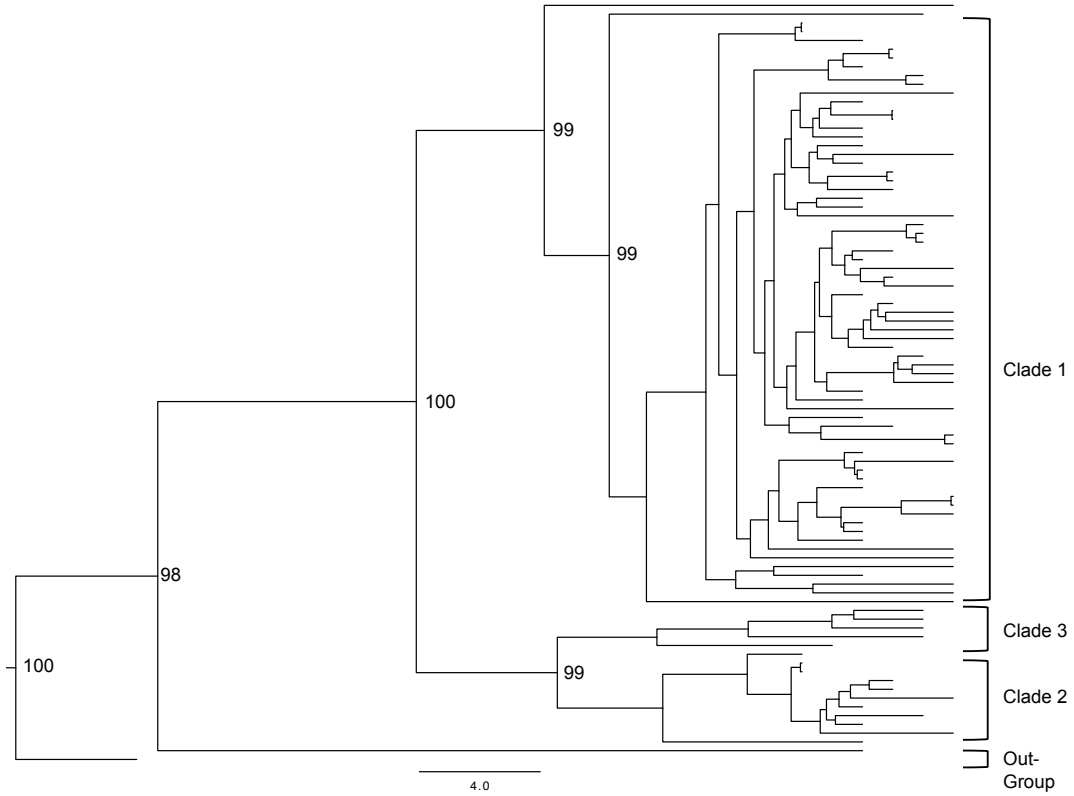

Figure 3.26 – Bayesian Phylogenetic Tree of Concatenated TuYV Genes for Codon Based Nucleotide Analysis (n=87).
Clades are based on significant nodes of $\geqslant 85\%$ indicating genetic groups after 1000 bootstrap replicates. Clades 1 and 2 contain European isolates. BrYV Isolate ABJ (Xiang *et al.*, 2011) (accession number HQ388348) and all Chinese isolates are located in Clade 3. Isolate TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) and TuYV isolate WA-1 (accession number JQ862472) (Wylie *et al.*, 2012) are the out-group. The scale bar signifies a genetic distance of 4.0 nucleotide substitutions per site.

One representative from each of the three European genetic clades defined in the P5 tree (Figure 3.22) were used to construct a P5 tree with other members of the *Polerovirus* genus (Figure 3.27). The UK Clade 2 isolate LAB-I was more closely related to TuYV-FL in a group with BWYV, BMYV and BChV, whereas both Clade 1 and 3 representatives (L1851-C and W-S121) were linked more closely to BrYV in comparison to the other European *Polerovirus* species. Each representative was significantly different from any other sequence with over 85% bootstrap value separating the isolates, this indicates species differentiation not only from the originally sequenced TuYV-FL but from each of the genetic clades defined by P5.

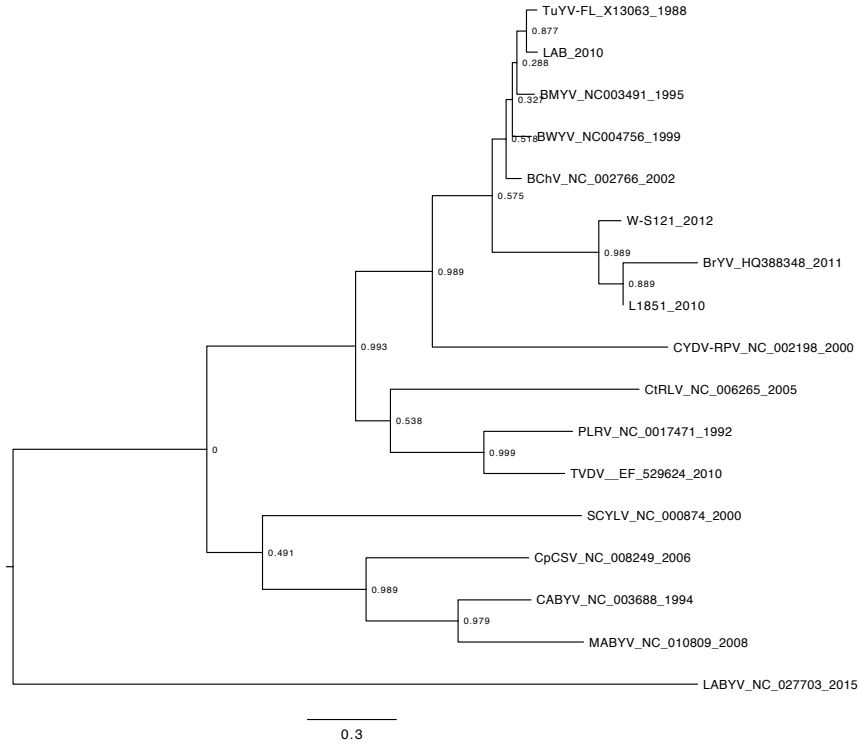

Figure 3.27 – Maximum Likelihood Phylogenetic Tree of P5 Amino acid Sequences (n=17) of members of the *Luteoviridae* genus with representatives of the three Distinct European Genotypes.
Brassica yellows virus (BrYV, accession number: HQ388348, Xiang *et al.* (2011)). Turnip yellows virus (TuYV-FL, accession number: X13063, Veidt *et al.* (1988)). Beet mild yellowing virus (BMYV, accession number: NC003491, Guilley *et al.* (1995)), Beet western yellows virus (BWYV, accession number: NC004756, Su *et al.* (1999)), Beet chlorosis virus (BChV, accession number: NC002766, Hauser *et al.* (2002)), Cereal yellow dwarf virus (CYDV-RPV, accession number: NC002198, direct submission), Carrot red leaf virus (CtRLV, accession number: NC006265, Huang *et al.* (2005)), Potato leaf-roll virus (PLRV, accession number: NC001747, Prüfer *et al.* (1992)), Tobacco vein distorting virus (TVDV, accession number: EF529624, Mo *et al.* (2010)), Sugarcane yellow leaf virus (SCYLV, accession number: NC000874, Moonan *et al.* (2000)), Cowpea chlorotic spot virus (CpCSV, accession number: NC008249, Guilley *et al.* (1994)), Cucurbit aphid-borne yellows virus (CABYV, accession number: NC003688, Abraham *et al.* (2006)), Melon aphid-borne yellows virus (MABYV, accession number: NC010809, Xiang *et al.* (2008)), Luffa aphid-borne yellows virus (LABYV, accession number: NC027703, Knierim *et al.* (2015)). The scale bar signifies a genetic distance of 0.3 amino acid substitutions per site.

3.3.7 Genetic Diversity of TuYV Sub-populations

To better understand the genetic groups outlined by P5 phylogenetic analysis the four sub-populations were compared to each other and their individual sub-population. Originally all European isolates' nucleotide identities were between 47.4 to 100% with the deduced amino acid sequence ranging between 58 and 100% making it a highly variable gene between isolates (Table 3.4). When each sub-population's P5 gene was compared within its sub-population there was above 90% nucleotide and amino acid identity within each clade (Table 3.9). However, when each clade is compared to one another the differences between them were evident, with nucleotide identity ranging from 66.9 to only 87.9% between European isolates, and 53 - 80.1% when comparing European nucleotide sequences to Chinese isolates. The nucleotide results are representative of the amino acid sequence identities of each gene, with all amino acid identities between clades being <90 % similar.

Table 3.9 – Nucleotide (nt) and Amino Acid (AA) Sequence Identities of TuYV Isolates within and between Clades of P5 Maximum Likelihood Phylogenetic Tree.

Clade	nt % Identity	AA % Identity	Clade	nt % Identity	AA % Identity
Within 1	92.3-100	90.5-100	Between 1 + 3	80.2-87.3	82.5-85.3
Within 2	97.3-99.9	97-100	Between 1 + 4	75.9-80.1	74.5-83
Within 3	97.8-100	98-100	Between 2 + 3	67.2-68.4	61.7-63.3
Within 4	98.4-99.2	98.7-99.6	Between 2 + 4	53-53.5	47.2-48.3
Between 1 + 2	66.9-68.3	60.6-63.2	Between 3 + 4	69.1-69.8	71.4-72.5

3.3.8 Mixed Genotype

There were 11 plants where mixed infections of TuYV isolates were detected by sequence polymorphisms with mixed signals in the aligned sequencing trace files. These 12 sequenced isolates were Ca2312(b), CHN15(b) (1-3100nt), FRA341(b), FRA381(b), K436(b), K505(b), POL1(b), S142(b), Wi1764(b), Wi1835(b) and Y2789(b). Of the 11 isolates 9 were quasispecies to each other with very high percent identity. However, FRA381b was significantly different due to it not being a recombinant isolate unlike FRA381 (Table 3.8), but both FRA381b and FRA381 were located within the common clade. FRA341 and FRA341(b) were also considerably different as they were located in the common and uncommon clades, respectively. Mixed infection of genotypes is therefore possible, although detected at a low frequency, and is the factor that gives rise to large number of recombinant TuYV isolates between the common and uncommon clade (Section 3.3.6).

3.4 Discussion

3.4.1 TuYV Incidence

Although TuYV is transmitted in a persistent manner, which can take up to 3 hours unlike non-persistant virus that can be transmitted seconds, incidence is not hindered by this method. High levels of TuYV have been found throughout Europe with some fields having 100% incidence (Table 3.2). This is a trait of the *Luteoviridae*, Barley yellow dwarf virus (BYDV) has also had high incidence levels documented. BYDV is a non-propagative persistent luteovirus infecting monocots, having average UK incidence levels of 50% in grasses and 58-65% maize (Irwin and Thresh, 1990), but BYDV incidence can be above 80% in the crops and wild grasses (Latch, 1977; Gray *et al.*, 1996). Viruses within the family *Luteoviridae* are prominent issues within agriculture due to the high yield penalties they incur on crops, as well as their ability to be widespread at high levels. Although incidence results are extremely high its not uncommon for this family of viruses and with the stringent ELISA result interpretation, specific monoclonal antibody and molecular testing, TuYV is clearly major problem within Europe (See Section 2.4.1).

UK incidences seemed closely linked to the number of *M. persicae* caught in Rothamsted insect survey suction traps (Figure 1.6) with the highest average infection in 2012 and 2015 (Table 3.1), as the aphid numbers were still increasing well into November in these years. This could be due to the effect of favourable climate, still, warm and dry weather being favourable conditions for aphid flights. This would also result in an early infection in the Winter OSR, which germinated around the time of the aphid flights, causing the virus to move throughout the plant as it develops and eventually produces seed (Asare-Bediako, 2011). The only cultivar of OSR tested that indicated reduced levels of TuYV was Amalie (Limagrain), this cultivar has reported resistance to TuYV (Limagrain, 2016). However, Amalie still had a TuYV incidence of 6.6%, although lower than other adjacent OSR plant cultivars (Incentive 62% was located within a mile radius), this could allow the development of resistance-breaking strains becoming more common. Highlighting the necessity of a full understanding of the genetic diversity of TuYV.

All samples in this study were from winter OSR and even in the colder areas of Europe, TuYV was detected. England had lower incidence than mainland

Europe, except Ukraine and Denmark (Table 3.2). These latter, colder areas are likely to have reduced numbers of vector, thus resulting in a lower incidence in these areas and reducing the virus' further spread. The crop in many of the fields tested had been treated with pesticide sprays and/or seed treatments, but TuYV was still present, indicating that TuYV is a widespread and a pathogen that is difficult to control, which requires better control and management. TuYV could become an even more important problem, spreading more consistently in previously less affected colder regions due to global warming, which will lead to an increase in average temperatures of 1 - 3.5 °C (Johns *et al.*, 2003), allowing more favorable conditions for the vector.

TuYV is a serious problem across much of Europe (Table 3.2); with such high incidences inevitably reducing yields to an economically damaging point. Pesticides appear to have little effect on infection levels but can help to reduce quantity of virus present in plants (Walsh *et al.*, 1989), which may help to improve yield. Contrary to this is, crops treated with Crusier such as fields in Aire still showed 97% incidence, as such the benefit from insecticides is clearly limited in some situations were disease pressure is potentially high (Table 3.2). This is a problem due to the fact that transmission of the virus can be achieved by a single aphid; if at any point a plant is not fully protected it will be susceptible to TuYV infection. This will be increasingly likely with the banning of some pesticides such as the neonicotinoid seed treatments (The European Commission, 2013), short persistence of other pesticides (Syngenta, 2014) and the fact that pesticide resistance is an increasing problem in aphid populations (Moores *et al.*, 1994a; IRAC-IRM, 2014).

3.4.2 TuYV Conserved Sites

RACE PCR was used to gain valuable information about the 5′ UTR sequences of UK isolates, as was RT-PCR, using previously designed primers to amplify a region of the TuYV 3′ UTR which could be used for more universal primer design. LAB-I and L1851-C underwent RACE PCR as these belonged to the two major groups of TuYV (based on P0 analysis) comprising up around 98% of isolates in these groups (Asare-Bediako, 2011). Sequences of the 5′ UTR revealed an insertion of a thymine and a substitution of guanine instead of thymine within the 5′ UTR (Figure 3.6) in some isolates. This indicated that primers should not be targeted to this area, consequently MN45/46 were designed, avoiding variable

regions within 5′ UTR for of all future PCR of TuYV isolates. There was even more variability in the 3′ UTR, with significant sequence divergence between sequenced isolates, however, a conserved region was discovered which was used in the design of primer MN48.

With new genetic information gathered from the UTRs from either end of the TuYV genome, RT-PCR can now be accomplished with MN48. As viral titre in plants can be very low due to the virus being limited to the phloem of plants, random hexamers were unable to amplify TuYV to a detectable level. The new primer designed during the course of this work thus allowed a standard protocol for RT-PCR and 5′ and 3′ PCR steps ready for sequencing. The production of these primers will be a valuable tool for further investigation not only for our work but will help other groups interested in TuYV or closely related poleroviruses.

A decision was made to amplify each isolates in two halves as it was not efficient to carry out a full genome amplification. The RT was possible with the use of MN48 to produce a single template for both PCR steps. The 5′ section amplified with MN46-MN59 was 1-3746nt and the 3′ section 3040-5595nt with MN37-MN48, this produced an overlap of over 700bp for sequencing confirmation the two halves were from the same isolate's genome.

It was apparent just from sequence comparison for primer design that TuYV-FL has relativity low genetic similarity to the vast majority of TuYV isolates from brassicas in Europe. The first fully sequenced genome (LAB-I) in my study was obtained from a UK isolate and blasted on NCBI, revealing the highest scoring sequences were that of the recently sequenced and characterised BrYV from China (Xiang *et al.*, 2011). This new information was helpful for alignment of new sequences and primer design at the start of this work.

The areas of divergent sequence of the TuYV genome in the genes P1 and P5 as well as the non-coding region are due to apparent deletions, divergent evolution and recombination (Figure 3.9). *Polerovirus* populations are known to be far more diverse than the closely related Luteoviruses, contributing possibly to their wider host range (Herrbach *et al.*, 2001; Pagán and Holmes, 2010). The recombinant nature of the TuYV genome along with the error prone nature of its RdRp seems to have given rise to distinct genetic areas between TuYV isolates.

3.4.3 Phylogenetic Relationships of TuYV

The genetic variability of TuYV populations infecting OSR of 13 counties in the UK as well as 5 countries in mainland Europe were analysed using the whole coding genome sequences, which encoded seven viral genes. The results revealed that European TuYV isolates can be divided into two distinct genetic groups based on amino acid sequences of P0, P1, P2, P3a, P3 and whole genome nucleotide sequences irrespective of the geographical origin, or year of sampling. However, P4 amino acids could differentiate isolates from European and Chinese origin. P5 defined three European genetic groups, these were designated the common and uncommon genetic groups that had a mix of origins but it also included a "weed-like" group originating mostly from weed plant species which consisted of three weed and one TuYV isolate originating from OSR. This "weed-like" group was also identified in the nucleotide based trees of P1, P3, and P4 (Figures 3.13, 3.19 and 3.21). The P5 maximum likelihood tree was also able to distinguish BrYV (along with isolates originating from China) and an out-group including TuYV-FL. Previous studies showed variation in the TuYV isolates from OSR and other brassica plant samples collected from England, France and Germany, these studies showed variation within P0 and P3 but without biological inference from groups detected (Miranda *et al.*, 1995; Schubert *et al.*, 1998; Hauser *et al.*, 2000; Asare-Bediako, 2011). The common genetic group comprises 79.1% of European isolates and the uncommon group comprises 20.8% of European isolates. The P5 Gene discriminated a third group making up 3.7% of total European isolates derived from the uncommon group, reducing it down to 11% of the total number of TuYV isolates sequenced (this is also after removal of recombinant isolates). This very low level of incidence of the "weed-like" group might be due to these isolates not being particularly well adapted to OSR, which does not thrive or is out-competed by other more pathogenic isolates (Viganó and Stevens, 2007). This phenomenon has been referred to as cross-protection (Gal-On and Shiboleth, 2006). It could also be due to less efficient transmission by aphids (Power, 1996) as P5 has been associated with aphid uptake (Brault *et al.*, 2005), TuYV restriction to the phloem (Peter *et al.*, 2009), and recently long-distance movement as well as viral accumulation (Rodriguez-Medina *et al.*, 2015). Weed isolates sequenced from around the UK were also located in both of the other two clades (common and uncommon), indicating some are able to infect OSR, and that weed species are an active host and reservoirs of TuYV (See Chapter 4).

3.4.4 Recombination and Evolution of TuYV

Recombination can have a significant effect in driving the evolution of virus populations (García-Arenal *et al.*, 2003) and generating genome diversity (Gibbs *et al.*, 2010). The results of this analysis show two very strongly supported points of recombination, suggesting that recombination plays a significant role in driving evolution and survival of TuYV populations. Out of the 179 full genomes sequenced, 89 had recombination points within their genome. These two major potential recombination points locate to 3488nt (P3a and P3) and 4823nt (P5) within the genome, which correlates with similar locations to those reported in BrYV, 3531nt and 4819nt (Lim *et al.*, 2014). The recombinations detected were between the two main European genotypes of TuYV common and uncommon, recombination happens in both directions.

The TuYV recombinant genotypes were detected from UK and mainland Europe OSR samples as well as in UK weed isolates; this was the result of recombination between two distinct genotypes: the common (Clade 1) and uncommon (Clade 2) (See Figure 3.10 - 3.18). The significant phylogenetic incongruence observed between genes of TuYV and its alleviation once recombinants were removed in this study lends support to the idea that recombination plays a role in the evolution of the virus. The detection of only one recombination break point within both coat protein genes could be due to the strong structural selection pressure acting on these genes (Mayo and Ziegler-Graff, 1996).

Other studies have suggested P0 was the most divergent gene between polerovirus species (Herrbach *et al.*, 2001), with amino acid identity as low as 24.7% when comparing sequenced European TuYV isolates with BMYV. However, in this study P5 was also found to be highly variable within the sequenced TuYV species and between polerovirus species with amino acid identity as low as 58% within sequenced TuYV isolates and 53.1% when compared to BrYV (Table 3.4). However, neutrality tests showed that P5 might not be under neutral mutation rates and the diverse populations found in the phylogenetic analysis might be fixed as P5 is a minor component of the coat protein so could be functionally constrained (Table 3.7). When each of the three European P5 genotypes percentage identity were compared, each sub-population of TuYV had >90% homology in both nucleotide and amino acid sequences, but <90% identity between genotypes and as low as 60.2% for amino acids between genotype 1 and 2 (common and uncommon clades). This indicates that based on P5 there are

three set genetic groups of TuYV in Europe, a common and uncommon which have a mixed host origin (OSR or weed host) and location origins (from anywhere in Europe) and a "weed-like" population that was found in weeds at two locations in Suffolk (UK) and one OSR sample from Cornwall (UK) S-Cw330. This was further supported by population analysis (Figure 3.24).

The major coat protein is highly conserved in poleroviruses (Hauser *et al.*, 2000) including TuYV, which suggests strong functional constraints (Gray and Gildow, 2003) and as such it was expected to have a lower rate of evolution than other more variable genes (Pagán and Holmes, 2010). TuYV and other species of the family *Luteoviridae* had 90.6-100% amino acid identity when compared to the P3 of sequenced isolates, with the lowest being 89.6% with BMYV, which has very low homology for this very conserved region (Table 3.4). This is similar with the gene within the P3 sequence, P4, which had very limited codon selection with zero codons under positive selection, with only 8 negatively selected codons unlike P3's 14 codons. There is very strong selection pressure upon these genes, limiting variation and selection within them (Tables 3.5 and 3.6). The gene that was interesting to see very high positive and negative selection pressure in was P1 with 17 positively selected positions and 157 negatively selected codon positions (Table 3.6), as it is part of the RdRp, which is thought of as a essential gene which should confer a very strong selection pressure. This gene is also under neutral evolution (Table 3.7) therefore it is not under as strong selection pressure as P3 and P4. P1 also had a large number of nucleotide conflicts within its sequence (Figure 3.9 A) which could be attributed to clade 1 (common) and clade 2 (uncommon) having very divergent sequences in this area, which introduced gaps into the alignments made between isolates (Figure 3.9 A). Even with this highly divergent region only the two clades previously discussed were detected (common and uncommon genotypes). The recently defined gene P3a (Smirnova *et al.*, 2015) was under selective pressure as was not undergoing neutral evolution (Table 3.7), with limited codon selection 1 positive and 10 negative codon sites (Table 3.6). P3a had genetically distinct groups with very low homology between the two clades (Table 3.4 and Figure 3.16). As such, P3a is does not offer much more insight into the biological background of the European isolates due to its short length and limited gene diversity compared to previously identified genes. From this study a new resouces which can be used is the sequence divergence, which could be used to design primers distinguishing between the genotypes, located in P1, the non-coding region and P5 (Figure 3.9).

3.4.5 Mixed TuYV Infection

There are several reports of high frequencies of mixed genotype infections in many host-pathogen interactions (García-Arenal *et al.*, 2001; Hodgson *et al.*, 2004), that can cause more serious diseases, but characterisation of single genotype infections does not predict the most competitive isolate in mixed infections. This study as well as others, demonstrated the low frequencies of mixed genotypes of polerviruses within a single host (Ahmad *et al.*, 2006; Knierim *et al.*, 2013). Only 11 samples containing discernible mixed infections in this study and of those only 2 samples had genetically diverse isolates within them separating into different clades and being recombinants (See Section 3.4.5). Mixed infected samples were from several locations within the UK and mainland Europe as well as one from a Chinese sourced sample, demonstrating mixed infection is not geographically limited, as expected due to knowledge of BrYV recombination potential (Lim *et al.*, 2014). Mixing of genotypes in a single host are important to virus evolution as demonstrated with FRA381, as it facilitates an environment for recombination, which may contribute to the appearance of more severe, or fitter virus strains (Kasschau and Carrington, 1998; Monci *et al.*, 2002). The mutation and recombination events in the P3 and P5 regions of the TuYV genome do not seem to be associated with the observed mixed genotype infections in this study, as the conserved nature of the P3 region does not introduce much variation into P3 or P3a, but instead later downstream from the recombination (in P5). In the P5 recombination site there was considerable variation between the genotypes found (See Table 3.9) and this might be responsible for increased viral fitness; the high proportion of recombinants in this region suggest this. This could possibly mean that changing agricultural and reservoir plant populations, such as those with quantitative resistance to TuYV could cause the viral emergence of a less commonly found genotype such as the "weed-like" group by either recombination due to mixed infection or the out competing of other genotypes in the new host landscapes (Elena *et al.*, 2011). This highlights the need to fully assess recombinant pathogenicity, especially as recombination seems to be prevalent in TuYV populations.

3.4.6 Population Diversity

The relatively higher haplotype frequency observed in P0, P1 , P2 and P5 (Table 3.5) could be due to infections of the OSR in the region with TuYV isolates transmitted from several host plants by *M. persicae* as well as other

vectors. This large amount of infected material and the rapidly evolving nature of those genes increases the likelihood of more distinct haplotypes and further subdivision of TuYV (Tables 3.4 and 3.7). Other wild hosts and brassica crops within the vicinity of OSR crops could introduce extra variation into the TuYV population due to the different host environments where TuYV might make adaptive changes (Hauser *et al.*, 2000). The large source of varied host plants each harbouring TuYV, which is undergoing gradual mutations, means TuYV has a very high proportion of haplotypes, as many genes can undergo neutral evolution which do not prove to be fatal (Table 3.6). Virus evolution through quasi-species populations within a single, which is on the edge of self destructive and beneficial mutation (Eigen *et al.*, 1988; Andino and Domingo, 2015).

European TuYV seems highly divergent from the TuYV-FL and according to genetic analysis (Figures 3.10, 3.22 and 3.25). This is grounds to recategorise the species as with other poleroviruses before for BChV which had below 90% amino acid similarity to TuYV in two genes (Hauser *et al.*, 2002). The scarcity of TuYV-FL like isolates in brassicas in Europe and China suggests that TuYV-FL is not a representative of *Brassica*-infecting TuYV isolates and hence based on its origin (lettuce) and the original and recent descriptions of TuYV should be classified as a different species. The Australian isolate WA-1 was also genetically distinct from European strains, often falling in the out-group along with TuYV-FL .Due to geographical isolation Australian strains of TuYV might be substantially different in biological activity as well such as host range and pathogenicity, which warrants further investigation in light of the phylogenetic groups these isolates fall into. There is also the need to assess whether the three TuYV genotypes detected in the UK and two in mainland Europe might also need further reclassification due to low amino acid and nucleotide identity between many of TuYV genes, which could cause differences in pathogenicity, phenotype or host range. This should be further investigated when representatives of each genotype biological activity can be studied, along with an expanded study of the *Luteoviridae* family over more plant families and geographical locations.

Chapter 4

Investigation into TuYV Host Range

4.1 Background

TuYV is one of the main factors reducing yields in OSR, ∼15% on average in the UK (Nicholls, 2013), but it can also inflict losses on other brassica crops such as Brussels sprouts by up to 65% (Walsh, 2012). The other crop that TuYV has been implicated in yield reductions is lettuce by up to 40% (Walkey and Pink, 1990). Brassicas and lettuce crops are the only economically important crops that TuYV is known to affect. However, the host range of TuYV has been reported to include many species of both crops and weeds (Table 4.1). The epidemiology of TuYV so far reported could be problematic for correctly identifying host species as some results could relate to the host range of the American polerovirus BWYV, rather than European TuYV; some host range studies have been performed with BWYV isolates from the USA to understand the host range (Duffus and Russel, 1970; Beuve *et al.*, 2008). This is complicated further, as many studies investigating wild hosts use antisera techniques alone, which can have cross-reactivity with other virus species, possibly causing misrepresentation of the TuYV host range (Jaegle and Van Regenmortel, 1985).

This wide host range of TuYV is aided by the the large range of aphids that can transmit the virus (See Section 1.4), as well as the extensive host range exhibited by its main vector *M. persicae*. Many other aphid species do not usually exploit oilseed rape as a host under natural conditions and are often not seen as important vectors, yet they could contribute to an increased reservoir

of the virus in other plant species. *M. persicae* is considered the main vector due to its abundant numbers, large host range and >90% transmission efficiency (Stevens *et al.*, 1995). The *Luteoviridae* are not seed-borne and need to be constantly maintained in live plants or aphids, making the wide host range a necessity (Brault *et al.*, 2011).

As OSR is not present throughout the year and TuYV needs to be in constant circulation, a pathosystem of varied hosts maintaining the viral reservoir is needed for the survival of the virus (Robinson, 1976). This epidemiology needs to be understood so control methods can be improved utilising the increased knowledge of sources of infection that has been gained (Tomlinson, 1987). Control of wild host plants would offer one possible control of sources of infection. The other benefit of studying virus epidemiology is the possible discovery of some sources of resistance within wild populations (Duffus, 1971).

The aim of the work described in this chapter was to determine the host range of three TuYV isolates representing distinct genetic groups of UK *Brassica* isolates (See Chapter 3), as well as investigating the natural host range of UK TuYV and its implication for OSR.

Table 4.1 – Reported Plant Host Range of TuYV.

Latin Name	Common Name	Latin Name	Common Name
Asteraceae		*Cicer arietinum*	Chickpea
Chrysanthemum segetum	Corn marigold	*Lupinus albus*	Lupin
Conzya	Fleabane	*Pisum sativum*	Pea
Lactuca sativa	Lettuce	*Vicia faba*	Broad bean
Matricaria perforata	Mayweed	*Lupinus luteus*	Yellow lupin
Brassicaceae		*Ornithopus sativus*	Pink serradella
Alliaria petiolata	Garlic mustard	*Trifotium dubium*	Suckling clover
Arabidopsis thaliana	Thale cress	*Trifotium repens*	White clover
Brassica carinata	Abyssinian cabbage	*Trifolium resupinatum*	Persian clover
Brassica juncea	Indian mustard	***Fumarianceae***	
Brassica napus	Rapeseed	*Fumaria officinalis*	Common fumitory
Brassica oleracea	Cabbage	***Hydrophyllaceae***	
Brassica rapa	Turnip	*Phacelia tanacetifolia*	Scorpion weed
Brassica nigra	Black mustard	***Lamiaceae***	
Capsella bursa-pastoris	Shepherds purse	*Lamium amplexicaule*	Henbit deadnettle
Camelina sativa	False flax	*Lamium purpureum*	Purple deadnettle
Coronopus didymus	Swine cress	***Papaveraceae***	
Lepidium sativum	Cress	*Papaver rhoeas*	Corn poppy
Lepidium campestre	Pepperweed	***Plantaginaceae***	
Lunaria annua	Honesty	*Plantago major*	Greater plantain
Raphanus raphanistrum	Wild radish	***Polemoniaceae***	
Raphanus sativus	Radish	*Navarretia squarrosa*	Stinkweed
Sinapis alba	White mustard	***Portulaceae***	
Sinapis arvensis	Wild mustard	*Montia perfoliata*	Miner's lettuce
Thlaspi arvense	Fanweed	***Polygonaceae***	
Caryophyllaceae		*polygonum convolvulus*	Bind weed
Stellaria media	Common chickweed	***Primulaceae***	
Spergula arvensis	Corn spurry	*Anagallis arvensis*	Scarlet pimpernel
Chenopodiaceae		***Scrophulariaceae***	
Spinacea oleracea	Spinach	*Veronica arvensis*	Corn speedwell
Compositae		*Veronica persica*	Common field speedwell
Anthemis cotula	Chamomile	***Solanaceae***	
Senecio vulgaris	Groundsel	*Nicotiana benthamiana*	Tobacco
Sonchus oleraceus	Sow thistles	*Physalis pubescens*	Hairy nightshade
Taraxacum officinale	Dandelion	*Solanum nigrum*	Blackberry nightshade
Zinnia peruviana	Peruvian zinnia	***Urticaceae***	
Cucurbitaceae		*Urtica urens*	Annual nettle
Citrullus lanatus	Melon	***Valerianaceae***	
Cueurbitaeeae		*Valerianella locusta*	Lewiston cornsalad
Bryonia dioica	Red bryony	***Violaceae***	
Fabaceae		*Viola arvensis*	Field pansy

Compiled from: (Duffus and Russel, 1970; Walkey and Pink, 1990; Stevens *et al.*, 1994; Graichen *et al.*, 1996; Thurston *et al.*, 2001; Pallett *et al.*, 2002; Coutts *et al.*, 2006)

4.2 Materials and Methods

4.2.1 PCR Primers for infection detection

For molecular testing and sequencing of TuYV in this chapter primers listed in Table 4.2 were used with conditions outlined in Section 2.5. Crop primers were used to detect TuYV laboratory strains coat protein for molecular detection. Where as the weed primers were used on an area of genetic variability (P5) for confirmation of results to ensure no cross contamination.

Table 4.2 – Details of Primers for TuYV Detection and Sequencing.

Plant	Primer Name	Sequence (5′-3′)	Orientation	Target Region Gene
Crop	MN37	GGACAACTGGAATTCTGCTCTC	Forward	3040-3062nt (ORF3)
	MN42b	GRACCAGCTATCGATGAAGAACC	Reverse	4027-4049nt (ORF3)
Weed	MN66	ATCGTTTTATCGTCTATACTGGAGTC	Forward	4200-4225nt (ORF5)
	MN48	GTTTAATGTCTCTGGCTTGACTTTAT	Reverse	5569-5595nt (ORF5)

4.2.2 Crop Plants

The crops used in this host range study included: Carrot (cultivar Red Cored Chantenay), Field Bean (cultivar Fuego), Iceberg Lettuce (cultivar Lakeland), Sugar Beet (cultivar Master) and Potato (cultivar Charlotte). Lettuce and carrot seeds were sourced from Tozer Seeds. Field bean and sugar beet seed were provided by Dr. Simon Berry (Limagrain UK Ltd). Potato tubers were acquired from Co-operative Food. For TuYV transmission see Section 2.3.2. Crop lines were challenged with each representative of the three genetic groups of TuYV, L1851-C, LAB-I, Cau74-R, in separate experiments due to space limitations and to avoid co-infection of more than one isolate.

4.2.3 Weed Species

Weed species were selected from both the literature and results from ELISA positive weed samples in Chapter 3. Each species, if possible, had several different lines selected to take into account of the natural genetic diversity of each weed (Table 4.3). Weeds lines were challenged with each representative of the three genetic groups of TuYV, L1851-C, LAB-I, Cau74-R, in separate experiments due to space limitations and to avoid co-infection of more than one isolate.

Table 4.3 – Weed Species Selected for Host Range Study from Kew Seed Bank.

Serial No	Family	Species	Common Name	Location origin	Year of collection
49720	*Polygonaceae*	*Rumex crispus*	Dock	Suffolk	1983
36669	*Polygonaceae*	*Rumex crispus*	Dock	East Sussex	1977
480170	*Polygonaceae*	*Rumex crispus*	Dock	West Sussex	2008
53121	*Apiaceae*	*Anthriscus sylvestris*	Cow Parsley	Suffolk	1984
49616	*Apiaceae*	*Anthriscus sylvestris*	Cow Parsley	Wiltshire	1983
39420	*Apiaceae*	*Anthriscus sylvestris*	Cow Parsley	Essex	1982
39213	*Resedaceae*	*Reseda luteola*	Weld	Suffolk	1982
78173	*Resedaceae*	*Reseda luteola*	Weld	Hertfordshire	1989
131209	*Resedaceae*	*Reseda luteola*	Weld	Wiltshire	1998
120692	*Geraniaceae*	*Geranium dissectum*	Wild Geranium	Cornwall	1997
671596	*Geraniaceae*	*Geranium molle*	Wild Geranium	Norfolk	2012
134819	*Geraniaceae*	*Geranium pyrenaicum*	Wild Geranium	Avon	1999
79284	*Brassicaceae*	*Lepidium didymum*	Swine Cress	Greater London	1989
533652	*Brassicaceae*	*Lepidium densiflorum*	Common Pepperweed	Kansas (USA)	2009
126373	*Compositae*	*Taraxacum officinale*	Dandelion	West Sussex	1998
615071	*Compositae*	*Taraxacum officinale*	Dandelion	South Glamorgan	2011
126535	*Compositae*	*Taraxacum officinale*	Dandelion	Avon	1998
63362	*Compositae*	*Sonchus arvensis*	Sow Thistle	Suffolk	1986
142115	*Rosaceae*	*Rubus cotteswoldensis*	Bramble	Gloucestershire	1999
59226	*Rurticaceae*	*Urtica dioica*	Nettle	West Sussex	1985
64794	*Caprifoliaceae*	*Dipsacus fullonum*	Teasel	Greater London	1986
678274	*Caprifoliaceae*	*Dipsacus fullonum*	Teasel	Norfolk	2012
130741	*Caprifoliaceae*	*Dipsacus pilosus*	Teasel	Oxfordshire	1998
400448	*Asteraceae*	*Cirsium vulgare*	Spear Thistle	East Sussex	2007
59318	*Asteraceae*	*Cirsium vulgare*	Spear Thistle	Shropshire	1985
1231321	*Asteraceae*	*Cirsium vulgare*	Spear Thistle	Avon	1997

4.2.4 Experimental Design

All weed and crops lines were sown in excess numbers then thinned out to try to allow the correct number of germinated plants with a target of 5 plants per line, four of which were to be TuYV challenged. The plant lines (Section 4.2.2 and Table 4.3) were inoculated with the three representative isolates (L1851-C, LAB-I, Cau74-R) independent from each other to avoid co-infection. Aphids were allowed to feed for one week, with each individual isolate challenged group being contained within the same insect proof cage to aid aphid movement and feeding on all plants. Negative controls, due to limited insect confinement space available, were not feed on by non-virerliferous aphids or sprayed with insecticide.

4.3 Results

4.3.1 Wild TuYV Weed Host Range

Sampling of the field sites in the UK outlined in Chapter 3 included the collection of any possible weed hosts (existing hosts outlined in Table 4.1). Sampling also included many herbaceous weed species not yet reported as host of TuYV: weld, cleavers, bramble, ribwort plantain, wild geranium, dock, spear thistle, verbascum, teasel, cow parsley, corn mint and ochre. This was to assess the wild host range of TuYV that exists around OSR field locations, to investigate the epidemiology of the virus.

A variety of weeds species that had positive ELISA results from UK sampling were taken forward to investigate if they could act as a reservoir for isolates that infect OSR (Table 4.4). This type of study for wild sources of TuYV has not been undertaken before. Several reported weed hosts of TuYV collected had no ELISA positive samples. These species were: annual nettle, dandelion, common fumitory, corn poppy, greater plantain, miner's lettuce and bind weed.

The ELISA results clearly showed that TuYV isolates infect a variety of weed species, many of these TuYV isolates are also capable going on to infect OSR as well (Table 4.4). Negative results are less clear in that aphids could have "found" weed leaf samples unpalatable due to the age or condition at time of feeding, rather than isolate host range limitations. Five *Brassicacae*, three *Asteraceae*, two *Compositae* and two *Lamiaceae* species were found to be hosts at several locations in the UK. New host species were also identified during this work including dock (*Polygonaceae*), verbascum (*Scrophulariaceae*), teasel (*Caprifoliaceae*), cow parsley (*Apiaceae*), weld (*Resedaceae*), spear thistle (*Asteraceae*), wild geranium (*Geraniaceae*). This adds four new plant families to the host range of TuYV, *Apiaceae* and *Caprifoliaceae*, *Resedaceae*, *Geraniaceae*, from three of which it was possible for aphids to transmit TuYV to OSR (Table 4.4).

Table 4.4 – TuYV-Infected Weed Species from which it was Possible to Transmit TuYV to Oilseed Rape, Indicated by ELISA Results.

Plant Number	Sample Location	Weed Origin	Transmission to [a] Oilseed Rape
51	Suffolk	Shepherds Purse	+
52	Suffolk	Groundsel	+
53	Suffolk	Shepherds Purse	-
62	Suffolk	Weld	-
121	Suffolk	Shepherds Purse	+
127	Suffolk	Garlic Mustard	-
261	Cheshire	Common Chickweed	+
412	Cheshire	Shepherds Purse	+
547	Kent	Ribwort Plantain	-
548	Kent	Purple Deadnettle	-
553	Kent	Ribwort Plantain	+
559	Kent	Ribwort Plantain	+
560	Kent	Winter Speedwell	-
616	Kent	Mayweed	-
626	Kent	Cleavers	-
683	Cornwall	Field Pansy	-
684	Cornwall	Winter Speedwell	+
694	Cornwall	Shepherds Purse	-
695	Cornwall	Shepherds Purse	+
1035	Warwickshire	Common Pepper Weed	+
1039	Warwickshire	Spear Thistle	+
1102	Angus	Charlock	-
1119	Angus	Dock	-
1247	Angus	Winter speedwell	+
1251	Angus	Wild Geranium	-
1316	Stirlingshire	Dock	+
1381	Stirlingshire	Common Chickweed	+
1390	Stirlingshire	Dock	+
1457	Stirlinghsire	Wild Geranium	-
1458	Stirlingshire	Common Chickweed	-
1467	Stirlingshire	Spear Thistle	-

[a] + = Successful Transmission Determined by ELISA Detection, - =Unsuccessful Transmission determined by lack of ELISA Detection

Plant Number	Sample Location	Weed Origin	Transmission to [a] Oilseed Rape
1522	Warwickshire	Wild Geranium	+
1525	Warwickshire	Mayweed	+
1532	Warwickshire	Charlock	+
1534	Warwickshire	Winter speedwell	-
1538	Warwickshire	Leafy spurge	-
1601	Warwickshire	Henbit Deadnettle	+
1672	Warwickshire	Swine Cress	-
1678	Warwickshire	Dock	-
1808	Wigtownshire	Hedge Mustard	-
1881	Wigtownshire	Common Chickweed	+
1889	Wigtownshire	Shepherds Purse	-
1941	Northumberland	Groundsel	-
1953	Northumberland	Cow parsley	-
1954	Northumberland	Teasel	+
2020	Northumberland	Hairy Bitter Cress	-
2027	Northumberland	Wild Geranium	-
2029	Northumberland	Dock	+
2091	Northumberland	Cow Parsley	+
2100	Northumberland	Bramble	-
2236	Lincolnshire	Smooth Sow Thistle	+
2304	Lincolnshire	Teasle	-
2306	Lincolnshire	Groundsel	-
2369	Ceredigion	Fleabane	+
2441	Ceredigion	Field Pansy	+
2505	Ceredigion	Swine Cress	+
2573	Somerset	Swine cress	+
2649	Somerset	Dock	-
2719	Somerset	Charlock	+
2782	Yorkshire	Verbascum	+
2798	Yorkshire	Hairy Bitter Cress	+
2856	Yorkshire	Suckling Clover	+

[a] + = Successful Transmission Determined by ELISA Detection, - =Unsuccessful Transmission determined by lack of ELISA Detection

To confirm that the OSR had been infected by TuYV originating in the weed species as indicated by ELISA, RNA was extracted then underwent PCR (Primers MN66 and MN49) and sequencing to confirm it was not the result of cross-contamination from other maintained isolates. The P5 region was very variable between weed sequences so ideal for confirmation of sequence homology between isolates. Sequence analysis of 12 weed isolates that were amplified and sequenced confirmed the known hosts: shepherd's purse, groundsel, common chickweed, fleabane, field pansy, swine cress, charlock, suckling clover (Figure 4.1). These results also included the newly identified host verbuscum. The lack of weed isolates that could be successfully amplified and sequenced with primers designed based on knowledge gathered in Chapter 3, may be hitherto unrecognised genetic diversity, or closely related virus species. Out of the 32 isolates shown to be able to infect OSR, it was only possible to amplify and sequence 12 from the weed host and OSR, these were limited to four UK regions, Suffolk, Cerdigion, Somerset and Yorkshire.

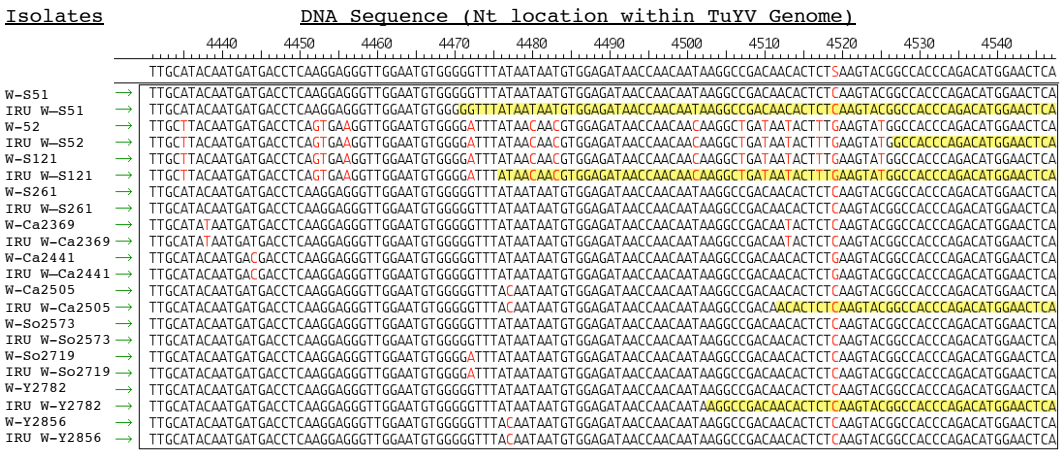

Figure 4.1 – P5 Sequence Confirmation of Weed TuYV Isolates Capable of Infecting OSR.
Sequences of each weed isolate aligned with sequence obtained from OSR inoculated with aphids after feeding on each corresponding infected weed (denoted by IRU).

4.3.2 The Weed Host Range of TuYV Crop Isolates

Section 4.3.1 provides information on the host range of TuYV in wild species and also the ability for those species to act as reservoirs. Due to the inability to amplify and sequence many of isolates infecting new weed hosts, more work was needed to investigate and confirm new and existing hosts. Section 4.3.2 investigated the ability of TuYV isolates representing the three previously

described genetic groups (L1851-C, LAB-I, Cau74-R), to infect weeds grown under controlled conditions. This experiment investigated the host range of crop-derived TuYV isolates, representing three phylogenetic groups, as well as attempting to confirm new weed hosts indicated by ELISA detection of TuYV in weeds from the field following ELISA and/or RT-PCR (Section 4.3.1).

Seed of many of the weed species of interest described in this chapter was obtained from the Kew seed bank (See Section 4.2.3). During the weed germination and growth for this study there was a growth cabinet failure causing some lines not to germinate or die, this accounts for the lack of target of 4 plants tested for each of the lines ordered. Each weed line was separately inoculated with all three TuYV genetic groups where possible (Table 4.6).

Dock (*Rumex crispus*) was ELISA positive for all three isolates, but there was variation between dock lines, with 49720 and 480170 only being positive for Cau74-R (Section 4.2.3). Weld (*Reseda luteola*) lines were positive for L1851-C and Cau74-R but there was no sign of infection by the LAB-I isolate (See Section 2.4.1). Geranium (*Geranium dissectum, G. molle, G. pyrenaicum*) was ELISA positive for all three TuYV isolates, except line 134819, for which L1851-C was not detected. The documented host species *Lepidiums* (Graichen *et al.*, 1996) plants were ELISA positive except for Cau74-R which was unable to infect common pepperweed. Teasel (*Dipsacus fullonum*) was a host for the L1851-C and LAB-I but Cau74-R was not detected. The smooth sow thistle (*Sonchus arvensis*) was only tested against L1851-C and was ELISA negative. The spear thistle (*Cirsium vulgare*) line 59318 was only susceptible to Cau74-R, and the 400488 line was the only weed in this experiment that was not susceptible to any TuYV isolate. This work identifies species *Rumex*, *Reseda*, *Dipsacus* and *Cirsium* as susceptible hosts for TuYV for the first time. It also indicates that not only are there biological differences between TuYV isolates from the three genetic groups, but there is also variation in the susceptibility between different accessions of some weed species.

Aphid transmission of TuYV gave inconsistent results, certain susceptible lines did not show uniform ELISA results (Table 4.6). Some dock, weld, geranium, lepidium and dipsacus plants gave positive ELISA results, whereas other individuals gave negative ELISA results. Utilising aphids to infect plants can results in false negatives, however it is the only method of transmitting TuYV and it was not possible to use clip cages as the leaves of the weeds plants

were too small to attach them to.

Symptom severity within the weed lines were muted, as previously reported for TuYV. Muted symptoms included yellowing and reddening of outer leaves. Unchallenged healthy control, infected and non-infected dock plants exhibited leaf yellowing and sometimes stunting with no correlation with ELISA results (Figure 4.2). However dock line 36669 did have consistent yellowing for all plants tested (Figure 4.2); this line was susceptible to all three TuYV isolates (Table 4.6). Infected weld (*Reseda*) lines presented the consistent symptom of stunting (Table 4.6, Figure 4.5). All weld plants inoculated with LAB-I remained similar to healthy controls with no infection detected by ELISA (Figure 4.5). All geranium lines exhibited yellowing and reddening of their leaves regardless of what isolate was challenged with or the ELISA results. However, infected plants in geranium line 671596 did show considerable stunting when compared to the healthy controls (Figure 4.3). Lepidium plants presented the known TuYV post-infection phenotypes of stunting, leaf curling and yellowing (Graichen *et al.*, 1996) (Figure 4.5), however these symptoms were also observed in the ELISA negative common pepperweed challenged with Cau74-R (Figure 4.5). The Teasel plants infected with L1851-C had slight leaf yellowing but no other symptoms. LAB-I and Cau74-R infection produced no symptoms (Figure 4.4). Sow thistle plant showed no symptoms and appear less stunted and healthier than the control (Figure 4.4). The spear thistle line 59318 had yellowing symptoms in all plants except the healthy controls, however line 400448 only showed yellowing leaf symptoms in plants inoculated with Cau74-R, but were ELISA negative (Figure 4.4). Symptoms would be difficult to attribute directly with TuYV infection for any weed species investigated, as many of the symptoms were most likely due to stress caused by aphids and being pot bound. This is demonstrated further as infected teasel plants had no visible symptoms of TuYV.

Table 4.6 – Inoculation of Several Weed Species with Different TuYV Isolates.

Weed Species Information				Number of ELISA Positive Plants (Number of Plants Tested) by each virus isolate		
Serial No	Family	Species	Common Name	L1851-C[a]	LAB-I[b]	Cau74-R[c]
36669	*Polygonaceae*	*Rumex crispus*	Dock	3(4)	3(4)	1(4)
49720	*Polygonaceae*	*Rumex crispus*	Dock	0(4)	0(4)	2(3)
480170	*Polygonaceae*	*Rumex crispus*	Dock	0(1)	0(1)	2(4)
39213	*Resedaceae*	*Reseda luteola*	Weld	2(2)	0(1)	1(1)
78173	*Resedaceae*	*Reseda luteola*	Weld	1(1)	0(1)	1(4)
131209	*Resedaceae*	*Reseda luteola*	Weld	0(1)	N.t.[d]	0(1)
120692	*Geraniaceae*	*Geranium dissectum*	Wild Geranium	1(1)	2(2)	1(3)
134819	*Geraniaceae*	*Geranium pyrenaicum*	Wild Geranium	0(4)	1(4)	2(4)
671596	*Geraniaceae*	*Geranium molle*	Wild Geranium	1(2)	N.t.	1(1)
79284	*Brassocaceae*	*Lepidium didymum*	Swine Cress	1(1)	2(3)	1(1)
533652	*Brassocaceae*	*Lepidium densiflorum*	Common Pepperweed	1(1)	N.t.	0(1)
64794	*Caprifoliaceae*	*Dipsacus fullonum*	Teasel	1(1)	2(2)	0(4)
678274	*Caprifoliaceae*	*Dipsacus fullonum*	Teasel	4(4)	2(4)	0(4)
63362	*Compositae*	*Sonchus arvensis*	Sow Thistle	0(1)	N.t.	N.t.
59318	*Asteraceae*	*Cirsium vulgare*	Spear Thistle	0(1)	0(1)	4(4)
400448	*Asteraceae*	*Cirsium vulgare*	Spear Thistle	0(1)	0(1)	0(2)

[a]L1851-C isolate representative of the common phylogenetic group
[b]LAB-I isolate representative of the intermediate phylogenetic group
[c]Cau74-R isolate representative of the rare phylogenetic group
[d]N.t denotes lines not tested

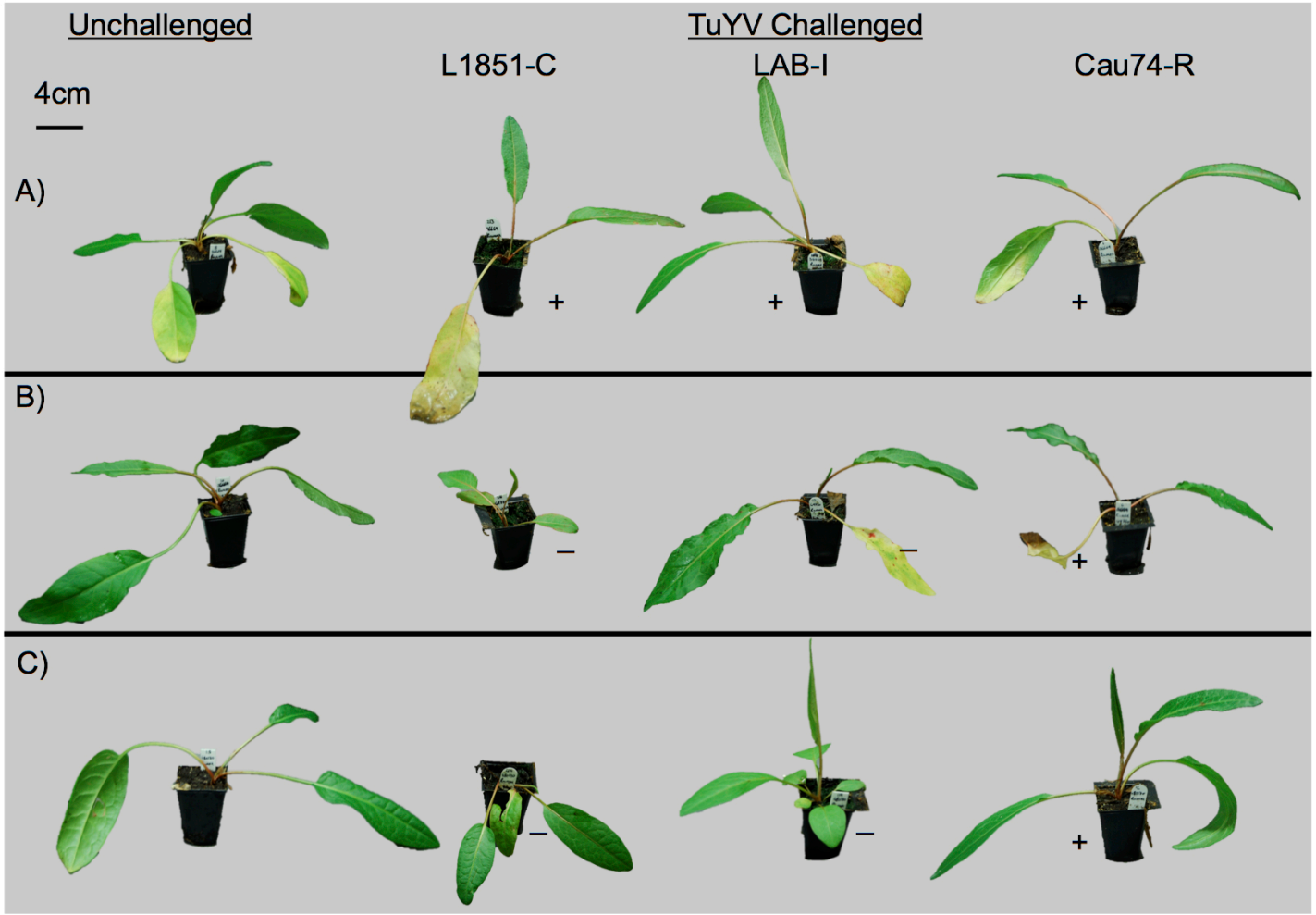

Figure 4.2 – *Rumex* Weed Host Range Phenotypes Following Challenge with Three TuYV Isolates.
Weed plants incoluated with L1851-C, LAB-I, Cau74-R representing the three phylogenetic groups of TuYV. Phenotypes images taken 30 days post inoculation (DPI) of challenged plants compared to healthy controls. Positive ELISA result indicated by +, Negative ELISA result indicated by -. N.t. lines not tested. A) *Rumex crispus* (36669). B) *Rumex crispus* (49720). C) *Rumex crispus* (9480170).

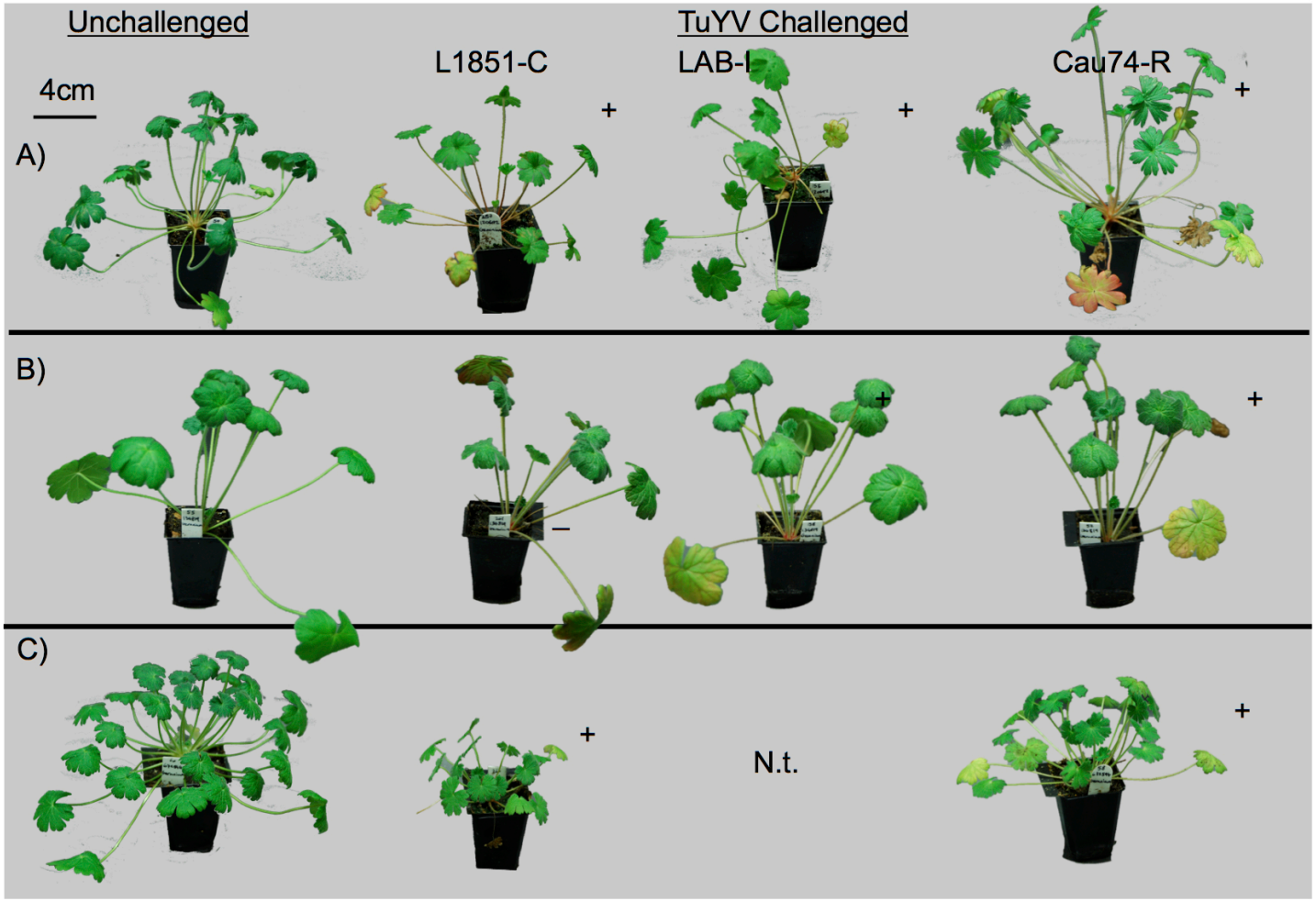

Figure 4.3 – *Geranium* Weed Host Range Phenotypes Following Challenge with Three TuYV Isolates.
Weed plants incoluated with L1851-C, LAB-I, and Cau74-R representing the three phylogenetic groups of TuYV. Phenotypes images taken 30 DPI of challenged plants compared to unchallenged controls. Positive ELISA result indicated by +, Negative ELISA result indicated by -. N.t. lines not tested. A) *Geranium dissectum* (120692). B) *Geranium pyrenaicum* (134819). C) *Geranium molle* (671596).

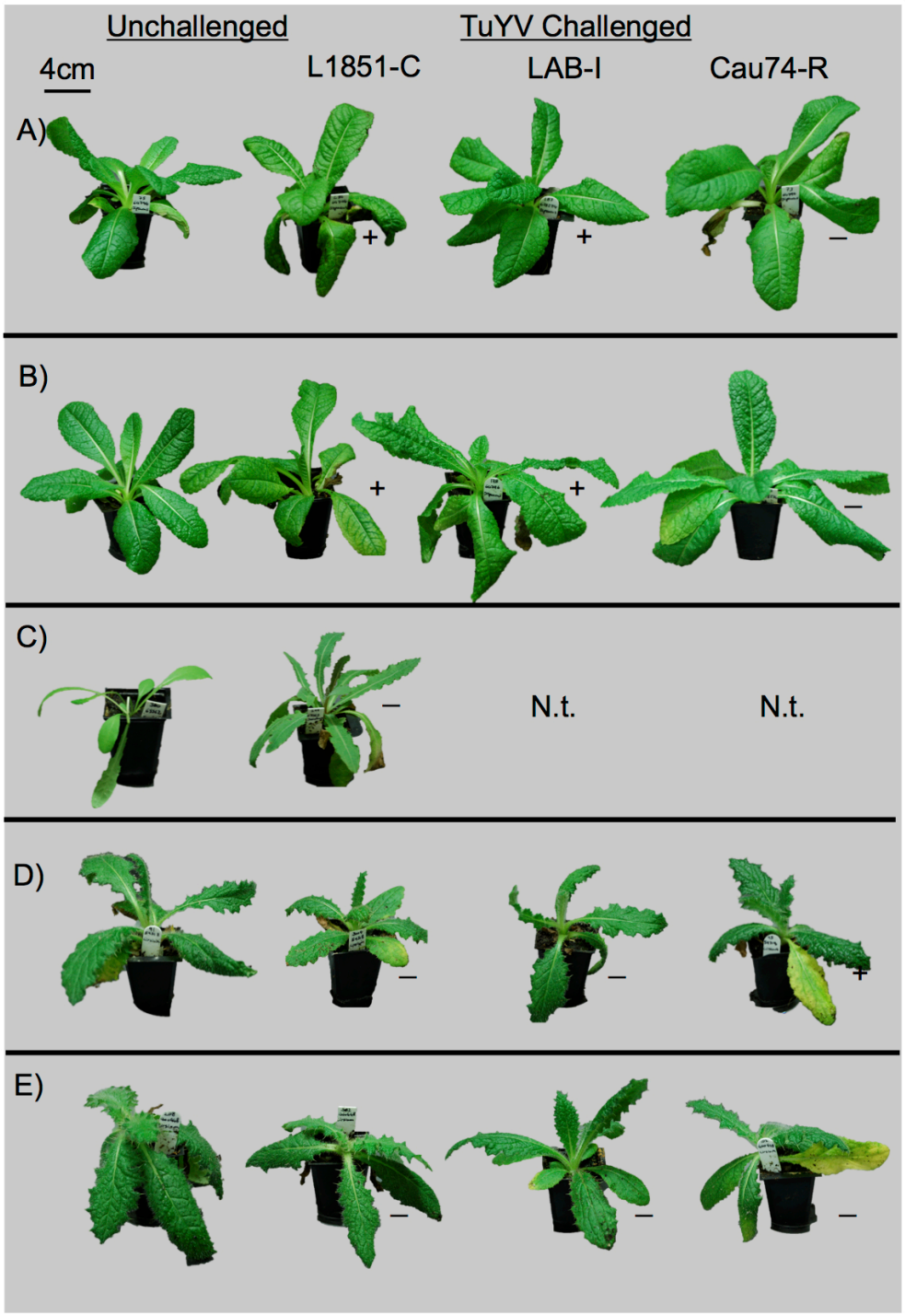

Figure 4.4 – *Dipsacus*, *Sonchus* and *Cirsium* Weed Host Range Phenotypes Following Challenge with Three TuYV Isolates.
Weed plants incoluated with L1851-C, LAB-I, and Cau74-R representing the three phylogenetic groups of TuYV. Phenotypes images taken 30 DPI of challenged plants compared to unchallenged controls. Positive ELISA result indicated by +, Negative ELISA result indicated by -. N.t. lines not tested. A) *Dipsacus fullonum* (64794). B) *Dipsacus fullonum* (678274). C) *Sonchus arvensis* (63362). D) *Cirsium vulgare* (59318). E) *Cirsium vulgare* (400448).

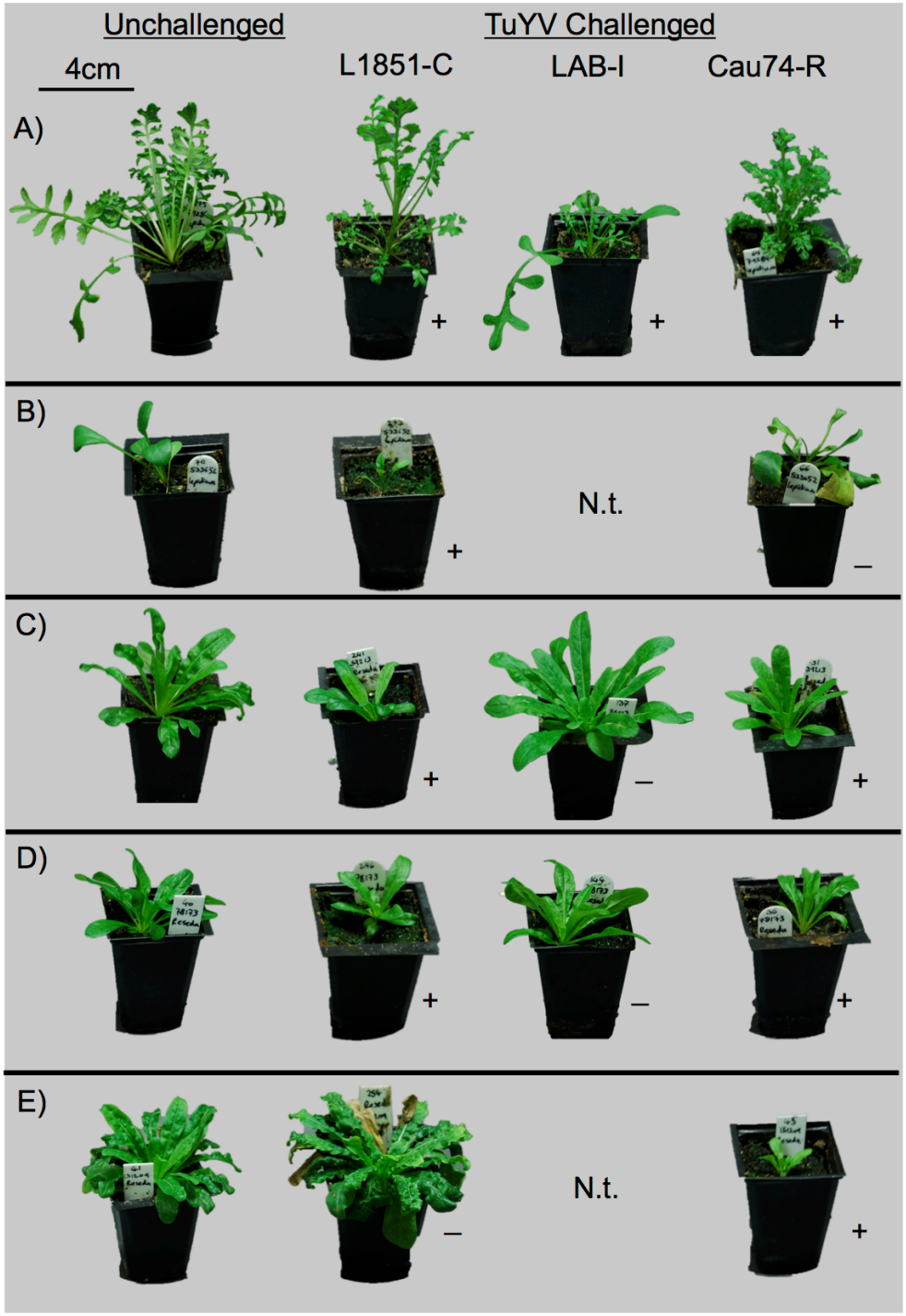

Figure 4.5 – *Lepidium* and *Reseda* Weed Host Range Phenotypes Following Challenge with Three TuYV Isolates.
Weed plants incoluated with L1851-C, LAB-I, and Cau74-R representing the three phylogenetic groups of TuYV. Phenotypes images taken 30 DPI of challenged plants compared to unchallenged controls. Positive ELISA result indicated by +, Negative ELISA result indicated by -. N.t. lines not tested. A) *Lepidium didymum* (79284). B) *Lepidium densiflorum* (533652). C) *Reseda luteola* (39213). D) *Reseda luteola* (78173). E) *Reseda luteola* (131209).

4.3.3 TuYV Crop Host Range

Further to the work investigating the epidemiology of TuYV in wild hosts, other crop species could be important sources of TuYV for OSR with their abundance and any proximity. This section describes several economically important crop plants that could be potential reservoirs for TuYV. This section also used the three genetic groups defined in Chapter 3 and Asare-Bediako (2011) to investigate any biological differences between the isolates, as well as investigating the host range of OSR-infecting TuYV isolates in other crops. This helped define the host range as some conclusions of the TuYV host range have previously been based on isolates from the USA which are likely to be BWYV.

TuYV was found to infect all crops tested, which were field bean (*Vicia faba)*, sugar beet (*Beta vulgaris*), lettuce (*Lactuca sativa*), carrot (*Daucus carota subsp. sativus*) and potato (*Solanum tuberosum*) (Table 4.7). However, there were differences in the host range between the three genetic groups of TuYV. The isolate L1851-C infected all crops except sugar beet consistent with previous research (Mayo, 2002) (Figure 4.6 A), LAB-I infected all but sugar beet and field bean (Figure 4.6 B), however Cau74-R was able to infect all crops (Figure 4.6 C). These crops are within plant families which are potential hosts of TuYV: *Fabaceae*, *Solanaceae*, *Asteraceae*, *Apiaceae* (previously shown in Section 4.3.1 as plant families TuYV could possibly infect). However, sugar beet belongs to the family *Amaranthaceae*, and is thought not to be a host; which is one of the biological factors defining TuYV as a species distinct from BMYV. The discovery of a UK *brassica*-infecting polerovirus isolate infecting sugar beet suggests the definition of these two species needs re-assessment.

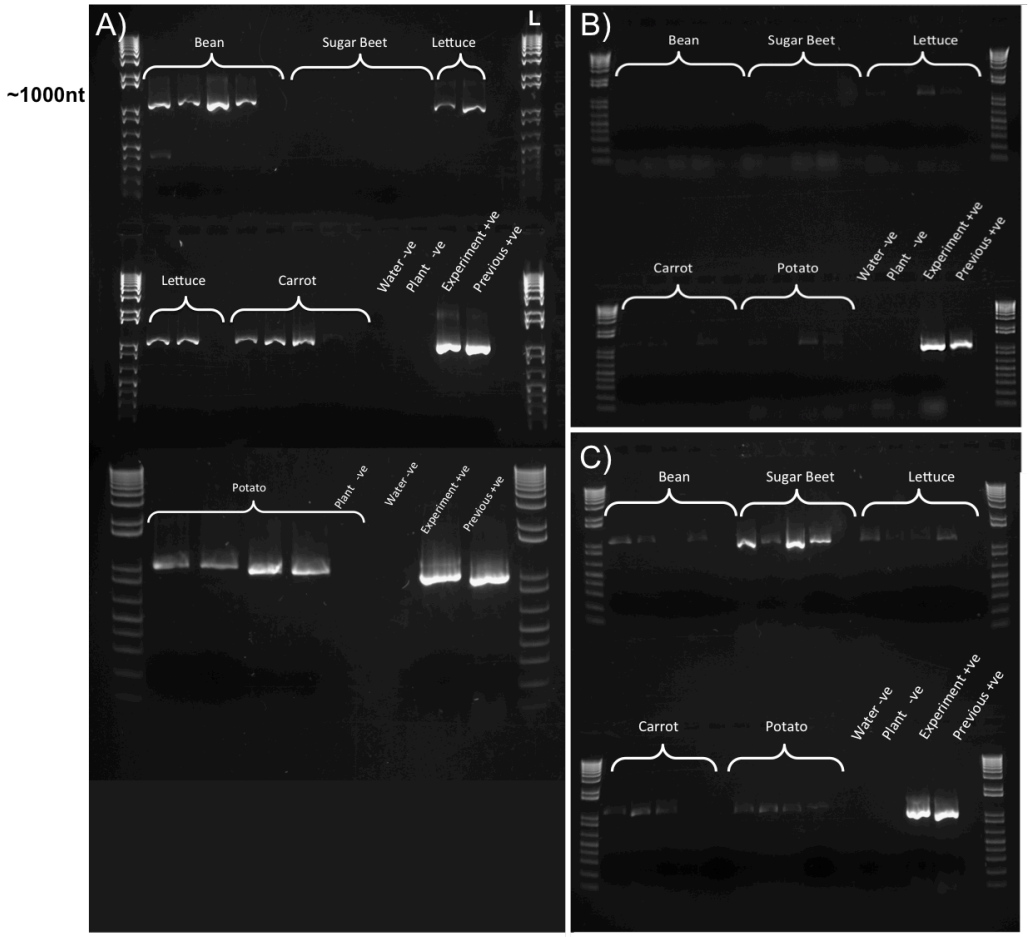

Figure 4.6 – Crop Host Range PCR Detection of Three TuYV Isolates. All plants were tested for TuYV with P3 specific primers, producing 1000bp product. This included 4 TuYV challenged plants per line and one unchallenged control for each; field bean, sugar beet, lettuce, carrot and potato. Challenged with TuYV: A) L1851-C isolate. B) LAB-I isolate. C) Cau74-R isolate. Against 1Kb+ Ladder (L).

The three isolates of TuYV produced different symptoms and phenotypes in the crop plants tested. In potato the Cau74-R induced very stunted plants. LAB-I infected plants had yellowing of the leaves; but this might be due to leaf senescence as the LAB-I group potatoes established faster. L1851-C did not produce visible symptoms post challenge (Figure 4.7 A). Infected lettuce plants had symptoms that would make them unmarketable as leaf curling and disrupted morphology of the leaves would be undesirable to the consumer. The L1851-C and LAB-I isolates produced stunted plants with some leaf curling (Figure 4.7 B). The lettuce infected with the Cau74-R were not as stunted but did have an unusual morphology and yellowing of the leaves. Infected field bean plants were visibly smaller than healthy controls, but LAB-I caused less stunting but did exhibit yellowing of the leaves (Figure 4.7 C). Field beans inoculated with Cau74-R also had rust-like

Table 4.7 – RT-PCR Results of Crop Species with Challenged with Different TuYV Isolates.

Crop Species Information			Number of ELISA Positive Plants (Number of Plants Tested) by each virus isolate		
Family	Species	Common Name	L1851-C[a]	LAB-I[b]	Cau74-R[c]
Fabaceae	*Vicia faba*	Field bean	4(4)	0(4)	3(4)
Amaranthaceae	*Beta vulgaris*	Sugar beet	0(4)	0(4)	4(4)
Asteraceae	*Lactuca sativa*	Lettuce	4(4)	3(4)	4(4)
Apiaceae	*Daucus carota subsp. sativus*	Carrot	4(4)	3(4)	3(4)
Solanaceae	*Solanum tuberosum*	Potato	4(4)	4(4)	4(4)

[a]L1851-C isolate representative of the common phylogenetic group
[b]LAB-I isolate representative of the intermediate phylogenetic group
[c]Cau74-R isolate representative of the rare phylogenetic group

symptoms (Figure 4.7 C). Only sugar beet plants infected with Cau74-R had the discernible symptoms of fewer leaves and stunted growth, consistent with PCR results (Figure 4.8 A). Carrot plants infected with each isolate showed less vigorous growth, with LAB-I isolate producing stunted plants, and carrot plants challenged with Cau74-R also had rust like symptoms (Figure 4.8 B). Generally the only symptom in carrots infected with TuYV is stunted growth. The one caveat of these symptoms is that they are also typical of abiotic stresses, aphid feeding and insecticide treatments the plants were subjected to. The control plants, due insect containment space restraints, did not have non-viruliferous aphids feeding on them, or pesticide treatments. Future experiments that are investigating the phenotype more thoroughly rather than purely host range would have a more robust testing system. This would include more reps for each TuYV isolate treatment with each plant and more controlled environment with controls which undergo the same care regime involving non-viruliferous aphids and pesticide treatments.

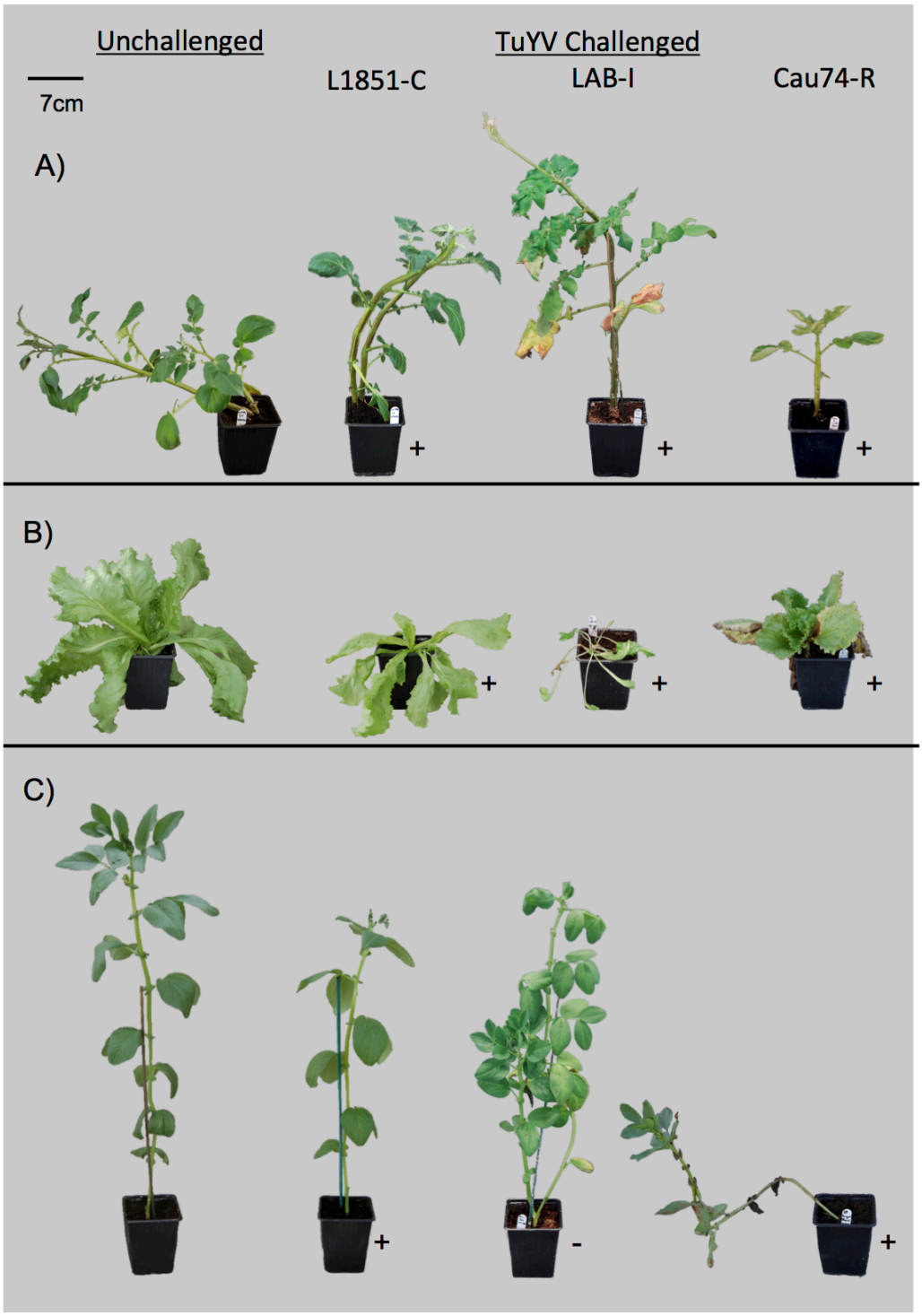

Figure 4.7 – Potato, Lettuce and Field Bean Crop Host Range Phenotypes Following Challenge with Three TuYV Isolates.
Crop plants incoluated with L1851-C, LAB-I, and Cau74-R representing the three phylogenetic groups of TuYV. Phenotypes images taken 45 DPI of challenged plants compared to unchallenged controls. Positive ELISA result indicated by +, Negative ELISA result indicated by -. A) Potato. B) Lettuce. C) Field Bean.

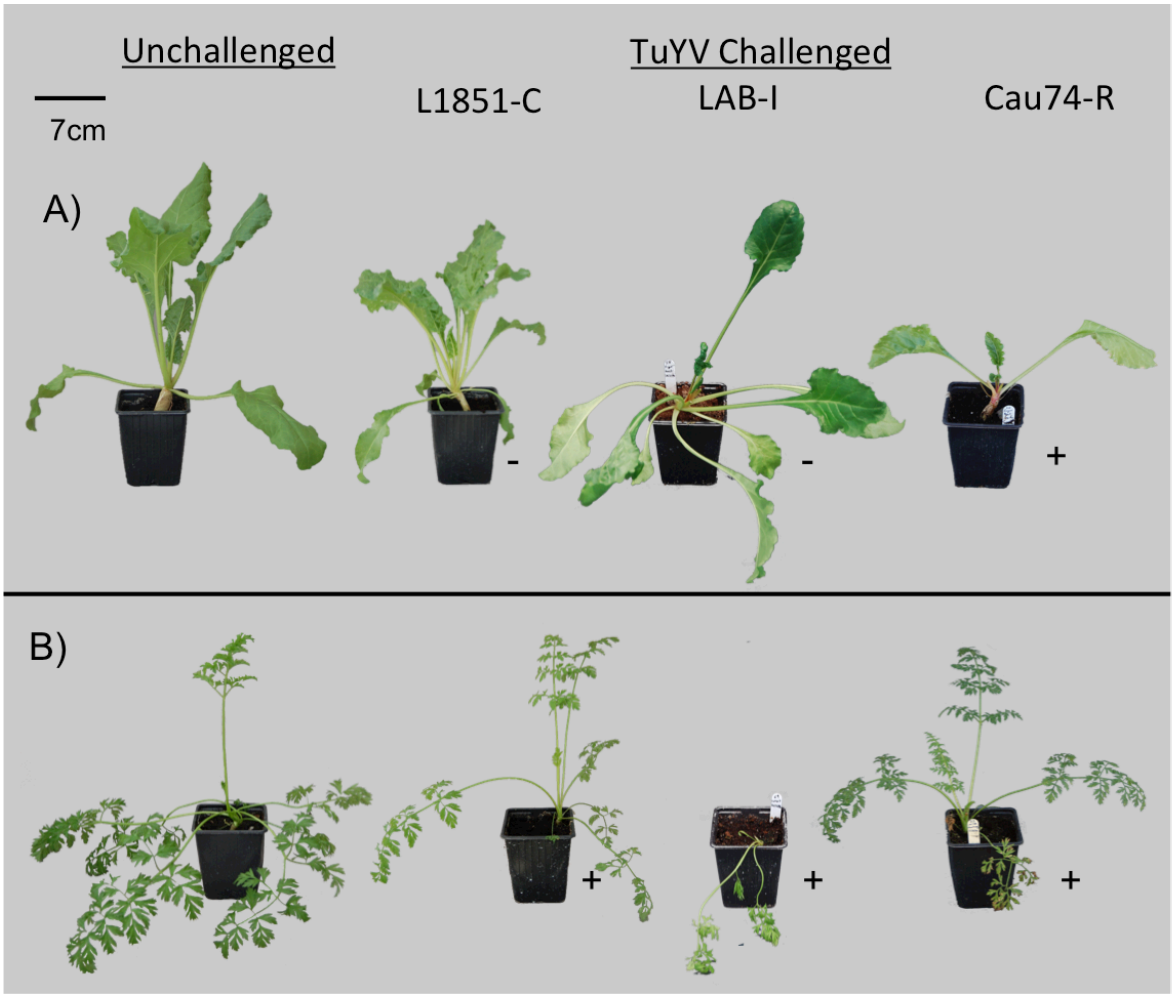

Figure 4.8 – Sugar Beet and Carrot Crop Host Range Phenotypes Following Challenge with Three TuYV Isolates.
Crop plants incoluated with L1851-C, LAB-I, and Cau74-R representing the three phylogenetic groups of TuYV. Phenotypes images taken 45 DPI of challenged plants compared to unchallenged controls. Positive ELISA result indicated by +, Negative ELISA result indicated by -. A) Sugar beet. B) Carrot.

Following the crop host range study, confirmation was needed of the sugar beet results due to it being previously classified as a non-host for TuYV (Duffus and Russel, 1970). The virus infecting the sugar beet was sequenced and aligned with the genome sequence of Cau74-R and sequences of TuYV from a OSR plant infected with sugar beet infecting isolate. The isolate Cau74-R was originally sequenced in 2012 during a host range study conducted by an undergraduate student. It appears within this time (2012-2015) there have been 4 point mutations over the 900bp sequenced, but these were shared between the TuYV from the original OSR and sugar beet plants (Figure 4.9). These results confirm the ELISA results (Table 4.7 that a TuYV isolate was able to infect sugar beet.

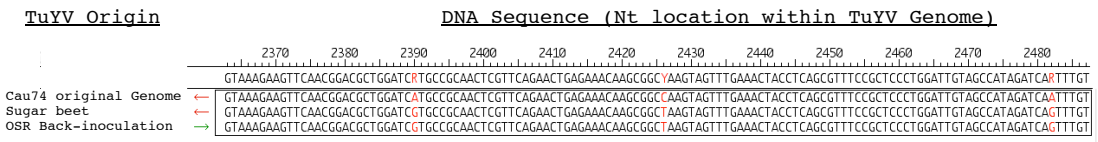

Figure 4.9 – Sequence confirmation of TuYV in sugar beet and sugar beet infecting isolate from OSR.
Sequence alignment of Cau74-R genome and TuYV isolates amplified from Cau74-R infected sugar beet and an OSR back-inoculated with a sugar beet infecting isolate.

4.4 Discussion

4.4.1 TuYV Wild Species Host Range

It is clear that the UK host range of TuYV is wide, including many weed species that can act as active reservoirs for OSR crops (Table 4.4). Section 4.3.1 confirmed several previously reported TuYV host weed families *Lamiaceae*, *Plantaginaceae*, *Scrophulariaceae*, *Violaceae* (Stevens *et al.*, 2008b). This was confirmed by sequencing of isolates found in several plant families: *Asteraceae*, *Brassicaceae*, *Caryophyllaceae*, *Compositae*, *Fabaceae*, demonstrating them as active reservoirs and hosts of the virus. In addition to these confirmed hosts there were four new, previously unreported host plant families, *Apiaceae*, *Caprifoliaceae*, and *Resedaceae*, *Geraniaceae*, three of which were demonstrated to harbour TuYV isolates capable of infecting OSR. These species were sampled from around UK fields of OSR and these results demonstrate the ability of TuYV to naturally infect a wide host range which could be sources of TuYV for OSR crops. This constant presence of TuYV in the environment will reduce the ability of crop rotation to reduce sources of infection. The main weed families were found to be infected by TuYV *Brassicacae*, *Asteraceae*, *Compositae*, *Lamiaceae* which are very abundant in hedgerows and fields in the UK and mainland Europe (Wilson *et al.*, 1999).

Many ELISA-positive weed samples in the wild host range experiment could not have TuYV amplified sufficiently for sequencing. This poses the question why TuYV could not be amplified from these samples. The possible reasons for this could be there is a genetically diverse isolates of TuYV that could not be amplified with the primers designed in Chapter 3, or the anitsera used in ELISA are cross-reacting with closely related viruses, some of which could also infect OSR (Table 4.4). This has been seen before with members of the *Polerovirus* genus with antisera cross-reacting and detecting unknown viruses (Asaad *et al.*, 2009). This is an area that might prove interesting for further research, to understand the diversity of viruses that are present in these weed species, which could be closely related to or divergent from TuYV. Generic *Luteoviridae* primers along with a host range study using *M. persicae* to transmit these unknown viurses would be necessary to ascertain their classification.

The TuYV ELISA results varied within a given weed line due to false negative results. This is most likely due to viruliferous aphids not feeding

successfully on all plants, as all weed plants per isolate were kept in close proximity of each other, introducing choice. Aphids can become conditioned to feed on the species of plant they are initially feed on, resulting in a preference for these plants, which could help explain the lack of consistent results (Nikolakakis *et al.*, 2003). Each plant was checked for the presence of aphids within the first week of challenge and all plants did have aphids present, but successful virus transmission requires hours of aphid feeding, which might not have occurred, or non-viruliferous aphid progeny with no conditioning could of colonised the weeds. The efficiency of TuYV ELISA positive weed samples able to transmit TuYV to OSR via *M. persicae* was 52% compared to the reported efficiency of OSR to OSR of 96% (Stevens *et al.*, 1995). 52% efficiency still allows weeds to be a viable source of TuYV inoculum.

The pathogenicity of the TuYV weed specific isolates is presumably limited due to only one isolate within that group was found to infect OSR within field conditions (Figure 3.24). The TuYV minor coat protein P5 has been linked to causing disease symptoms, virus accumulation and virus spread through the plant and aphid (Brault *et al.*, 2005). This gene could be involved in determining host range due to the activities it controls. To investigate the host range and fitness of different TuYV isolates a study would have to inoculate weeds and OSR with a mixed infection of weed and OSR TuYV isolates, including passaging the infection over several plants to see if there is competitive selection (García-Arenal and Fraile, 2013). Yield studies would also be an interesting avenue for investigation to see if there is significant variation in yield losses, dependent on which genetic group the isolate belongs to.

To help confirm wild host range results, a selection of the newly identified weed (Table 4.4) hosts of TuYV were challenged with different isolates (L1851-C, LAB-I and Cau74-R) representing the three genetic groups of TuYV (Table 4.6). In turn, this demonstrated that OSR infecting TuYV isolates can infect weeds, using them as hosts during periods when OSR is not grown. Unfortunately not all weed lines selected for this experiment could be tested to investigate their ability to infect OSR. The host range study was also to designed to investigate the reported hosts that were not found to harbour the virus in my sampling study; this could not be accomplished as these selected lines did not germinate. The weld, teasel, spear thistle and geranium species were confirmed as hosts, but there were differences in susceptibility depending on the isolate and the weed lines within a species. Cau74-R was unable to infect teasel plants,

but was the only isolate that infected Spear thistle. LAB-I did not infect weld plants. This is the first account of biological differences between TuYV isolates representing different genetic groups of TuYV. Interestingly spear thistle 400448 was not susceptible to any isolate of TuYV, even though it has been shown to be susceptible with line 59318, offering a potential source of resistance from line 400448. Further investigation of this accession or investigating other *Asteraceae* species for resistance that have better molecular characterisation, such as lettuce, is a potential research avenue for resistance work.

4.4.2 TuYV Crop Species Host Range

Investigating not only weed species as reservoirs of TuYV, but crop species as well will allow better understanding and management of TuYV spread and hopefully its control. All crops tested against TuYV were susceptible. The crops potato (*Solanaceae*) and lettuce (*Asteraceae*) are both within plant families that have been reported to be hosts of TuYV (Table 4.1). However carrots are in the family *Apiaceae*, which was not previously identified as a host. Carrots are related to cow parsley which was also an ELISA positive sample in the weed host experiment (Table 4.4), adding credence to the *Apiaceae* family being a potential host to TuYV and would be interesting to investigate further. The ability of one TuYV isolate to infect sugar beet is an interesting result, as it has implications for the Poleroviruses species classification. This is not the first time this has been suggested as TuYV-FL was also able to infect sugar beet (Beuve *et al.*, 2008), but not previously confirmed by sequencing. Definition of BMYV and TuYV will need clarification in the future to establish what the biological differences are, plus the genetic determinants of those differences.

TuYV infection of lettuce is known to cause chlorosis in the leaves with varying degrees of severity (Walkey and Pink, 1990). The UK brassica TuYV isolates that were tested seemed however to cause stunting and yellowing of leaf tips and not chlorosis (Figure 4.7 B). In potato symptoms were muted, the only isolate of TuYV that induced consistent symptoms was Cau74-R causing stunting of the plants (Figure 4.8 A). The effect on potato by TuYV is unreported but the closely related PLRV causes leaf curling, yellowing and necrotic discolouration on the tubers, all leading to yield losses, but certain strains of PLRV have been reported to cause no symptoms (Jayasinghe, 1988; Alvarez *et al.*, 2007). With cross-reactivity of ELISA antisera and possible parallels in TuYV and PLRV

symptoms, it might be possible that PLRV ELISA positive potato plants in the field are actually effected by TuYV instead. BMYV causes loss of yield and yellowing of the leaves in sugar beet (Smith and Hallsworth, 1990; Stevens *et al.*, 2005), the Cau74-R isolate induced stunting but no yellowing in infected plants (Figure 4.7 A). BMYV might cause a more severe disease than TuYV in sugar beet, TuYV infection causes stunting and is known to reduce yields in other crops means is a credible threat. Field beans have been found to be infected by TuYV in Africa, following viral detection in yellowed and stunted plants (Abraham *et al.*, 2008). The three UK TuYV isolates seem to cause stunting symptoms without yellowing in field bean so will be hard to detect visually which could lead to the lack of accreditation of TuYV impact on this crop type (Figure 4.7 C). TuYV could be a potential cause of yield loss in beans. Carrots have no published symptoms or effects of TuYV infection, in this study the symptoms varied, LAB-I caused severe stunting, but L1851-C and Cau74-R isolates infected plants showed limited symptoms (Figure 4.8 B). TuYV is able to infect a variety of crops but induces muted or inconclusive symptoms, allowing it to be overlooked as a pathogen to these species. TuYV could be an important pathogen in all crops investigated in this study. Yield and phenotype symptoms need to be assessed more thoroughly now the expanded host range of TuYV is known. This should then clarify the sources yield losses in these species, which have been previously attributed to other *Luteoviridae* viruses or have no known explanation.

Genetic groups test included a representative isolate from the most common and the less common OSR infecting TuYV genetic groups and a recombinant isolate of these two clades. Each of the TuYV representatives both in weeds and crops had variation in their host ranges, indicating the genetic variation uncovered in Chapter 3 does have a biological implication and adds power to the argument of further categorising of the the TuYV species. A tool to investigate such determinates of pathogenicity are infectious clones, which can incorporate different genetic areas of each genetic group to identify regions responsible for changes in host range (See Chapter 5). This future work could be extended with a infectious clone with a tractable marker such as GFP, which could visualise TuYV infection and if TuYV is cellular location is changed in different species of host or with co-infectious of other species of viruses. Investigation of the variation in cellular tropism that might account for viral titre variation found between less efficient weed hosts and brassicas might proved useful for understanding TuYV infection.

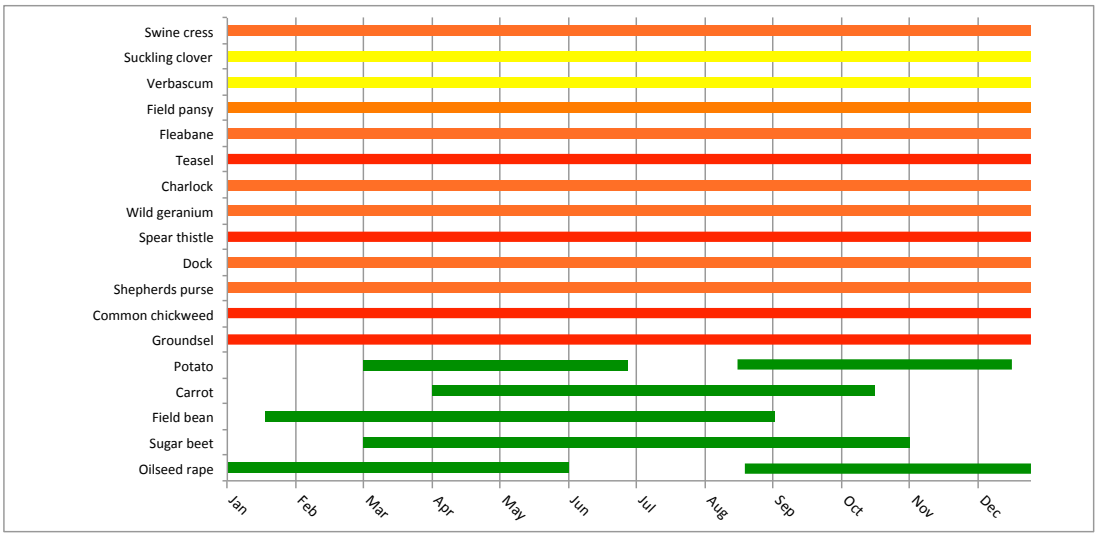

Figure 4.10 – Weed and Crop Presence within the UK Throughout a Year. Bars represent the presence of plants type during certain months of the year. Crops represented in Green, based on UK cultivation. Weeds severity in agriculture for prevalence and yield impact: Red = very important, Orange = moderately important, Yellow = not very important. Based on species discovered as hosts in this study and information cultivation data from Horticultural Development Company (2009). Constructed in Excel.

Understanding reservoirs of TuYV infection and the ability of the virus to be transmitted to different crops will be important if management schemes are to be designed to combat infection. With all confirmed weeds and crops hosts being present during the germination of OSR it clear to see the importance of these reservoirs (Figure 4.10). Weed populations could even more important reservoirs than other crops, with multiple generations per annum, as well as being annuals allowing time for high titre TuYV accumulation. TuYV isolates of the all three UK based genetic groups can infect both OSR and weeds (See Chapter 3). This will make control difficult as this wide host range and effectiveness of aphid transmission is causing 60% of OSR UK acreage to contain TuYV (Nicholls, 2013) meaning it is ubiquitous in the environment, hindering full control. In future, management schemes including: applications of insecticides timings, weed management and crop rotation might be able to include strategies to control aphids at certain times of the year, reducing potential TuYV weed reservoirs and transmission to OSR. In both weed and crop species it is hard to identify TuYV, as symptoms of infected plants are muted. Crop hosts have not been studied fully; as shown in this chapter the host range is still growing. If management of TuYV infection is effective during the emergence of OSR this will heavily reduce the effect on yield (Walsh, 2011). It is evident from the abundance of TuYV in weed and crop species, management of TuYV will be difficult with current approaches,

as hosts are ubiquitous in the UK environment present throughout the year as both crops and weeds species.

Chapter 5

Construction of an Infectious Viral Clone

5.1 Background

Viral clones have been key in understanding the roles and interactions of viral genes. The first fully sequenced TuYV isolate now known as TuYV-FL was successfully made into a viral clone using *Agrobacterium tumefaciens* to infect several plant lines (Leiser *et al.*, 1992). Infectious full-length cDNAs have also been established for several other viruses in the *Luteoviridae* family: Barley yellow dwarf virus (BYDV) (Young *et al.*, 1991), Cucurbit aphid-borne yellows virus (CABYV) (Prüfer *et al.*, 1995), PLRV (Prüfer *et al.*, 1997), BMYV (Stephan and Maiss, 2006) and BrYV (Zhang *et al.*, 2015).

Studies with infectious clones *in vitro* and *in vivo* have demonstrated several key functions of *Polerovirus* genes. ORF0 is necessary for the greater accumulation of viral RNA (Ziegler-Graff *et al.*, 1996). ORF1 and ORF2, together with the virus genome promoter sites for transcription initiation, sufficient and required for efficient replication (Reutenauer *et al.*, 1993; Mohan *et al.*, 1995). ORF3a was discovered to be involved in long-distance movement in plants (Smirnova *et al.*, 2015). ORF3 and ORF5 are required for the formation of progeny virions (Reutenauer *et al.*, 1993; Filichkin *et al.*, 1994; Brault *et al.*, 1995; Prüfer *et al.*, 1995). ORF4 is necessary for movement within plants (Chay *et al.*, 1996; Ziegler-Graff *et al.*, 1996). Aphid transmission is dependent on the expression of the ORF5 (Jolly and Mayo, 1994; Brault *et al.*, 1995). Infectious clones have been instrumental in a deeper understanding of the *Luteoviridae*

family.

Agrobacterium that contain a Ti plasmid can efficiently transfer a portion of this plasmid into the plant genome. It is surrounded by Right (RB) and Left (LB) boundary sequences, this section of the plasmid is called T-DNA (De Block *et al.*, 1984; Deblaere *et al.*, 1985). This property has provided the basis for a technique called agroinfection or agroinoculation in which the viral genome is inserted into the T-DNA ready for plant transcription to initiate an infection when it is inside a plant cell (Grimsley *et al.*, 1986). At first agroinfection was limited to viruses with DNA circular genomes (Boyer and Haenni, 1994), until the introduction of Cauliflower mosaic virus (CaMV) derived 35S promoters and self cleaving ribozymes which could be used to produce ssRNA viral transcripts (Leiser *et al.*, 1992).

The transmission of poleroviruses occurs in a circulative non-propagative manner via aphid vectors (King *et al.*, 2012). Poleroviruses are also limited to the phloem of plants and as such are not able to be transmitted by mechanical inoculation (Mayo and dArcy, 1999). This causes issues when investigating this range of viruses, as fresh material or constant cultures of infected plants with aphids are necessary to maintain the *Polerovirus* isolates. Both methods of maintaining poleroviruses pose issues; there is no possibility of resurrecting virus isolates from small amounts of stored samples and cultures are costly in size and could become cross-contaminated. Thus, the production of infectious clones of TuYV would solve many of these issues.

The genomic RNA of the fully sequenced common TuYV isolate L1851 (L1851-C) is 5.728 kb in length, larger than the reported genome size of TuYV-FL at 5.641 kb (Veidt *et al.*, 1988) and the closely related BrYV isolates that vary between 5.666 - 5.678 kb (Xiang *et al.*, 2011). Phylogenetic analysis has shown variation in the genes of European TuYV, primarily the P0 and P5 genes (See Chapter 3). But the biological consequences of this genetic variation have not been fully investigated, thus an infectious clone would permit studies on host range determinates, gene function and pathogenic determinates.

This chapter describes the construction of an infectious clone of UK *Brassica* TuYV isolate infectious clone. This work will utilise TuYV L1851-C in a plasmid construct for *Agrobacterium* infiltration. This will represent the most common group of TuYV isolates sequenced (Chapter 3) with *Agrobacterium*

allowing effective inoculation of most plant species.

5.2 Materials and Methods

5.2.1 Viral clone PCR primers

For the production and verification of the infectious clone, a variety of primers were used (See Table 5.1). The 35S promoter used and amplified in this chapter was from pGRT121, a modified version of pBI121 (Jefferson *et al.*, 1987) developed in-house by Dr. Graham Teakle (obtained through personal communication).

5.2.2 BP and LR Recombination Reactions

Gateway cloning was used for the production of the infectious viral clone plasmid (Gateway® Technology with BP and LR Clonase^{TM} II; Invitrogen). RNA was extracted from TuYV-infected *B. napus* plants as described in Section 2.5.1. RT-PCR was performed to obtain cDNA (see Section 2.5.6). The primers contained *att*B adapters to aid the introgression of the target genes into entry clones (Table 5.1). A BP recombination reaction was performed with the *att*B-PCR products and pDONR221 to transform the host *E. coli*. The expression clone was generated using the LR recombination reaction, using the previously mentioned entry clone and Gateway destination vector, PEarleyGate100 (Tair stock No. CD3-724) (Earley *et al.*, 2006). Constructs were sequenced after each step to ensure the correct sequence was present.

5.2.3 Media

YEB media (1 L) contained 5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L $MgCl_2$, pH 7.2, autoclaved. To prepare YEB plates bacterial agar 1.5% was added to 20ml per petri dish of YEB media.

Table 5.1 – Details of Primers Used for Production and Confirmation of the TuYV Infectious Clone.

Primer Name	Sequence (5′-3′)	Orientation	Target Region
attB1[a]	GGGGGACAAGTTTGTACAAAAAAGCAGGCT	Forward	attB adapter
attB2[a]	GGGGACCACTTTGTACAAGAAAGCTGGGT	Reverse	attB adapter
M13 F[a]	GTAAAACGACGGCCAG	Forward	N-terminal sequence of lacZ
M13 R[a]	CAGGAAACAGCTATGAC	Reverse	N-terminal sequence of lacZ
MN37	GGACAACTGGAATTCTGCTCTC	Forward	3040nt-3062nt TuYV
MN42b	GRACCAGCTATCGATGAAGAACC	Reverse	4027-4049nt TuYV
MN57	GACCACAACCACTGGCTGAG	Reverse	3690nt-3709nt TuYV
MN78	ACACCGAAGTGCCGTAGG	Reverse	5624nt TuYV
MN79	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGTCAACATGGTGGAGCACGACA	Forward	*attB* + pGRT121
MN80	GATTCCCTCCTGGTTTCTTTTGTTCCTCTCCAAATGAAATGAACTTCCT	Reverse	pGRT121 + TuYV
MN95	GGGGACCACTTTGTACAAGAAAGCTGGGTCGATCGTGGAGCTCGGTGTTTCGTC CTCACGGACTCATCAGTAGACATGTGAATCATGTCTAGACACCGAAGTGCCGTAGG	Reverse	5624nt TuYV + Ribozyme + *attB*
MN96-2	GGGGACCACTTTGTACAAGAAAGCTGGGTCGATCGTGGAGCTCGGTGTTTCG TCCTCACGGACTCATCAGTAGACATGTGAATC	Reverse	Ribozyme + *attB*

[a]Kit primer from Invitrogen

5.2.4 Generation of *Agrobacterium tumefaciens* Competent Cells

Agrobacterium tumefaciens strain GV3101 was inoculated into 5 ml of YEB media containing rifampicin 100 μg/ml and gentamicin 30 μg/ml and grown at 28°C with 220 rpm shaking overnight. The following morning, the overight culture was inoculated into 200 ml of YEB medium containing Rif^{100} and $Gent^{30}$ and incubated at 28°C (220 rpm shaking) for 4-6 hours. The flask was chilled on ice for 10 minutes with occasional shaking, followed by centrifugation of the culture at 1000 G at 4°C for 20 minutes. The pellet was washed by re-suspension in ice cold TE buffer (10 mM Tris/HCl, 1 mM EDTA pH 8.0). This centrifugation and washing step was then repeated. Cells were then centrifuged at 1000 G at 4°C for 20 minutes and re-suspended in ice cold YEB medium. After this, 500 μl aliquots of *Agrobacterium* cells were mixed with 500 μl of 50% glycerol, then flash frozen in liquid nitrogen and stored at -70°C.

5.2.5 *Agrobacterium tumefaciens* Transformation

Agrobacterium competent cells were thawed on ice. Approximately 150-200 ng of plasmid DNA was added to 100 μl of *Agrobacterium* cells and mixed, followed by incubation on ice for 5 minutes. Cells were heat shocked by freezing at 5 minutes in liquid nitrogen followed by 5 minutes at 37°C in a water bath. They were then left on ice for 2 minutes followed by the addition of 900 μl of YEB medium containing no antibiotics. The *Agrobacterium* cells were then incubated for 2-3 hours at 28°C shaking, followed by centrifugation at 1000 G on a desktop microfuge for 5 minutes. Approximately 900 μl of medium was removed and cells were re-suspended in the remaining medium (approximately 100 μl). Cells were then plated on YEB containing appropriate antibiotics and incubated for up to two days at 28°C to allow colonies to form.

5.2.6 *Agrobacterium tumefaciens* Infiltration

An overnight culture of transformed *Agrobacterium* in 10 ml YEB media with 50 μg/ml kanamycin was prepared. To perform the infiltration a 2.5 ml syringe was used. The syringe (no needle) was pressed against the underside of the leaf (avoiding the cotyledons), the plunger was gently depressed, wetting the leaf. A single 1 mm deep incision was also made on the plant stem around 2 cm long with

a sterile scalpel. 20 μl of the culture was added to the top and bottom of the incision. All inoculated areas were marked and 3-4 weeks later infiltrated plants were tested for TuYV (See Sections 2.4.1 and 2.5.6) infection above the area of inoculation.

5.2.7 Experimental Design

For *Agrobacterium* infiltration of both OSR and *Nicotiana benthamiana* (*N. benthamiana*) un-inoculated and mock inoculated controls were kept within the same insect proof cage and checked periodically for aphid contamination. Presence and absence of TuYV was determined with RT-PCR (see Sections 2.5.6 - 2.5.7) along with sequence analysis 30 days post inoculation. For aphid transmission of the viral clone, aphids were allowed to feed for three weeks then removed (see Section 2.3.2). All plants were kept within the same insect proof cage to aid aphid movement and feeding on all plants. Negative controls with non-viruliferous aphids were kept in a separate insect proof cage. Aphid transmission of the infectious clone was investigated with ELISA (see Section 2.4.1) three weeks after aphids were introduced.

5.3 Results

5.3.1 Sequence Confirmation

Full sequence knowledge was necessary to accomplish the construction of the infectious clone to verify sequence homology with the isolate L1851-C. This was necessary as the planned construction of the TuYV infectious clone utilised primers at the extremities of the TuYV genome with tag sequences to incorporate other elements into the final sequence (Figure 5.1). The design utilised a 35S promoter amplified from the plasmid GRT121, along with a ribozyme sequence (Leiser *et al.*, 1992). 5′ RACE was performed on L1851-C (Figure 3.8) to determine the sequence of the 5′ UTR for primer design, which would not impact on the coding region of P0 for later phylogenetic analyses.

The 3′ sequence of TuYV genome L1851-C was not previously known. TuYV does not possess a poly A tail, thus to attain the full sequence of the 3′ UTR region, a poly A tail was added to the total RNA extracted from L1851-C infected plants to allow 3′ RACE to be carried out (See Section 2.5.5). This polyadenylated RNA was used for RT-PCR with anchor primers (Table 2.3) and an internal TuYV forward primer (MN49, Table 2.2) to produce an amplicon which could be sequenced to determined the 3′ sequence and allow homologous primer design in this region (Figure 5.2).

The 3′ product (Figure 5.2, lane 6) product was sequenced (Figure 5.3), determining the the sequence homology to BWYV genomic RNA 3′-end sequence (Accession X13062.1) and not TuYV-FL (Accession X13063.1). This data helped to design primers MN78 and MN95, which had a reverse primer site of TuYV that allowed full reverse transcription of the genome with no nucleotide alterations. The MN95 primer contained: a 3′ primer site of TuYV, a ribozyme and an *attB* site for Gateway incorporation (Table 5.1). Primer MN95 was used to produce cDNA of TuYV that contained the full TuYV genome with the ribozyme sequence (Leiser *et al.*, 1992) and *attB* site.

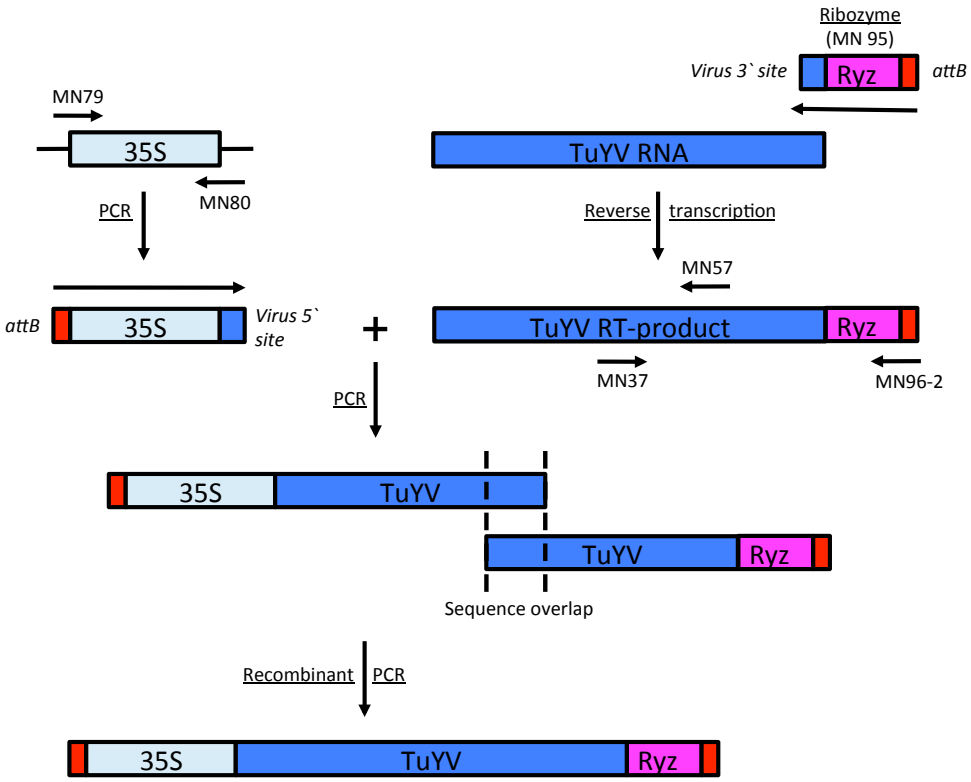

Figure 5.1 – Construction of Full-Length cDNA Infectious Clone of *Brassica* Infecting UK TuYV Isolate L1851-C.
TuYV infectious clone construction utilised primers with tag sequences to incorporate a variety of different elements into the final construct. This method ensured that there was no unnecessary nucleotide additions to the TuYV sequence. Multiple primers used in this method contained primer sites for the TuYV sequence for subsequent recombinant PCR and *attB* sites to allow incorporation into a Gateway system. Templates and primers used for each step of the viral clone construction are outlined, with the final overlapped sequence of ~600bp.

5.3.2 Infectious clone Amplification and Construction

The first step in the construction of the infectious clone was to construct a 35S promoter that would act as the forward primer for amplifying TuYV by PCR. The 35S promoter was amplified between -431bp to -1bp relative to the CaMV transcription initiation site from pGRT121 (Figure 5.4). All amplicons produced were used during the construction of the infectious clone. The amplicon produced also incorporated a TuYV primer site based on the MN45 sequence gained from 5′ RACE, and an *attB* site. This allowed the transcription initiation to avoid the incorporation of additional non-virally derived nucleotides within the 5′ sequence.

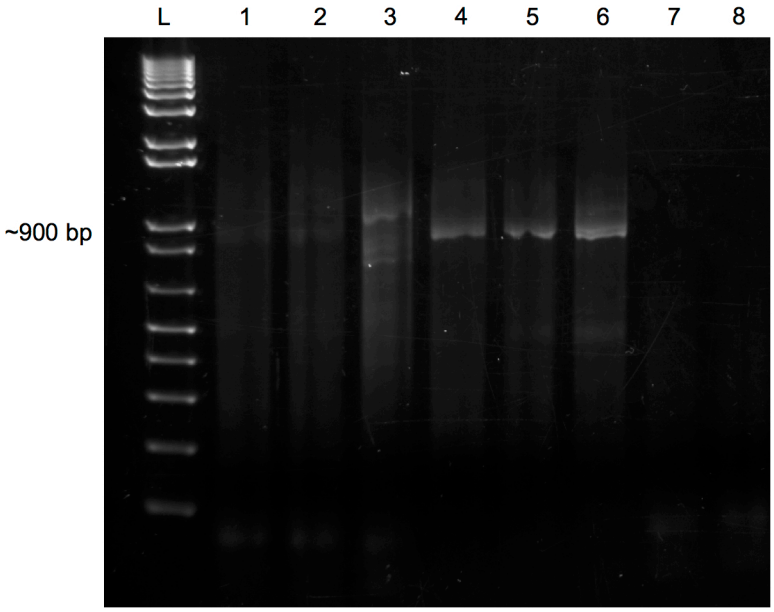

Figure 5.2 – PCR Amplification of the 3′ region of TuYV from TuYV RNA with poly A tail addition.
L) 1KB^{+} ladder. 1)-6) Polyadenylated TuYV PCR amplified with MN49 and TAG Primers at varying annealing temperatures (54 - 64 °C, increasing at 2 °C increments), ∼900bp target amplicon. 7) Plant RNA negative control. 8) dH_2O negative control.

The amplified 35S promoter was used as the forward primer, the reverse primer was MN57 located within ORF3. This produced a recombinant amplicon which contained both the promoter and 5′ TuYV sequence amplifying up to 3709nt of the TuYV genome (Figure 5.5). Sequencing of this amplicon confirmed the successful incorporation of both genetic elements: the 35S promoter and TuYV genome (Figure 5.6).

For a complete and infectious clone the 3′ portion of the L1851-C genome was needed, plus the addition of a ribozyme. The ribozyme was added to avoid the addition of any non-TuYV sequence to the 3′ UTR region post transcription, which could reduce the virus's pathogenicity. The reverse primer MN95 that contained the tag sequence of both the ribozyme and *attB* was used to produce the cDNA TuYV genome after RT-PCR. This cDNA was amplified with MN96-2 and the P3a-located forward primer MN37 (Figure 5.7). This PCR produced a target-sized amplicon, which contained the 3′ half of the TuYV genome. Sequencing of lane 2 and 3 products revealed that it included the TuYV 3′ sequence (Figure 5.8), but the ribozyme and *attB* sequence was confirmed post gateway cloning (Section 5.3.3).

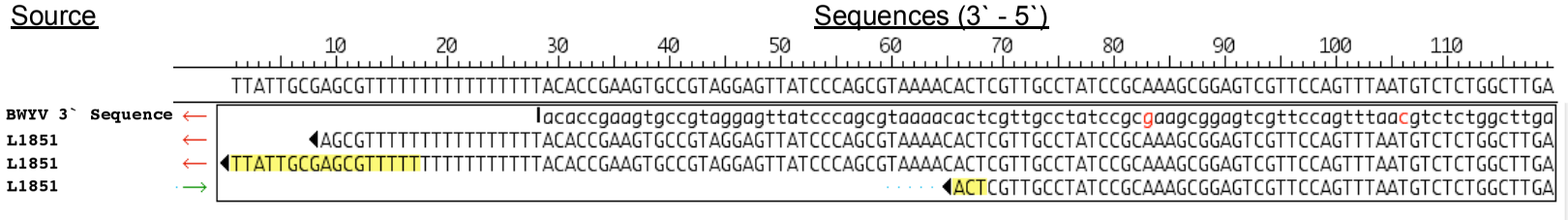

Figure 5.3 – Sequencing data from PCR Amplification of 3′ region of TuYV after poly A tail addition (3′ RACE).
L1851-C sequences aligned to BWYV genomic RNA 3′-end sequence (Accession X13062.1). L1851-C sequences obtained from lane 6 product Figure 5.7.

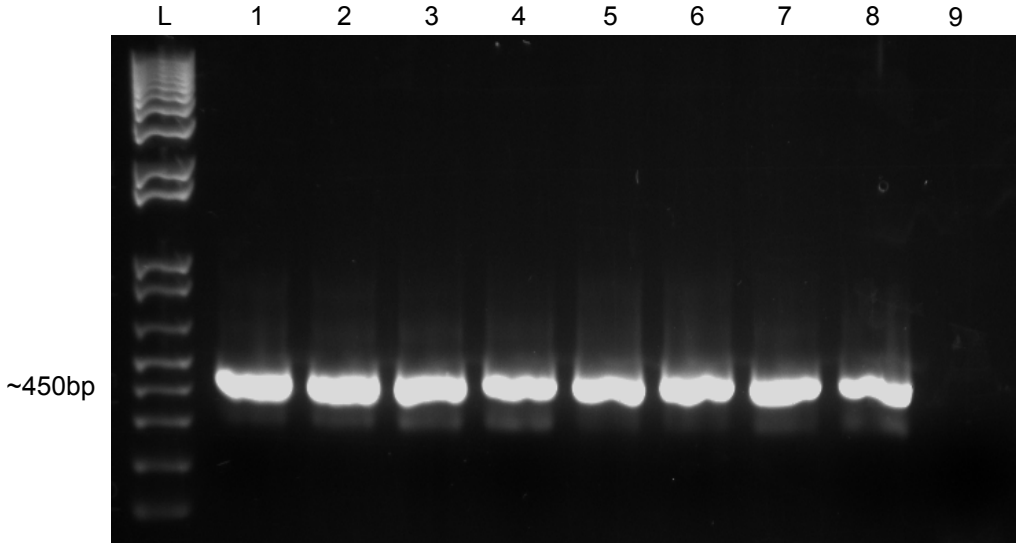

Figure 5.4 – PCR Amplification of 35S Promoter from pGRT121 Plasmid with *attB* Site and Viral Priming Site Addition.
L) 1KB^{+} ladder. 1)-8) 35S promoter amplified with MN79 and MN80 Primers at varying annealing temperatures (54 - 68 °C, increasing at 2 °C increments), ∼450bp target amplicon. 9) dH_2O negative control

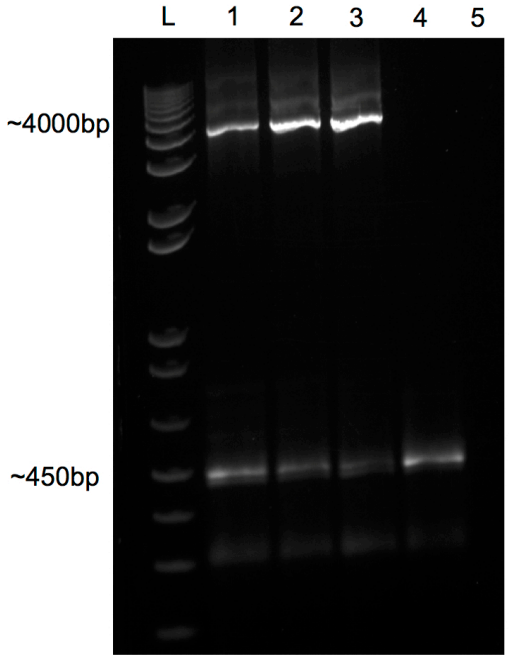

Figure 5.5 – Recombinant PCR of 35S PCR Product and TuYV Genome.
L) 1KB^{+} ladder. 1)-3) TuYV amplified with 35S promoter PCR amplicon and MN57 Primer at varying annealing temperatures (58 - 62 °C, increasing at 2 °C increments), ∼4000bp target amplicon, ∼450bp amplicon non-incorporated 35S promoter. 4) Plant RNA negative control. 5) dH_2O negative control.

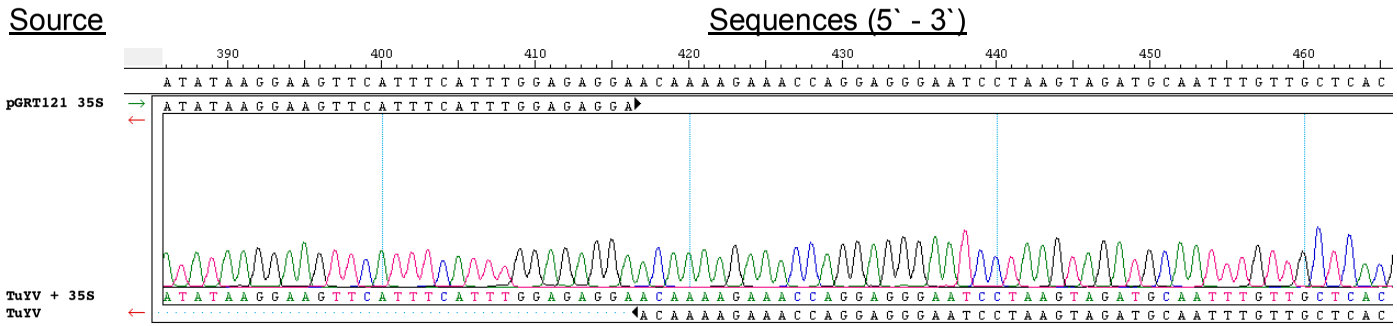

Figure 5.6 – Sequencing Data from Recombinant PCR Amplification of TuYV and 35S Promoter.
The trace file demonstrates the successful incorporation of the 35S with TuYV, with a single sequencing run across the recombinant region between the two genetic elements.

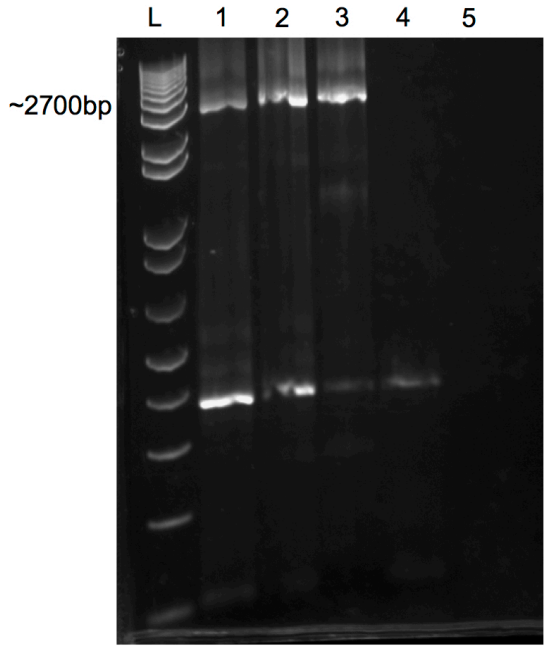

Figure 5.7 – PCR Amplification of the 3′ region of the TuYV with Ribozyme and *attB* site.
L) 1KB^{+} ladder. 1)-3) TuYV amplified with MN96-2 and MN37 Primers at varying annealing temperatures (58 - 62 °C, increasing at 2 °C increments), ∼2700bp target amplicon, ∼380bp amplicon non-specific amplification. 4) Plant RNA negative control with non-specific ∼380bp amplicon amplification from Plant RNA. 5) dH_2O negative control.

The conjoining of the 5′ and 3′ halves of the infectious clone was the last stage of the completing the construct. This was attempted with Sal1 restriction digestion and subsequent ligation, as there was a unique restriction site located within the 600bp over-lap between both sequences. However, this was not successful due to unexpected cleavage and ligations. Instead a similar method to incorporate the 35S promoter was used. Only the 5′ and 3′ infectious clone amplicons were used, as both the primers and the template producing a target-size product of ∼6100bp (Figure 5.9). Lane 3 products were taken forward for further infectious clone construction. Products of 4000bp and 2700bp were non-incorporated 5′ and 3′ infectious clone amplicons (Figure 5.9). This completed construct was ready for insertion into a binary vector for Angro-inoculation.

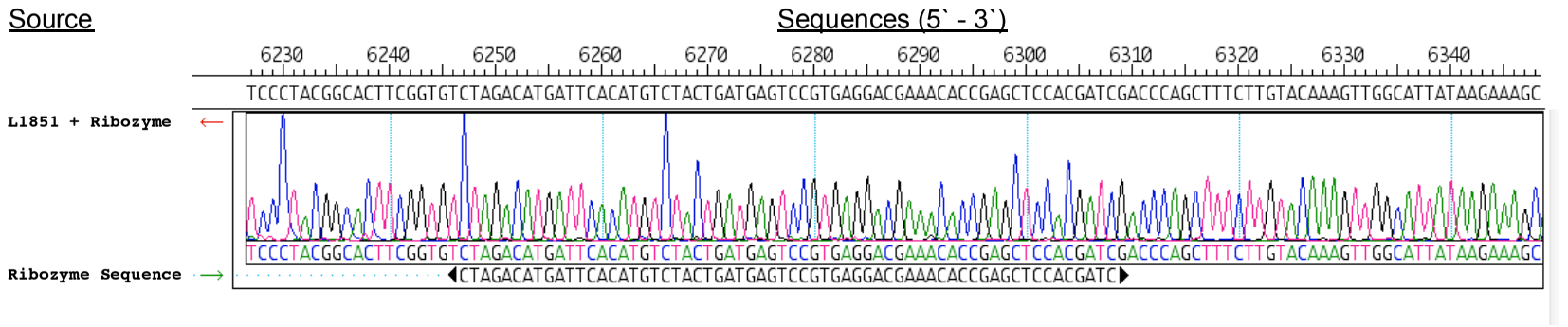

Figure 5.8 – Sequencing data of TuYV with Ribozyme Addition.
L1851-C sequence data across the 3′ portion of TuYV genome then entering the ribozyme and *attB* sequence. The trace files shows incorporation of each element successfully into the infectious clone construct.

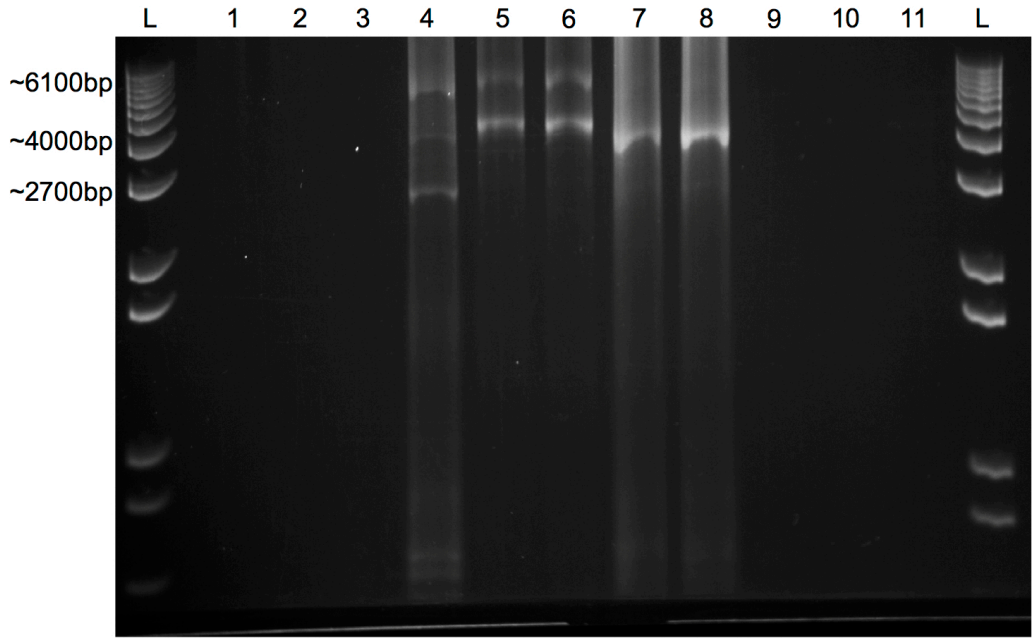

Figure 5.9 – Recombinant PCR of 35S 5′ TuYV and 3′ TuYV with Ribozyme products.
L) 1KB$^+$ ladder. 1)-2) Gel extracted 5′ and 3′ infectious clone amplicons with 62 - 64 °C annealing steps. 3)-4) Gel extracted 5′ and 3′ infectious clone amplicons with 62 - 64 °C annealing steps, plus MN79 and MN96-2 primers. 5)-6) PCR mix 5′ and 3′ infectious clone amplicons with 62-64 °C annealing steps. 7)-8) PCR mix 5′ and 3′ infectious clone amplicons with 62-64 °C annealing steps, plus MN79 and MN96-2 primers. 9) Plant RNA negative control. 10) dH_2O negative control. 11) Empty lane. L) 1KB$^+$ ladder.

5.3.3 Gateway Cloning

The infectious clone construct included *attB* sequences at either end of its sequence, allowing insertion into an entry clone via Gateway cloning. This facilitated the infectious clone construct being inserted into a binary vector which was used for agro-inoculation of plants (Figure 5.10). The entry clone used was pDONR221, which was incubated overnight with BP clonase and infectious clone PCR product. This mix was heat-shock transformed into competent *E. coli* and grown on kanamycin plates, for selection of transformed *E. coli* colonies.

Successful overnight *E. coli* colonies were tested for the TuYV insert with PCR detection of the TuYV P3 gene and pDONR221 plus TuYV P5 detecting primers. Each transformed plate has 20 colonies tested for both the plasmid and TuYV insertion (Figure 5.11). PCR detection of P3 also confirmed the recombinant PCR was successful between the two halves of the viral clone, as amplification could only occur if both halves were amplified together. Selected

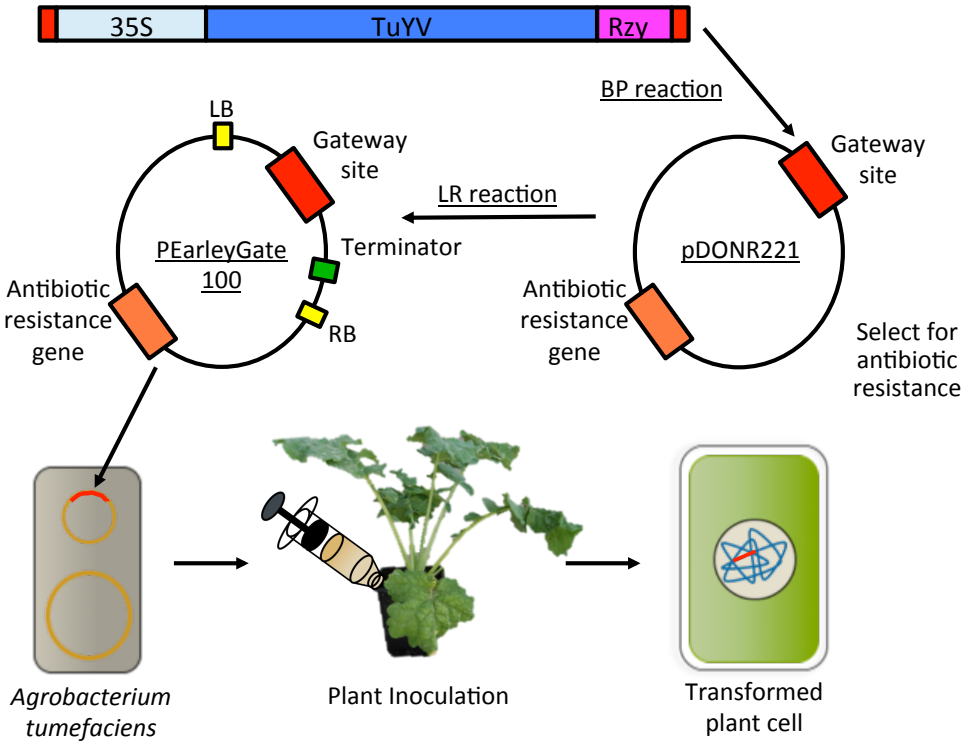

Figure 5.10 – Schematic Diagram showing Gateway Cloning of Infectious TuYV Clone and Plant Inoculation.
L1851-C viral clone BP reaction with Gateway entry vector pDONR221 forms the entry clone with the kanamycin selection gene. pDONR221 entry clones mixed with pEarleyGate100 destination clone and LR clonase produced the final destination clone. The destination clone included Left (LB) and Right Boundaries (RB) necessary for *Agrobacterium* to incorporate it into plant cells after Agro-inoculation.

colonies were grown overnight in liquid culture and their plasmids extracted, in preparation for LR reaction into the destination vector (See Section 5.2.2).

Following the LR reaction and heat shock of *E. coli* with LR reaction mix, successful colonies were again tested by the same PCR amplification protocols, as the destination clone had the same kanamycin resistance gene as the entry clone. As such, selective media plates would not distinguish between the two different plasmids. The destination vector pEarleyGate100 did not contain the M13 primer sites so this absence was used to detect *E. coli* colonies with successful LR transformed plasmids (Figure 5.12). Three colonies showed no amplification indicating successful destination vectors, two of these were taken forward to test for TuYV construct insertion.

The two colonies of *E. coli* which showed no indication of pDONR plasmid were

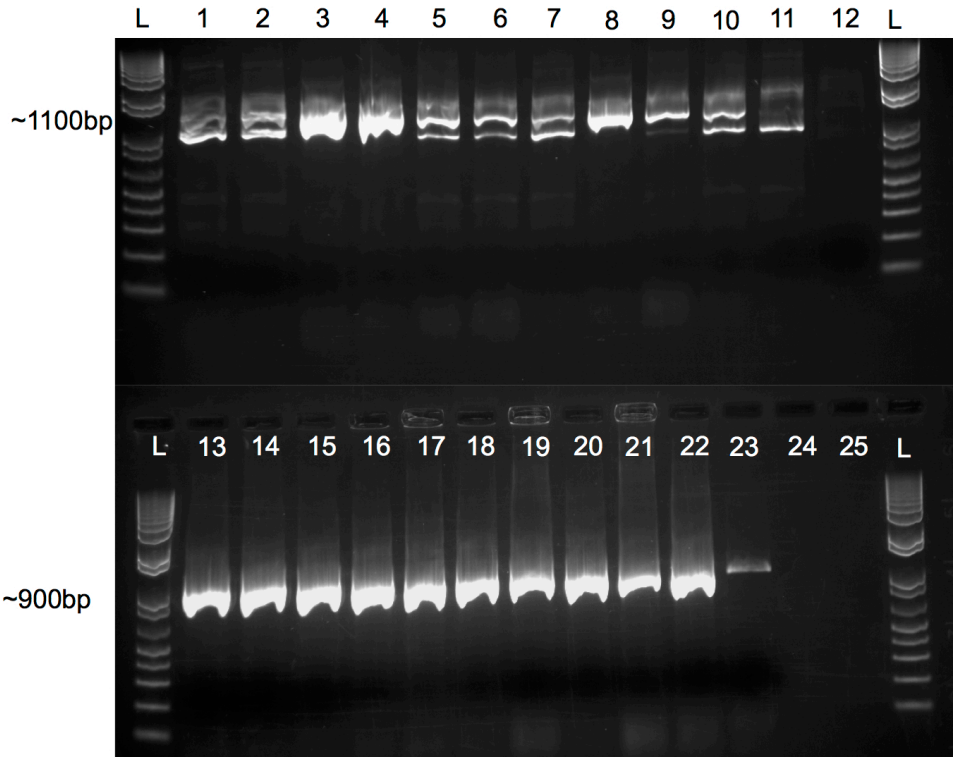

Figure 5.11 – PCR Amplification of Entry clones detecting the presences of TuYV Insert.
L) 1KB$^+$ ladder. 1)-10) Colony PCR of *E. coli* with primers M13F and MN36 detecting the pDONR221 plasmid and TuYV, target-size amplification of 1100bp (larger band). 11) pDONR not containing TuYV construct producing only the smaller band. 12) dH_2O negative control with M13F and MN36. L) 1KB$^+$ ladder. 13)-22) MN37 and MN42B amplifying TuYV P3. 23) Positive control of TuYV cDNA. 24) Plant RNA negative control. 25) dH_2O negative control. L) 1KB$^+$ ladder.

tested for the TuYV insert and the entry plasmid again. One colony did not contain the entry clone and included the TuYV construct (Figure 5.13). This colony was grown overnight in liquid culture and the plasmid extracted. The plasmid was then heat shock transformed into *Agrobacterium* and grown in selective liquid media after PCR detection of the TuYV insert. The destination clone pEarleyGate100 has an internal 35S promoter, causing the infectious clone to have two 35S promoters giving the potential for higher expression of the virus in agro-infected cells, aiding expression of the construct.

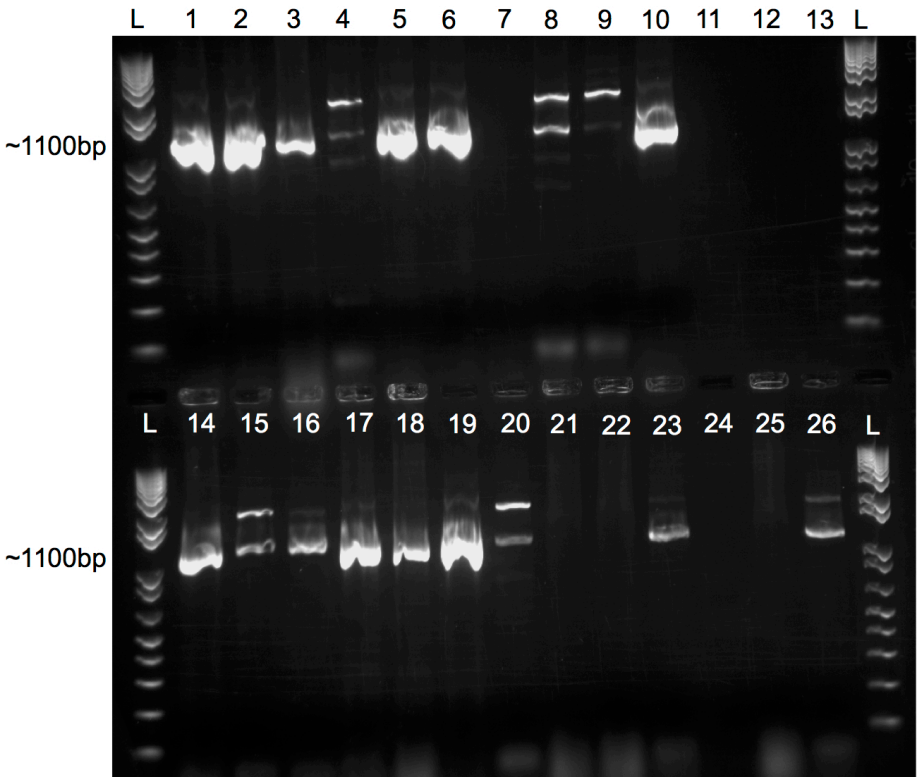

Figure 5.12 – PCR Amplification to check for successful LB reaction of destination clones for the presences of Entry Clone Plasmid.
L) 1KB$^+$ ladder. 1)-10) Colony PCR of *E. coli* with primers M13F and MN36 detecting the pDONR221 plasmid and TuYV. 11)-13) Empty lanes. L) 1KB$^+$ ladder. 14)-23) Colony PCR of *E. coli* colonies with primers M13F and MN36 detecting pDONR221 plasmid and TuYV. 11)-13) Empty lanes. 24) dH_2O negative control with M13F and MN36. 25) pEarleyGate100 Ecoli colonies without insert negative control with M13F and MN36. 26) Entry Clone positive control with M13F and MN36. L) 1KB$^+$ ladder.

5.3.4 Agro-inoculation

Both OSR and *N. benthamiana* plants (2 each) were used to test the infectious clone. *N. benthamiana* has previously been used to test viral clones due to the vascular nature of its leaves which can absorb a large volume of liquid cultured *Agrobacterium*. Following agro-inoculation, plants were tested 4 weeks later by PCR to detect the TuYV genome in new leaves produced the above site of inoculation using primers MN37 and MN48. This also produced a large enough portion of the TuYV for sequence confirmation to confirm results were not due to contamination from another TuYV isolate from aphid feeding.

At 4 weeks post-inoculation OSR plants agro-inoculated with the infectious clone exhibited reddening and yellowing of the leaves (Figure 5.14 A). The

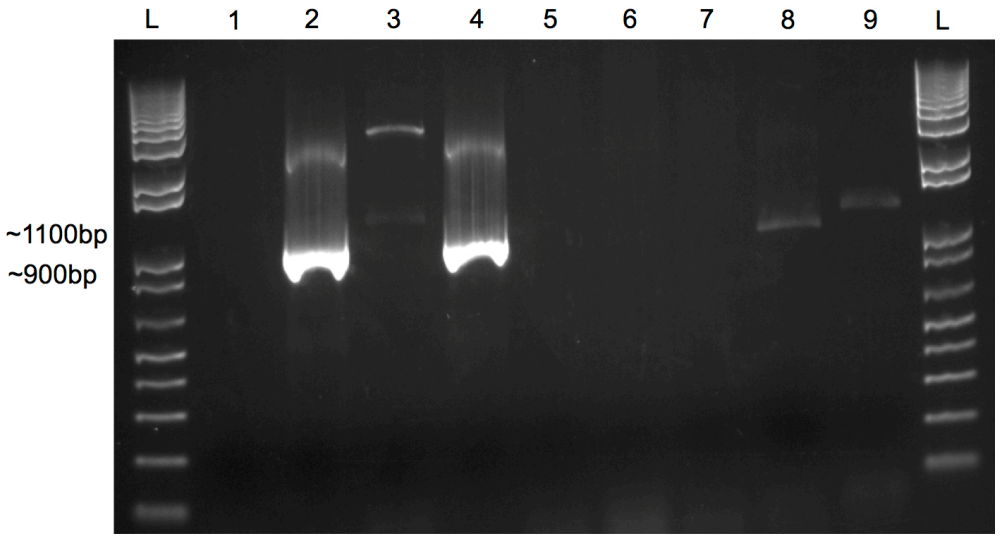

Figure 5.13 – PCR Amplification of Successful Destination Clones for Detecting TuYV Insertion.
L) 1KB$^+$ ladder. 1) Colony PCR of *E. Coli* 1 with primers M13F and MN36 detecting pDONR221 plasmid and TuYV. 2) Colony PCR of *E. Coli* 1 with primers MN37 and MN42b detecting TuYV P3 gene. 3) Colony PCR of *E. Coli* 2 with primers M13F and MN36 detecting pDONR221 plasmid and TuYV. 4) Colony PCR of *E. Coli* 2 with primers MN37 and MN42b detecting TuYV P3 gene. 5) dH_2O negative control with M13F and MN36. 6) pEarleyGate100 PCR with M13F and MN36 primers detecting pDONR221 plasmid and TuYV. 7) pEarleyGate100 PCR with MN37 and MN42b primers detecting TuYV P3 gene. 8) Colony PCR of *E. Coli* containing successful entry clone with primers M13F and MN36 detecting pDONR221 plasmid and TuYV. 9) Colony PCR of *E. Coli* containing successful entry clone with primers MN37 and MN42b detecting TuYV P3 gene. L) 1KB$^+$ ladder.

leaves of infectious clone-inoculated plants also became more brittle than the negative or mock controls with some slight stunting. The mock and negative OSR controls also showed some yellowing of the leaves but this could be due to being pot bound and stressed. *N. benthamiana* plants inoculated and mock inoculated exhibited stunting when compared to the negative control plant but no other visual symptoms (Figure 5.14 B).

To assess the ability of the infectious clone to infect both OSR and *N. benthamiana*, inoculated plants were tested by RT-PCR with P3 primers MN37 and MN42b. Both OSR plants inoculated with the infectious clone gave positive results for the presence of TuYV (Figure 5.15), with no TuYV present in mock inoculated and negative OSR plants. However, no TuYV was detected in the *N. benthamiana* plants inoculated with the infectious clone. Primers MN37 and MN49 were used to amplify across the recombinant region and allowed over

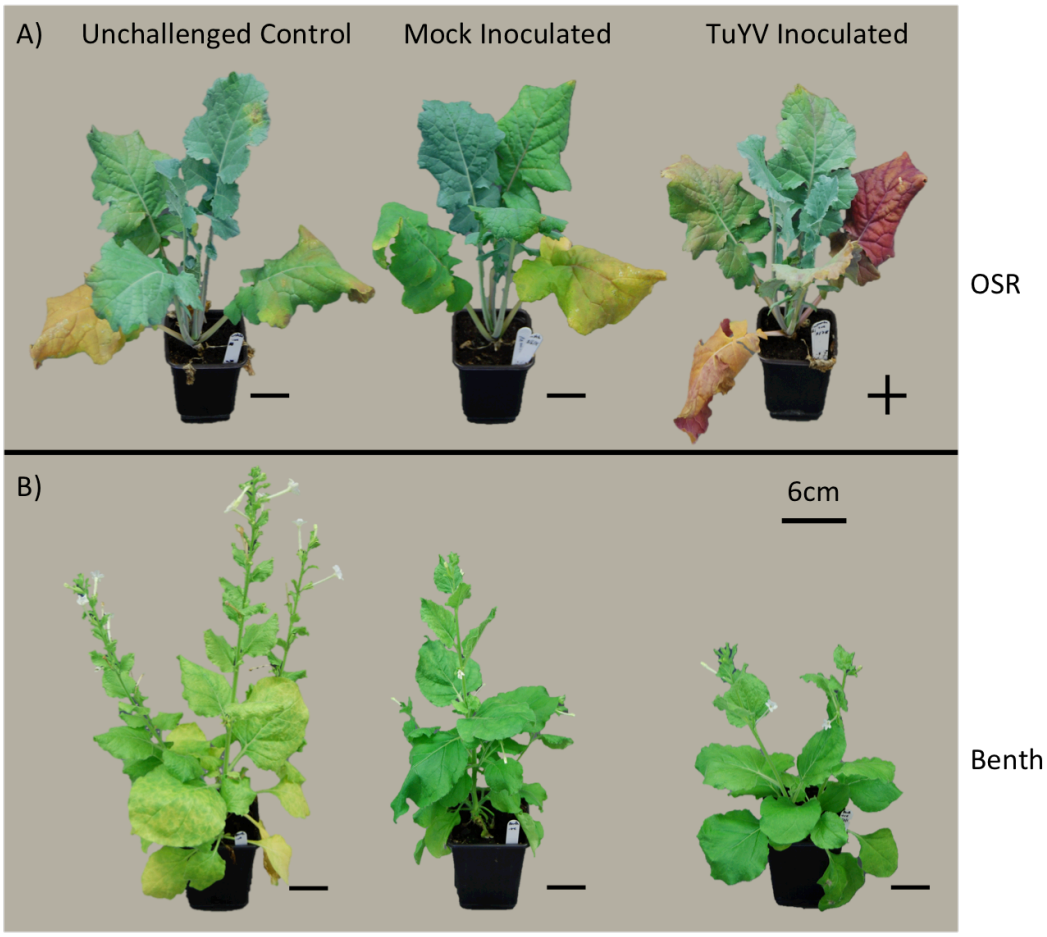

Figure 5.14 – Phenotypes of Unchallenged Control, Mock Inoculated and Infectious Clone Inoculated Plants.
A) Oilseed rape plants 30 DPI with infectious clone of TuYV, compared to negative and mock controls. B) *N. benthamiana* plants 30 DPI with infectious clone of TuYV, compared to negative and mock controls.

2500nt for sequence homology analysis for the infected OSR plant. This sequence analysis revealed the infectious clone Agro-inoculated plants had 99.8% homology with original infectious clone construct, this small difference could be attributed to sequencing error or spontaneous mutations, however Sanger sequencing has error rates of 0.001% to more than 1% so the more likely option (Richter *et al.*, 2008).

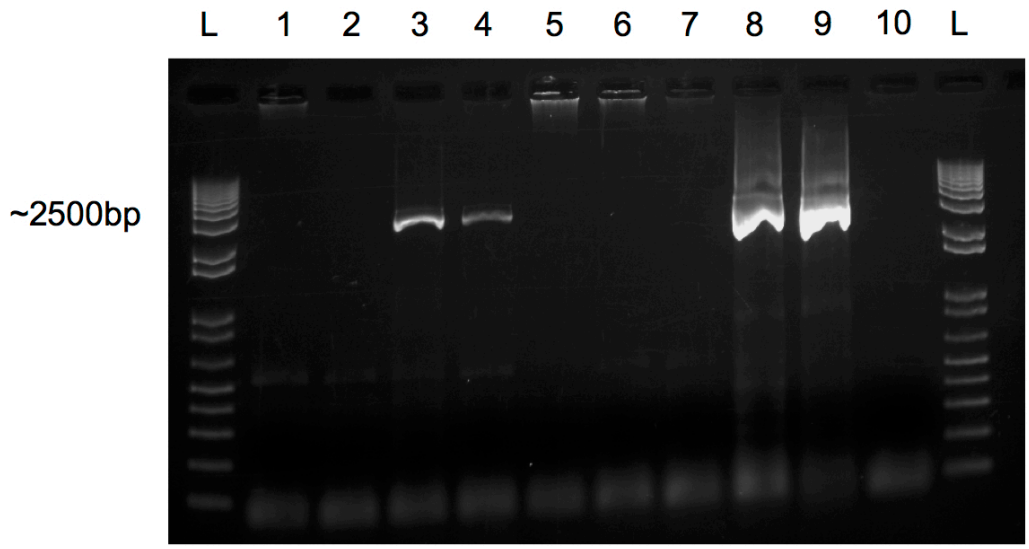

Figure 5.15 – RT-PCR Amplification of the 3′ Section of the TuYV Genome for the Detection of Infection by the Infectious Clone of the Upper Leaves. Total RNA extracted from inoculated, mock inoculated and negative control *N. benthamiana* and OSR plants, RT-PCR with primers MN37 and MN48. L) 1KB$^+$ ladder. 1) OSR un-inoculated negative control. 2) OSR mock inoculated control. 3) OSR inoculated with the infectious clone. 4) OSR inoculated with the infectious clone. 5) *N. benthamiana* un-inoculated negative control. 6) *N. benthamiana* mock inoculated control. 7) *N. benthamiana* inoculated with the infectious clone. 8) OSR TuYV-infected positive control. 9) *N. benthamiana* TuYV-infected positive control. 10) dH_2O negative control. L) 1KB$^+$ ladder.

To assess whether the virus derived from the infectious clone could be transmitted by aphids from systemically infected OSR leaves, TuYV positive plants were colonised with non-virliferous *M. persicae* and left to spread to neighbouring uninfected plants. Three weeks later, plants were tested by ELISA to see if TuYV was present in the uninfected plants that had be introduced. The infectious clone was transmitted by aphids from the agro-inoculated OSR to both *N. benthamiana* and OSR plants (Table 5.2). The unsuccessful attempts to agro-inoculate *N. benthamiana* with the infectious clone were ineffective due to the agro-inoculation of *N. benthamiana* or the inability to detect TuYV rather than the infectious clones ability to use *N. benthamiana* as a host.

Table 5.2 – Aphid Transmission of the TuYV Infectious Clone Dectection by ELISA.

Inoculum	Challenged Plant	A_{405} Absorbance[a]
TuYV positive aphids	Oilseed rape	0.707
Agro-inoculated infectious clone	Oilseed rape	2.494
None	Oilseed rape	0.552
Non-viruliferous aphids	Oilseed rape	0.474
Aphids fed on infectious clone	Oilseed rape	0.781
TuYV positive aphids	*N. benthamiana*	0.810
None	*N. benthamiana*	0.624
Non-viruliferous aphids	*N. benthamiana*	0.601
Aphids fed on infectious clone	*N. benthamiana*	1.041

[a]Individual plant results

5.4 Discussion

In this study, a full-length infectious cDNA clone of TuYV-L1851-C was successfully developed. The infectious clone gave full systemic infection of OSR but no infection in *N. benthamiana* was detected (Figure 5.15) following agro-infection. However, the infectious clone-produced virus was transmissible by aphids to both OSR and *N. benthamiana* plants (Table 5.2). The infectious clone and agro-infection will be useful for further studies of TuYV using reverse genetic approaches, such as for determining host range and determinates of pathogenicity, viral gene functions, virus-plant-vector interactions and especially for discerning any differences between the three TuYV phylogenetic groups based on P0. This is the first TuYV infectious clone with a *Brassica* host origin and the first TuYV infectious clone derived utilising a gateway cloning system. This will help facilitate future work if different destination vectors are required for different applications of the TuYV-L1851-C infectious clone.

The infectious clone could be used to investigate TuYV in a more controlled manner, which could improve understanding as a standardised and quantified inoculation could be used. Although in this work the Agro-inoculation appeared to be variable this is more likely due to avoidable human error. This method of inoculation will avoid aphid selective feeding causing false negative results. Specific genetically variable regions can now be investigated to discover their influence on TuYV host range and pathogenicity. This could be achieved by exchanging genetic regions between different genotypes, ensuring any biological differences observed are due to specific known sequence differences.

Many of the gene functions of TuYV and related species are known (Section 5.1) but with new genes or functions being discovered (Smirnova *et al.*, 2015), there is a need to confirm function of the *Polerovirus* genes in all species. Point or nonsense mutations might shed light on not only gene functions but the role of RNA secondary structure, which could be involved in transcription and infection of cells. For closely related viruses the use of viral clones has been instrumental in understanding gene functions utilising theses techniques. This has uncovered other traits of TuYV gene expression, such as selection against overexpression of P0 from the viral genome as enhanced translation caused instability during multiplication(Dunoyer *et al.*, 2002).

The infectious clone will be a valuable tool for future research on TuYV,

as many problems encountered during the course of this work in other chapters such as, keeping isolates pure and standardised inoculation will be either removed or reduced. The archive of isolates collected during this work (Chapter 3) can now be investigated fully, as previously isolate revival was not possible and thus any biological differences could not be investigated. This is due to the fact TuYV can not be mechanically inoculated nor have aphids take up TuYV from small amounts of frozen material. Molecular copying of these isolates will also allow future testing of any sources of TuYV resistance against a much broader range of TuYV isolates, such as the "weed-like" TuYV species uncovered in this study or the different recombinant isolates (Chapter 3).

Not only have infectious clones been used to investigate the characteristics of viruses (as mentioned in Section 5.1), but they have had other more experimental uses, and in the process uncovered other information such as identifying the importance of $5'$ sequence homology in the pathogenicity of viruses (Boyer and Haenni, 1994). One of the new areas viral clones are being utilised for is the production of Virus-Like Particles (VLPs). These are multi-subunit self-assembly competent protein structures (Zeltins, 2013). An example of a successful plant virus VLP was the manipulation of the CP of TMV infectious clones to produce nanotubes to house nickel (nanowires) in the hope that it will produce a robust basis for electronics in the future (Kadri *et al.*, 2011). Infectious clones have more potential then just to investigate the virus they are created from, and are a valuable source of bioengineering future technologies, with plants being able to accumulate large amounts of infectious clone products. A more experimental use of the TuYV infectious clone could be a possibility.

Chapter 6

Novel Sources of Resistance to TuYV Utilising *Arabidopsis thaliana* Knock-out Mutants

6.1 Background

6.1.1 Use of *Arabidopsis thaliana* to Investigate Sources of Resistance

Most efforts so far to breed lines of OSR resistant to TuYV have aimed to introduce genes to improve specific resistance traits against TuYV (Graichen and Peterka, 1999). The function or interaction of these genes with TuYV are still unknown. *Arabidopsis* as a member of the *Brassicaceae* family is the perfect model plant to look for resistance genes in the context of investigating possible sources of resistance that might be usable in OSR, as it is susceptible, has closely related gene orthologs and has very short generations times (Meyerowitz and Pruitt, 1985; Stevens *et al.*, 2005). An alternative method of investigating possible sources of resistance is by utilising knock-outs gene lines, genes that might be essential for virus replication or genes in pathways common to pathogen resistance *R* genes. Gene knock-outs are readily available in *Arabidopsis thaliana* due to the extensive genome annotations and T-DNA insert and EMS mutant lines available and have the benefit of introducing genetic variation that is not naturally present in the population.

It is known that TuYV causes reductions in fitness in *Arabidopsis* (reduced seed production, rosette size); these effects have been shown to be associated with virus titre within infected leaf tissue, assessed by ELISA after TuYV infection (Asare-Bediako, 2011). There are no known wild ecotypes of *Arabidopsis* that have resistance to TuYV (Stevens *et al.*, 2005). Tolerance, the ability of a plant to prevent itself from being damaged from infecting parasites, is also a desirable trait to combat disease (Salomon, 1999). The only known *Arabidopsis* ecotype with increased tolerance to TuYV is Ler-1, which had reduced yield losses, despite similar viral titres within leaf tissue four weeks after infection with TuYV, when compared to Col-0 and other ecotypes (Creissen *et al.*, 2015). However, tolerance seen in Ler-1 still lead to yield loss following TuYV infection and also has the potential for TuYV isolates over coming the tolerance. So far no classic [*R*] genes are known to be effective against TuYV in *Arabidopsis*.

This lack of natural resistance in both OSR and *Arabidopsis* means that this plant model can be used to investigate resistance via knock-out genes as no natural resistance is available. *Arabidopsis* is appropriate for such studies because of its highly annotated and sequenced genome with the availability of abundant knock-out mutants. Columbia (Col-0) is a widely used wild-type *Arabidopsis* seed due to its highly fertility, vigour and sensitivity to environmental changes. It is also highly susceptible to TuYV and is the genetic background of many gene knock-out lines, allowing for direct comparison of TuYV plant gene interactions. One of the gene families to confer resistance to a large range of plant viruses particularly potyviruses are the eukaryotic initiation factors (eIFs) of plants. They were found to interact with virus proteins directly in yeast two-hybrid binding assays (Chatel *et al.*, 1997). Interaction of essential functions such as virus replication if halted could stop the ability of viruses to infect plants could provide a valuable source of resistance, via a recessive form of resistance.

6.1.2 Loss of Function Resistance

Gene knock-outs in plants can results in recessive resistance if all copies of virus crucial genes are altered or removed conveying resistance due loss of function for key virus functions. The key observation in the understanding of the molecular nature of recessive resistance was that the virus protein genome-linked protein (VPg) was shown to have a direct interaction with plant eIFs. In contrast to plant cellular mRNAs that possess a m7G cap structure at their 5′ end and a

poly-A tail at their 3′ end, ssRNA viruses such as the poleroviruses have a VPg covalently attached to the 5′ terminus, mimicking plant RNA's m7G cap (Mayo and Ziegler-Graff, 1996), but no poly-A tail. Because poleroviruses exhibit vascular tissue tropism, virus replication and movement were thought to be limited to companion cells, phloem parenchyma cells, and sieve tubes (Mutterer *et al.*, 1999). However, all known recessive resistances to plant viruses involve eIFs (Le Gall *et al.*, 2011), the proteins involved in translation of plant and some viral RNA into proteins. The involvement of *eIF4E*, its isoform *eIF(iso)4E* (Robaglia and Caranta, 2006) and to a lesser extent *eIF4G* (Le Gall *et al.*, 2011; Tavert-Roudet *et al.*, 2012) have been identified in the investigation of recessive resistances to plant viruses. This was predominantly evident for recessive resistance to members of the *Potyviridae* family. Robaglia and Caranta (2006) concluded that the diversity of the resistance phenotypes was the result of a few amino acid changes in the *eIF4E* proteins conveying recessive resistance. The amino acid changes associated with resistance were clustered near the cap-binding pocket and at the surface of the protein (Robaglia and Caranta, 2006). Now it is known that this type of resistance can occur with viruses within the family of *Polerovirus* (Daughenbaugh *et al.*, 2003; Reinbold *et al.*, 2013), proving that interaction of poleroviruses' VPg is possible between the virus and the plant eIFs opening possible avenues of investigation (Chatel *et al.*, 1997; Kawaguchi and Bailey-Serres, 2002; Michon *et al.*, 2006; Jiang and Laliberté, 2011).

Both OSR and *Arabidopsis thaliana* have several copies (homologues) of the same gene, which for recessive resistance all homologues of a gene of interest need investigating to ensure resistance is viable and maintained. *Arabidopsis* encodes three genes for the *eIF4E* family (*eIF4E.1*, *eIF4E.2*, and *eIF4E.3*). One gene codes for *eIF(iso)4E* (Duprat *et al.*, 2002), and one for *eIF4E*-like protein, known as a novel cap-binding protein (*nCBP*) (Ruud *et al.*, 1998; Reinbold *et al.*, 2013). There is only one copy of eIF4G and two of the *eIF(iso)4G* subfamily (*eIF(iso)4G.1* and *eIF(iso)4G.2*) (Robaglia and Caranta, 2006; Treder *et al.*, 2008; Gallois *et al.*, 2010). Another sub-family of eIF that has been shown to be involved with viral replication are the *eIF3d* subunits, which interact with the VPg, initiating translation of calicivirus RNA, demonstrating another potential source of viral resistance if altered/removed (Daughenbaugh *et al.*, 2003).

A distinctive feature of *eIF4E* and *eIF4G*, in comparison with other host factors required for plant virus interactions, is that they show natural polymorphism associated with recessive resistance in many crops. The *eIF4E* factor

has been implicated in natural resistance to several potyviruses in diverse plant species (Le Gall *et al.*, 2011). This gene was also shown to be effective against the carmovirus *Tombusviridae* in melon. Carmoviruses are uncapped and non-polyadenylated viruses much like poleroviruses (Nieto *et al.*, 2006). It is thought that different plants regulate translation in unique ways with diverse and different eIF factors (Browning, 2004). This might be one factor behind host specificity in viruses, with the 3′ UTR region of viruses interacting with these factors in place of a poly-A tail (Truniger *et al.*, 2008). Termed the 3′ cap-independent translation enhancer (3′ CITE), this viral region functions by recruiting either translation initiation factors or the 60S ribosomal subunit to the viral RNA in members of both *Tombusviridae* and *Luteoviridae* families (Nicholson and White, 2011). Alteration or removal of this gene leads to passive resistance, also known as a loss of susceptibility (Lellis *et al.*, 2002). It is possible to mutate some of these eIF genes and produce resistance, while still allowing healthy plant growth (Duprat *et al.*, 2002).

There are also alternative proposed roles of eIF (Wang and Krishnaswamy, 2012). eIF4E, viral factors and eIF4G may form a complex that binds to VPg to mediate intracellular trafficking of the viral genome for targeting to plasmodesmata for cell-to-cell movement and, further, for systemic infection (Arroyo-García *et al.*, 1996). Secondly the VPg-eIF4E complex may be involved in the suppression of eIF4E-mediated transport of mRNA from the nucleus to the cytoplasm for translation and in the disturbance of siRNA and microRNA processing in the nucleus (Rajamäki and Valkonen, 2009).

Other genes which are not in the eIF family but might offer similar recessive resistance are *ASK1* and its otherlog *ASK2*, which are related to S-phase kinase-related protein 1 (*SKP1*) (Pazhouhandeh *et al.*, 2006), a component of the SCF family of ubiquitin E3 ligases. These contain a F-box-like motif that the P0 of poleroviruses is known to interact with, stopping post-transcriptional silencing of viral dsRNA in plants (Bortolamiol *et al.*, 2007). Knockdown of these types of genes have rendered *N. benthamiana* plants resistant to *Polerovirus* infection (Pazhouhandeh *et al.*, 2006). However, like eIF genes, the removal or alteration of *SKP1* genes could also prove to be detrimental to the plants as they are key for plant development (Zhao *et al.*, 2003). Other potential resistance genes are *SUC1* and *SUC2*. These are energy-dependent H^+/sucrose symporters that actively load sucrose into phloem companion cells (Truernit and Sauer, 1995). This is an essential process in apoplastic loaders, such as

Arabidopsis or *N. benthamiana* and is even used by symplastic loaders such as melon under certain stress conditions (Wippel and Sauer, 2012). In melon it was found that Cucumber mosaic virus (CMV) enhanced loading by increasing SUC gene expression increasing the potential for viral movement (Gil *et al.*, 2011). Therefore due to TuYV being limited to companion cells and with the knowledge of how it moves between these cells within plants being limited, SUC genes might be involved with its systemic infection of plants, thus loss of gene function could confer resistance. The involvement of SUC genes is affected further by members of the *AKT2/3* family, which have been identified as a gene encoding photosynthate-induced phloem K^+ channels, where potassium regulates the H^+/sucrose symporters via the phloem potential (Deeken *et al.*, 2002). Removal of these regulators reduce pholem sucrose by half, demonstrating the effect these regulator could have on reducing sucrose loading and potentially TuYV movement through the symporters (Hipper *et al.*, 2013). TuYV use of symporters has not been previously been demonstrated. It is known which viral genes are necessary for movement, however plant mechanisms that aid this movement are unknown (Hipper *et al.*, 2013). Knowledge of how TuYV undertakes movement within cells is also unclear, however Tobacco mosaic virus (TMV) proteins appear to use the macromolecular trafficking capacity of plasmodesmata to act as non-cell-autonomous proteins (Citovsky, 1999). Evidence consistent with this notion has been gained linking this trafficking to the MP of TMV (Ben-Nissan *et al.*, 2008). Plasmodesmal-associated protein kinase (*PAPK*) has been shown to be involved with this transport of TMV by specifically phosphorylating the C-terminal residues of TMV MP. *PAPK* is a member of the casein kinase I family. *PAPK* represents a novel plant protein kinase that is targeted to plasmodesmata which TuYV is now know to be associate with (Smirnova *et al.*, 2015) and may play a regulatory role in macromolecular trafficking between plant cells (Lee *et al.*, 2005).

In this chapter *Arabidopsis* genes that could be potentially be involved in TuYV replication, movement or post-transcriptional silencing were investigated to identify possible novel sources of resistance. Loss of function resistance is a recessive form of resistance, as such, *Arabidopsis* lines tested were genotyped for homozygous individuals. The lines selected can not only be used to assess resistance, but results could reveal novel plant-virus interactions.

6.2 Materials and Methods

6.2.1 Arabidopsis Growth Methods

Arabidopsis plants were grown in 6:1:1, Seed and Modular - F2S compost (Levington):sand:vermiculite fine grade in P40 modules. Each knock-out line had 10 modules sown with 3-4 seeds in each pot to ensure germination of a sufficient number of plants allowing 10 plants per line. Seeds were also germinated on 1% agar in petri dishes if germination was poor (below 50% germination). Seed were kept at room temperature for 1-2 days to break dormancy from cold storage. After this the seeds were stratified to achieve higher and more uniform germination rates; the pots were covered in tin foil and placed at 4°C for three days and petri dishes placed in plastic bags at 4°C. Seeds were moved to 10-hour day length growth cabinets at 10°C ± 2°C to allow better germination rates, as well as stop premature flowering and senescence. Multiple P40 modules were placed in large trays and the plants were tray watered as required a minimum of three times a week. After about 2 weeks seedlings were transplanted to one plant per pot. At 6 weeks the plants were segregated to allow healthy unchallenged controls to be separated from aphid challenged plants, then TuYV aphid-transmission was carried out. At 10 weeks ELISA was performed on all plants, with 5 individuals per line randomly selected for DNA extraction and confirmation of T-DNA in knock-out genes and EMS mutations.

6.2.2 *Arabidopsis thaliana* Knock-out Lines

Knock-out lines of *Arabidopsis* were selected based on a literature review of TuYV plant interactions, thus selecting possible candidate genes for resistance study (Table 6.1). Knock-out lines were ordered from the the European Arabidopsis Stock Centre (uNASC).

Table 6.1 – Arabidopsis Knock-out Lines Selected for Resistance Study.

uNASC Code	Knock-out[a]	Segregation[b]	Gene	Genetic Background
AT08002	Col-0	N/A	N/A	Col-0
N6552[c]	*eIF4E* (*cum1*)	Homozygous	*AT4G18040*	Col-0
N663174	*eIF4E.2*	Homozygous	*AT1G29590*	Col-0
N663501	*eIF4E.3*	Homozygous	*AT1G29550*	Col-0
AT07001	*eIF(iso)4E*	Homozygous	*AT5G35620*	Col-0
N6553[c]	*eIF4G* (*cum2*)	Homozygous	*AT3G60240*	Col-0
N673021	*eIF(iso)4G.1*	Homozygous	*AT5G57870*	Col-0
N677427	*eIF(iso)4G.2*	Homozygous	*AT5G18110*	Col-0
N679102	*nCBP*	Homozygous	*AT4G18040*	Col-0
N861685	*SUC1*	Heterozygous	*AT1G22710*	Col-0
N683085	*SUC2*	Heterozygous	*AT1G71880*	Col-0
N653316	*ASK1*	Homozygous	*AT1G10940*	Col-0
N657974	*ASK2*	Homozygous	*AT5G08590*	Col-0
N679170	*AKT2/3*	Homozygous	*AT4G22200*	Col-0
N532011	*CK1 (PAPK)*	Heterozygous	*AT4G28540*	Col-0
N698299	*eIF3d.1*	Heterozygous	*AT4G20980*	Col-0
N668575	*eIF3d.2*	Heterozygous	*AT5G44320*	Col-0

[a]All T-DNA Mutants are SALK lines
[b]Predicted from uNASC
[c]Ethyl methanesulfonate mutates

6.2.3 Experimental Design and Virus Challenge

The 17 selected lines (Table 6.1) were challenged with the three representative isolates (L1851-C, LAB-I, Cau74-R) independently from each other to avoid co-infection. The TuYV isolates represented three genetically distinct genotypes based on P0, Common, Intermediate and a Rare recombinant group. This was to investigate any differences in pathogenicity of the genotypes. Aphids were allowed to feed for one week, with all *Arabidopsis* lines being contained within the same insect-proof cage to aid aphid movement and feeding on all plants. Negative controls were not fed on by non-virerliferous aphids or sprayed with insecticide, due to limited insect confinement space available. Four weeks after initial introduction of aphids (2-3 weeks after aphid removal) plants were tested by ELISA with samples randomised on each plate (see Section 2.4.1). Once a week after aphids were introduced until plants were destroyed (4 weeks post aphid introduction) for ELISA testing, visual assessments were carried out as described in Section 2.4.2. Plants were assessed for stunting, leaf yellowing,

purpling and curling, when compared to unchallenged controls (See Section 2.4.2).

6.2.4 Confirmation of Knock-out Lines

For confirmation of the homozygosity of knock-out and mutation of genes causing loss of function, primers were designed to detect the mutation. Primers were designed from *Arabidopsis* gene sequences taken for Gbrowse via the TAIR website in relation to each knock-out line ordered, forward and reverse gene primers flanking the T-DNA insertion site were designed. Each gene-specific primer pair was used in conjunction with the T-DNA primer AMN29 (SALK LB 1.3) to allow detection of mutated lines, except N6552, N6553 and AT07001 (Table 6.2). Lines N6552, N6553 are EMS mutants and were sequenced to identify EMS induced polymorphisms, genotyping of AT07001 plants detected the presence and absence of a T-DNA insertion. Five plants were genotype form each line. Primers were used in PCR (outlined in Section 2.5.7), using 3 - 5ng of gDNA (See Section 2.5.1). Homozygous individuals were selected for selfing, however this could not be completed due to space requirements, containment issues and premature plant death.

Table 6.2 – Details of Primers Designed for *Arabidopsis thaliana* Genotyping.

Primer Name	Sequence (5′-3′)	Orientation	Target Gene
AMN1	ACTGTTTAGATCGTTGTTTTT	Forward	*SUC2*
AMN2	AGTGTTGTGCAAATCCTAC	Reverse	*SUC2*
AMN3	ATAGTACATATAAATATACACACTGTT	Forward	*SUC1*
AMN4	TATGGCAAGGTAGGGACAC	Reverse	*SUC1*
AMN5	TAGTCTATGAAAAAGTGAACAAGC	Forward	*eIF3d.2*
AMN6	GAGCCACTCCCTGAAGATG	Reverse	*eIF3d.2*
AMN7	AGTCTACACCTTTCGTTTCT	Forward	*eIF3d.1*
AMN8	AATTTGGTGGCTATAAGAGTCA	Reverse	*eIF3d.1*
AMN9	ACGTTTCGGGTTTATTGAC	Forward	*PAPK*
AMN10	GGATATGTTGTCTTGTCTTTA	Reverse	*PAPK*
AMN11	CTGTTGGAAAGATGGAGT	Forward	*ASK2*
AMN12	ATTATTAAGCTTTGATTGAA	Reverse	*ASK2*
AMN13	CCTCCTCTTCCTCCTCCACG	Forward	*ASK1*
AMN14	GCTCAAGCTGCATATTTCAAGAA	Reverse	*ASK1*
AMN15	TAATGGGCTATAGTAATGAAACA	Forward	*eIF4E.3*
AMN16	TAGGGCTCGTCGTGGTG	Reverse	*eIF4E.3*
AMN17	CTACATAGGTATGTGCTATTGTGT	Forward	*eIF4E.2*
AMN18	GATAGAGCAACTAAAGGTCAT	Reverse	*eIF4E.2*
AMN19	TTGAATAAAGTAGAAAGGTGTC	Forward	*nCBP*
AMN20	GTATCTTCTTCAATAAACCAAC	Reverse	*nCBP*
AMN21	GCAGATAGATAGAGGTATATAGTG	Forward	*eIF(iso)4G.2*
AMN22	TTTGATATACAGAATATTTTCGTAA	Reverse	*eIF(iso)4G.2*
AMN23	CGACCTTTTGCCCTATG	Forward	*eIF(iso)4G.1*
AMN24	TAATGCTAGATACCAAAATAAAA	Reverse	*eIF(iso)4G.1*
AMN25	ACTCAGAATCGTGGACAGACTATG	Forward	*eIF4G*
AMN26	ACCAATCTCTGTCAATGTCACTACT	Reverse	*eIF4G*
AMN27	CCGGTTAAAGTCAATCGCTC	Forward	*eIF4E*
AMN28	AGAGGAAGTACATTAGTTTGGAGAAG	Reverse	*eIF4E*
AMN29[a]	ATTTTGCCGATTTCGGAAC	Forward	T-DNA
AMN30[b]	GCGTGGACCGCTTGCTGCAACT	Forward	T-DNA
AMN31	GCTGAGAGAAGAAGCATCATACTC	Forward	*AKT2/3*
AMN32	CGATCCCGTGTTAATTATTGAAG	Reverse	*AKT2/3*
K01[c]	TTGACCCAATAGAGTCCAGAAAT	Forward	*eIF(iso)4E*
DSPM1[b]	CTTATTTCAGTAAGAGTGTGGGGTTTTGG	Reverse	*eIF(iso)4E*

[a]LB 1.3 SALK left border primer of the T-DNA insertion
[b]LB 1 SALK left border primer of the T-DNA insertion
[c]Taken from (Duprat *et al.*, 2002)

6.2.5 Statistics

Statistical analyses were performed using both Excel and R. Variance within the ELISA data of each knock-out line was assessed and compared to Col-0 as a control susceptible population, to allow for balanced and unbalanced data analy-

sis. ELISA A_{405} data was normalised with (x+1)log transformation (as some values were below 0) to standardise the data into a normal distribution by removing skew with qqplot analysis of the residuals. Following this transformation one way analysis of variance (ANOVA) was conducted on the data to allow the least significant difference (LSD) to be calculated. This was applied to the knock-out lines to identify results that were significant at the 0.05 alpha probability. The R package Agricolae was used to implement the LSD test (Crossa *et al.*, 1990). Heterozygous lines were omitted from the statistical analysis, to avoid unfair comparisons between populations.

6.3 Results

6.3.1 Genotyping of *Arabidopsis* Knock-out lines

To ensure correct interpretation of ELISA results of challenged *Arabidopsis*, lines underwent PCR and sequencing for confirmation of the presence/absence of the T-DNA, or loss of function mutation in each line (respectively). The results of the PCR genotyping (Figures 6.1 - 6.2) were as expected for most lines (Table 6.1), however the *PAPK* and *eIF3d.1* mutant lines were not heterozygous but homozygous for the T-DNA insertion. Three knock-out lines were heterozygous: *eIF3d.2*, *SUC1* and *SUC2* (Figure 6.2). The *eIF3d.2* knock-out line had mixed genotypes of heterozygous, wild-type homozygous and homozygous knock-out (Figure 6.2 D). *SUC1* knock-out individuals were uniformly heterozygous for the T-DNA insert and wild-type functional copy (Figure 6.2 G). *SUC2* also had a mixed genotype, plants were both homozygous for knock-out and wild-type (Figure 6.2 F). Sequence confirmation of the nucleotide substitution in both *eIF4E* and *eIF4G* (*cum1* and *cum2*, Yoshii *et al.* (2004)) revealed homozygous presence of eIF4E amino acid tryptophan^{99} to stop codon substitutions (TGG to TGA) and an eIF4G amino acid proline^{1327} to serine (CCG to TCG) substitution (Figure 6.3). Lines *SUC1*, *SUC2* and *eIF3d.2*, were the only heterozygous lines, attempts were made to produce homozygous seed but due to time, space and premature death these lines could not be established.

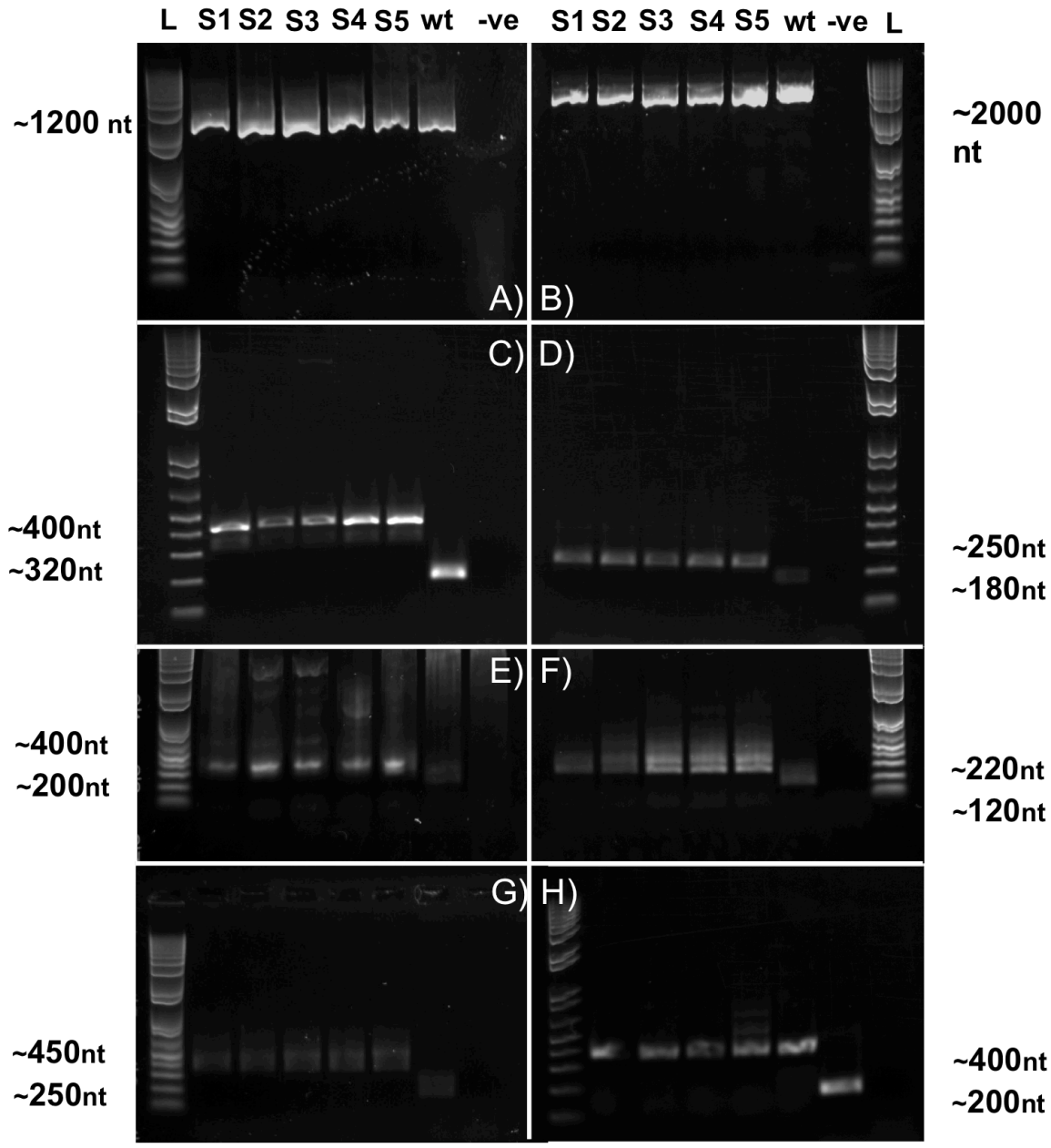

Figure 6.1 – Genotyping of *Arabidopsis* Knock-out Lines.
Five plants from each knock-out line were genotyped (S1-S5), along with a wild-type (wt) control (Col-0) and a dH_2O negative control was included along with KB^+ ladder (L). A) *eIF4E* gene amplification for genotyping by sequencing. B) *eIF4G* gene amplification for genotyping by sequencing. C) *eIF(iso)4G.1* T-DNA insert detection, homozygous knock-out detected when compared to wild-type genotype. D) *eIF(iso)4G.2* T-DNA insert detection, homozygous knock-out detected when compared to wild-type genotype. E) *nCBP* T-DNA insert detection, homozygous knock-out detected when compared to wild-type genotype. F) *eIF4E.2* T-DNA insert detection, homozygous knock-out detected when compared to wild-type genotype. G) *eIF4E.3* T-DNA insert detection, homozygous knock-out detected when compared to wild-type genotype. H) *ASK1* T-DNA insert detection, homozygous knock-out detected when compared to wild-type genotype.

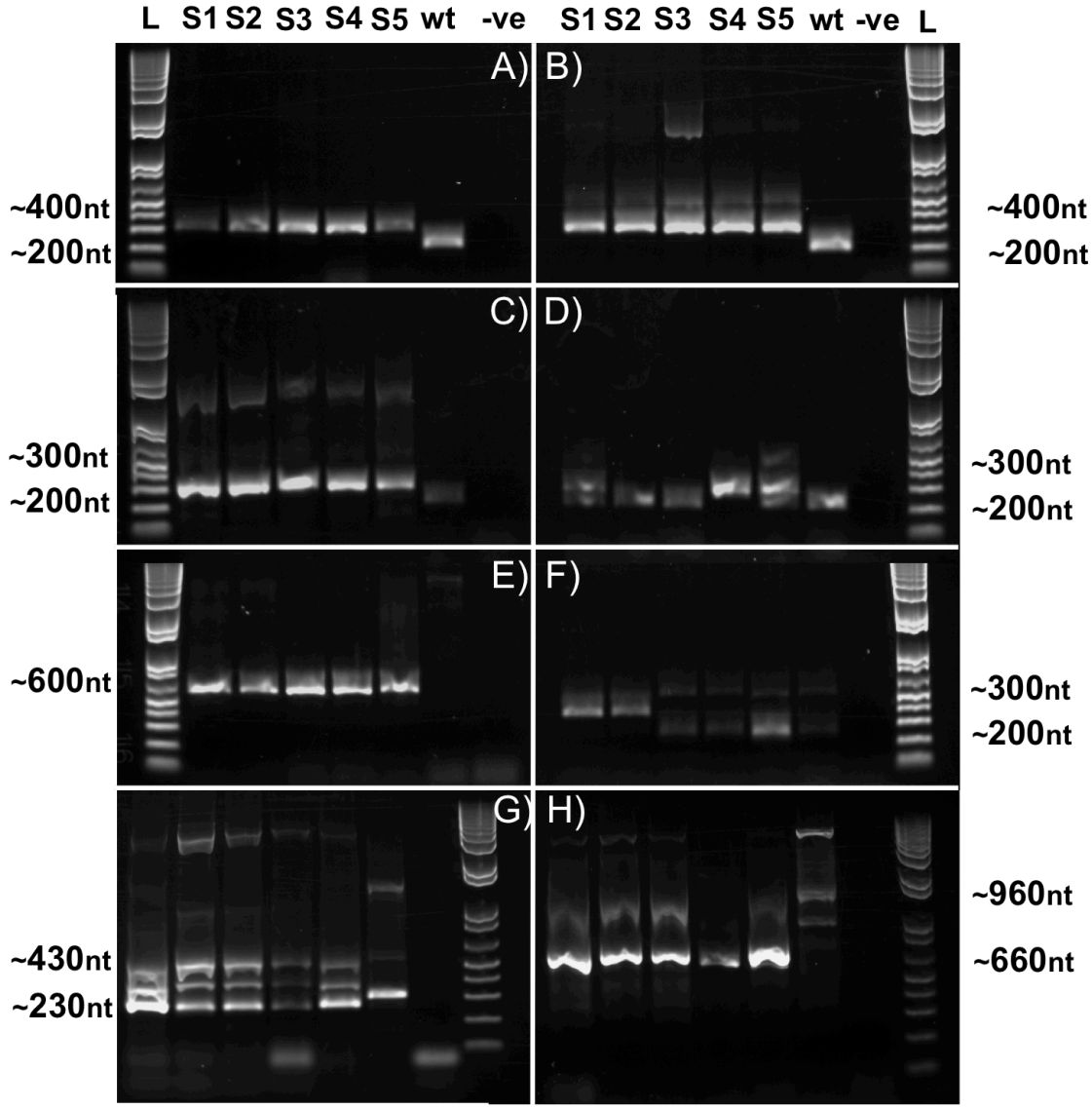

Figure 6.2 – Genotyping of *Arabidopsis* Knock-out Lines
Five plants from each knock-out line were genotyped (S1-S5), along with a wild-type (wt) control (Col-0) and a dH_2O negative control (-ve) was included along with KB^+ ladder (L). A) *ASK2* T-DNA insert detection, homozygous knock-out detected when compared to wild-type genotype. B) *PAPK* T-DNA insert detection, homozygous knock-out detected when compared to wild-type genotype. C) *eIF3d.1* T-DNA insert detection, homozygous knock-out detected when compared to wild-type genotype. D) *eIF3d.2* T-DNA insert detection, heterozygous knock-out detected when compared to wild-type genotype. E) *eIF(iso)4E* presence or absence detection of T-DNA insert, homozygous T-DNA insertion detected when compared to wild-type genotype. F) *SUC2* T-DNA insert detection, heterozygous knock-out detected when compared to wild-type genotype. G) *SUC1* T-DNA insert detection, heterozygous knock-out detected when compared to wild-type genotype. H) *AKT2/3* T-DNA insert detection, homozygous knock-out detected when compared to wild-type genotype.

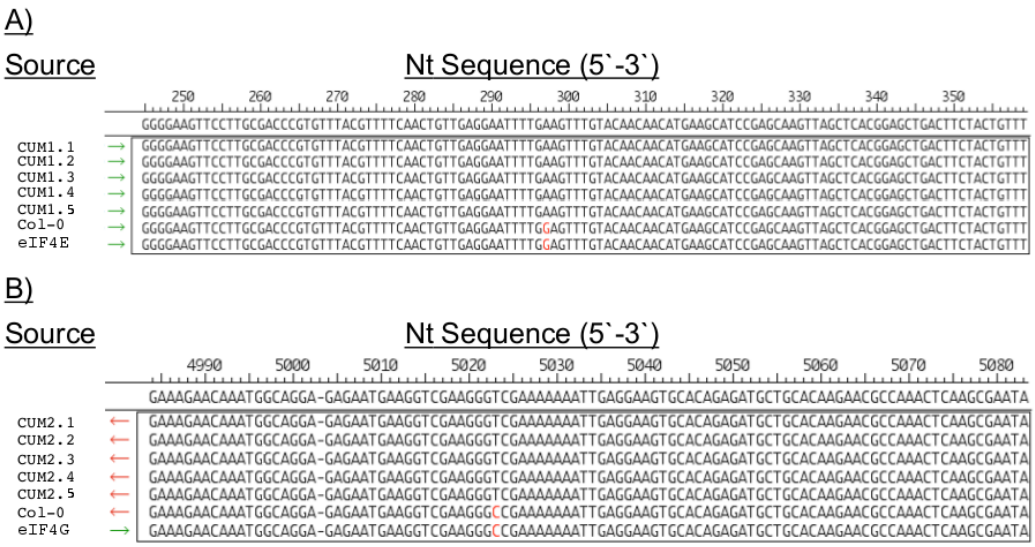

Figure 6.3 – Sequence Confirmation of Ethyl Methanesulfonate *Arabidopsis* Mutants.
A) *cum1* mutants aligned to coding sequence (CDS) of *eIF4E* with amino acid position Tryptophan^{99} - STOP TGG to TGA substitution. B) *cum2* mutants aligned to CDS of *eIF4G* with amino acid position proline^{1327} to serine CCG to TCG substitution.

6.3.2 *Arabidopsis* Knock-out lines ELISA results

6.3.2.1 Homozygous lines ELISA Results

Arabidopsis lines challenged with the three TuYV isolates (L1851-C, LAB-I, Cau74-R) were tested by ELISA. None of the lines investigated demonstrated extreme resistance to TuYV (Tables 6.3 - 6.5), but some lines exhibited reduced susceptibility relative to Col-0. The lines that had reduced susceptibility varied between the three isolates, indicating biological differences between the isolates and their interaction with the different *Arabidopsis* lines.

Arabidopsis *eIF4E* knock-out lines had lower ELISA values than that of the Col-0 controls, with significantly reduced levels of virus titre when challenged with LAB-I and Cau74-R. Knock-out line *eIF4E.2* had lower ELISA values than the controls, with significantly reduced levels for L1851-C and Cau74-R. Knock-out line *eIF4E.3* had varying effect on TuYV titre, with equivalent ELISA values when challenged with L1851-C compared to the susceptible control, but when challenged with Cau74-R there was significantly reduced TuYV titre. *Arabidopsis* knock-out line *eIF(iso)4E* had lower ELISA values than the

control for all isolates, but not to a significant degree. Knock-out line *eIF4G* had lower ELISA values than the Col-0 controls with significant reductions when challenged with L1851-C and LAB-I. Knock-out line *eIF(iso)4G.1* was the only line that had significant reductions in viral titre against all TuYV isolates. Knock-out line *eIF(iso)4G.2* was susceptible to L1851-C but when challenged with LAB-I and Cau74-R there were significant reductions in viral titre when compared to the Col-0 control. The knock-out line *nCBP* had similar ELISA values to the control when challenged with L1851-C and LAB-I, however Cau74-R challenged plants had a significant reduction in viral titre. Knock-out line *ASK1* was susceptible to L1851-C and LAB-I but had significantly reduced levels of Cau74-R. Knock-out line *ASK2* had lower ELISA results than the control, with significant reductions of LAB-I and Cau74-R viral titre, ASK gene interaction with P0 seems selective not broad spectrum as results were all higher than non-challenged Col-0 negative controls with variation in significance of the reductions (Tables 6.3 - 6.5). Knock-out line *AKT2/3* was susceptible to all TuYV isolates, with ELISA results being slightly lower or equivalent to the control, therefore it did not appear to reduce the level of virus throughout the plant to a significant degree. Knock-out line PAPK was susceptible to L1851-C but had lower ELISA results when challenged with LAB-I and Cau74-R, with Cau74-R showing a significant reduction in viral titre, indicating that its removal could effectively reduce but not stop TuYV's movement, replication or other factors, further investigation would be needed. Knock-out line *eIF3d.1* showed a significant reduction in the TuYV titre levels of L1851-C and Cau74-R, but was susceptible to LAB-I. No single gene investigated was able to give broad spectrum extreme resistance, only quantitative resistance which could be effective at reducing the impact of TuYV infection (Figures 6.4 - 6.6).

6.3.2.2 Heterozygous lines ELISA Results

Knock-out line *SUC1* was susceptible to L1851-C and LAB-I but had large reductions in viral titre when challenged with Cau74-R, so potentially reduced viral movement. ELISA results for the *SUC1* heterozygous lines were relativity consistent (Figures 6.4 - 6.6), more so than some homozygous knock-out lines, however a large spread of ELISA results were seen when challenged with TuYV isolate L1851-C. This constancy is possibly because compared to other heterozygous lines investigated, *SUC1* is uniformly heterozygous for the knock-out (Figure 6.2 G). *SUC1* resistance would fit a recessive model, therefore creation of a homozygous line of *SUC1* would be of interest, especially as Cau74-R

TuYV-challenged *SUC1* had a mean A_{405} ELISA value of 0.483, far below Col-0 mean A_{405} ELISA value of 1.835 (Table 6.5).

Knock-out line *SUC2* was susceptible to all three TuYV isolates, LAB-I and Cau74-R challenged plants did, however, have lower ELISA results compared to the Col-0 controls but not to a significant degree. Within the *SUC2* line individuals were both homozygous and heterozygous for the knock-out. Homozygous plants' ELISA A_{405} values were 0.301-0.790, whereas the heterozygous genotype individuals range was 1.485-3.215. This spread of values highlights that the creation of a *SUC2* homozygous line to investigate its knock-out from TuYV ability to effect infection could provide information leading to a population with quantitative recessive resistance.

The last line with heterozygous T-DNA insertion was *eIF3d.2* had lower ELISA results than the susceptible control when challenged with all TuYV isolates, however these were not significant reductions. The individual *eIF3d.2* plant that had the homozygous knock-out genotype had an ELISA A_{405} value of 0.165 with heterozygous knock-outs ranging between 0.459-0.418 and the homozygous wild-type individuals 2.456-2.954. These results indicated reduced TuYV titre within homozygous *eIF3d.2* knock-out plants. The mean ELISA results for *eIF3d.2* against all isolates tested was consistently lower than that of the susceptible controls. This indicates homozygous *eIF3d.2* could confer resistance that could significantly reduce TuYV titre and as well as indicating a new interaction between poleroviruses and a member of the eIF gene family.

Table 6.3 – Phenotypic and ELISA Data for *Arabidopsis thaliana* Knock-out Lines Challenged with TuYV Isolate L1851-C

	No. of plants with phenotypes[a]		Mean ELISA optical density[b]	
Arabidopsis line	0[c]	S[d]	Challenged	Control
Col-0 (wild-type)	0	8	1.174	0.066
eIF4E	1	6	0.970	0.049
eIF4E.2	1	7	0.834*[e]	0.055
eIF4E.3	6	1	1.23	0.054
eIF(iso)4E	7	1	0.921	0.048
eIF4G	5	2	0.625*	0.051
eIF4(iso)G.1	7	1	0.590*	0.060
eIF4(iso)G.2	8	0	1.192	0.060
nCBP	7	1	1.248	0.061
SUC1	2	6	1.563	0.155
SUC2	2	6	1.187	0.112
ASK1	1	5	1.045	0.085
ASK2	0	8	0.998	0.088
AKT2/3	0	8	1.102	0.089
PAPK	1	6	1.249	0.169
eIF3d.1	1	7	0.604*	0.158
eIF3d.2	6	2	0.804	0.147

[a] Eight plants inoculated per knock-out lines, if total number tested is lower this was due to poor germination.

[b] ELISA absorbance measured at 405 nm.

[c] No symptoms observed.

[d] Showed yellowing, purpling and/or leaf curling symptoms.

[e] A_{405} values from ELISA of aphid challenged plants were significant different from challenged Col-0 controls (following LSD analysis)

Table 6.4 – Phenotypic and ELISA Data for *Arabidopsis thaliana* Knock-out Lines Challenged with TuYV Isolate LAB-I

	No. of plants with phenotypes[a]		Mean ELISA optical density[b]	
Arabidopsis line	0[c]	S[d]	Challenged	Control
Col-0 (wild-type)	0	8	2.065	0.180
eIF4E	1	7	1.127*[e]	0.295
eIF4E.2	1	6	1.286	0.265
eIF4E.3	1	7	1.427	0.194
eIF(iso)4E	2	6	1.291	0.227
eIF4G	0	8	1.010*	0.220
eIF4(iso)G.1	0	8	0.812*	0.294
eIF4(iso)G.2	0	8	1.204*	0.268
nCBP	0	8	1.707	0.176
SUC1	4	4	1.311	0.236
SUC2	2	6	1.626	0.191
ASK1	0	5	1.653	0.257
ASK2	1	7	1.150*	0.227
AKT2/3	1	7	1.758	0.246
PAPK	3	4	1.332	0.236
eIF3d.1	2	6	1.777	0.181
eIF3d.2	1	7	1.679	0.304

[a] Eight plants inoculated per knock-out lines, if total number tested is lower this was due to poor germination.

[b] ELISA absorbance measured at 405 nm.

[c] No symptoms observed.

[d] Showed yellowing, purpling and/or leaf curling symptoms.

[e] A_{405} values from ELISA of aphid challenged plants were significant different from challenged Col-0 controls (following LSD analysis)

Table 6.5 – Phenotypic and ELISA Data for *Arabidopsis thaliana* Knock-out Lines Challenged with TuYV Isolate Cau74-R

	No. of plants with phenotypes[a]		Mean ELISA optical density[b]	
Arabidopsis line	0[c]	S[d]	Challenged	Control
Col-0 (wild-type)	0	8	1.835	0.193
eIF4E	0	8	0.628*[e]	0.295
eIF4E.2	0	7	0.937*	0.182
eIF4E.3	0	7	0.935*	0.179
eIF(iso)4E	1	7	1.057	0.167
eIF4G	1	7	1.138	0.146
eIF4(iso)G.1	0	8	0.552*	0.266
eIF4(iso)G.2	0	8	0.846*	0.152
nCBP	0	8	0.905*	0.151
SUC1	0	8	0.483	0.231
SUC2	0	8	1.09	0.140
ASK1	0	4	0.884*	0.187
ASK2	0	8	1.036*	0.205
AKT2/3	0	8	1.811	0.189
PAPK	0	8	0.542*	0.190
eIF3d.1	0	8	0.723*	0.157
eIF3d.2	0	8	1.140	0.242

[a] Eight plants inoculated per knock-out lines, if total number tested is lower this was due to poor germination

[b] ELISA absorbance measured at 405 nm.

[c] No symptoms observed.

[d] Showed yellowing, purpling and/or leaf curling symptoms.

[e] A_{405} values from ELISA of aphid challenged plants were significant different from challenged Col-0 controls (following LSD analysis)

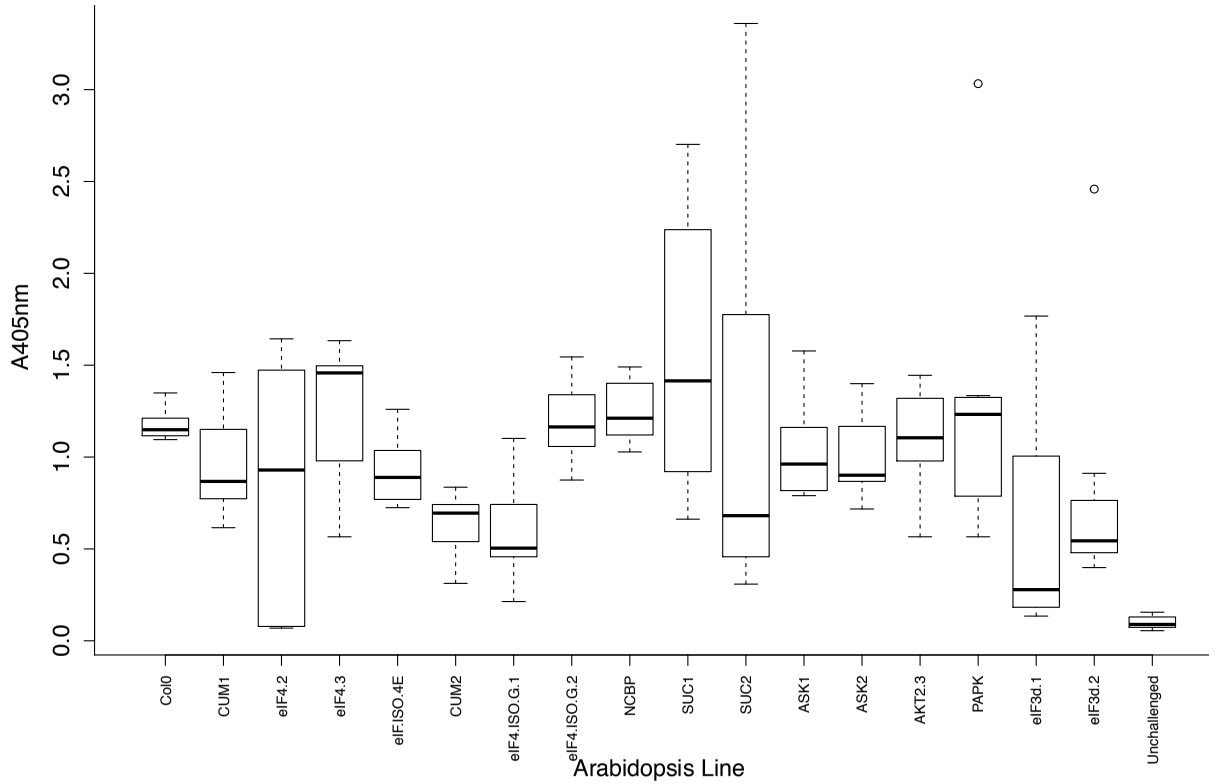

Figure 6.4 – Box and Whisker-Plots of ELISA Results of *Arabidopsis* knock-out line challenged TuYV Isolate L1851-C.
Box and Whisker plots representation of the A_{405} ELISA data collected 4 weeks after plant inoculation with L1851-C. Circles represent outlying results. Whiskers indicate the highest and lowest values. The ends of the box represent the upper and lower quartiles, the bar is the median (Constructed with R-Studio).

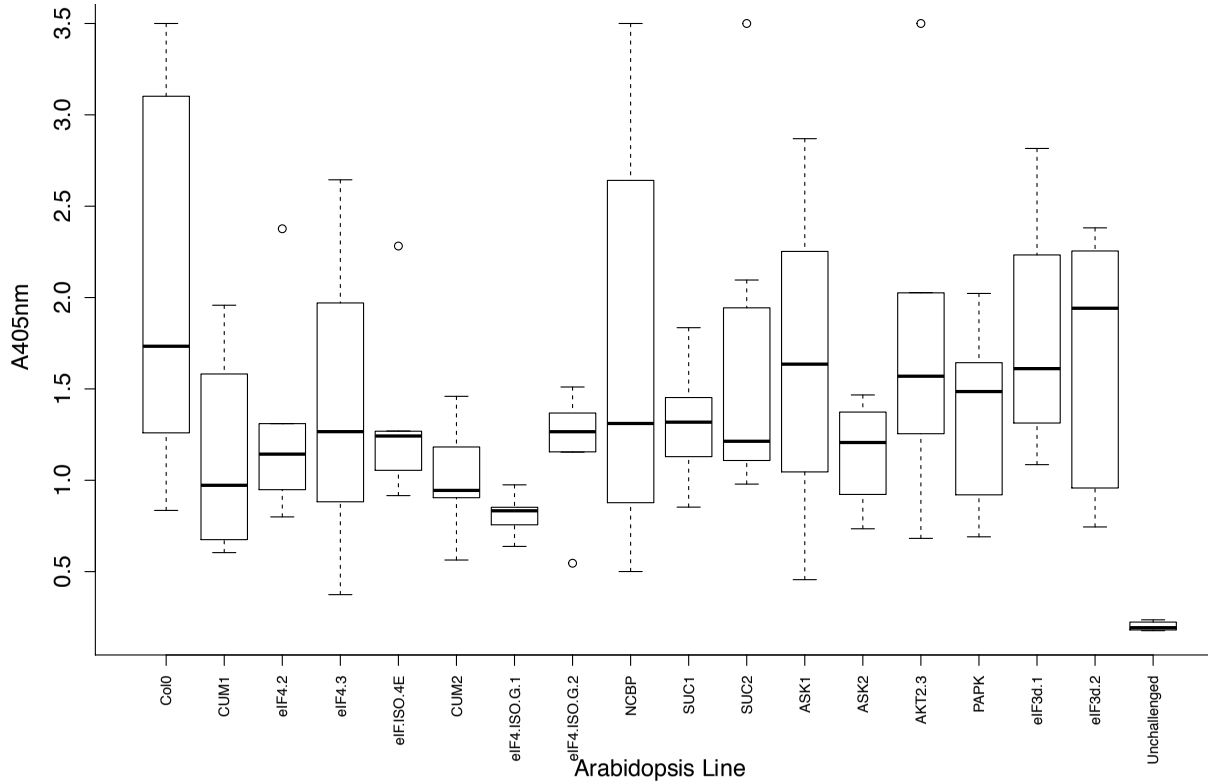

Figure 6.5 – Box and Whisker-Plots of ELISA Results of *Arabidopsis* knock-out line challenged TuYV Isolate LAB-I.
Box and Whisker plots representation of the A_{405} ELISA data collected 4 weeks after plant inoculation with LAB-I. Circles represent outlying results. Whiskers indicate the highest and lowest values. The ends of the box represent the upper and lower quartiles, the bar is the median (Constructed with R-Studio).

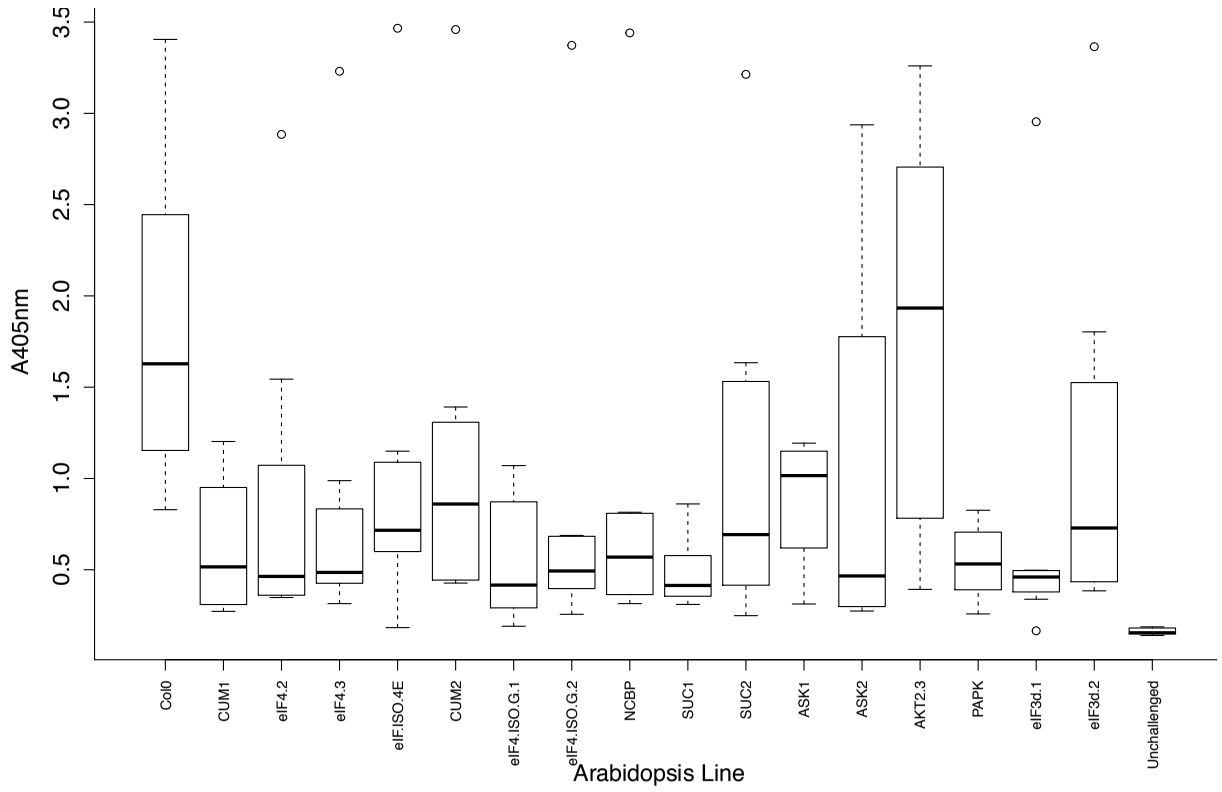

Figure 6.6 – Box and Whisker-Plots of ELISA Results of *Arabidopsis* knock-out line challenged TuYV Isolate Cau74-R.
Box and Whisker plots representation of the A_{405} ELISA data collected 4 weeks after plant inoculation with Cau74-R. Circles represent outlying results. Whiskers indicate the highest and lowest values. The ends of the box represent the upper and lower quartiles, the bar is the median (Constructed with R-Studio).

6.3.3 Arabidopsis Knock-out lines Phenotypes

The phenotype of TuYV-challenged *Arabidopsis* lines (Figures 6.7 - 6.9) are hard to attribute to TuYV infection, as symptoms of TuYV are muted and mimic stress symptoms also some are heterozygous (Stevens *et al.*, 2008b). This problem is compounded by the caveat that in this experiment the unchallenged control plants could not undergo the same aphid and insecticide treatments as the TuYV challenged plants stopping direct comparison. Areas of necrosis and leaf curling of plants could be due to the week of aphid feeding, as symptom assessment of each *Arabidopsis* line indicated a recovery from symptoms after the removal of aphids. Purpling and yellowing symptoms (Figures 6.7 - 6.9) in some lines could be due to growth conditions resulting in abiotic stresses, possibly due to being pot bound, aphid feeding. There were a number of plants in certain lines which did not show any symptoms, but this was not consistent between the different TuYV isolates each line was challenged with. TuYV L1851-C challenged plants resulted in the most symptomless plants (Table 6.3) with TuYV Cau74-R having the least (Table 6.5); these results do not correlate with ELISA results (Figures 6.4 - 6.6). This suggested that the symptoms could be largely due to growing conditions, with TuYV infection being an additional stress adding to the stress-like symptoms. However, all Col-0 positive control plants showed symptoms compared to the variability seen in the other lines so this could be discounted and symptoms seen as the result of TuYV titre or lack of within the knock-out lines.

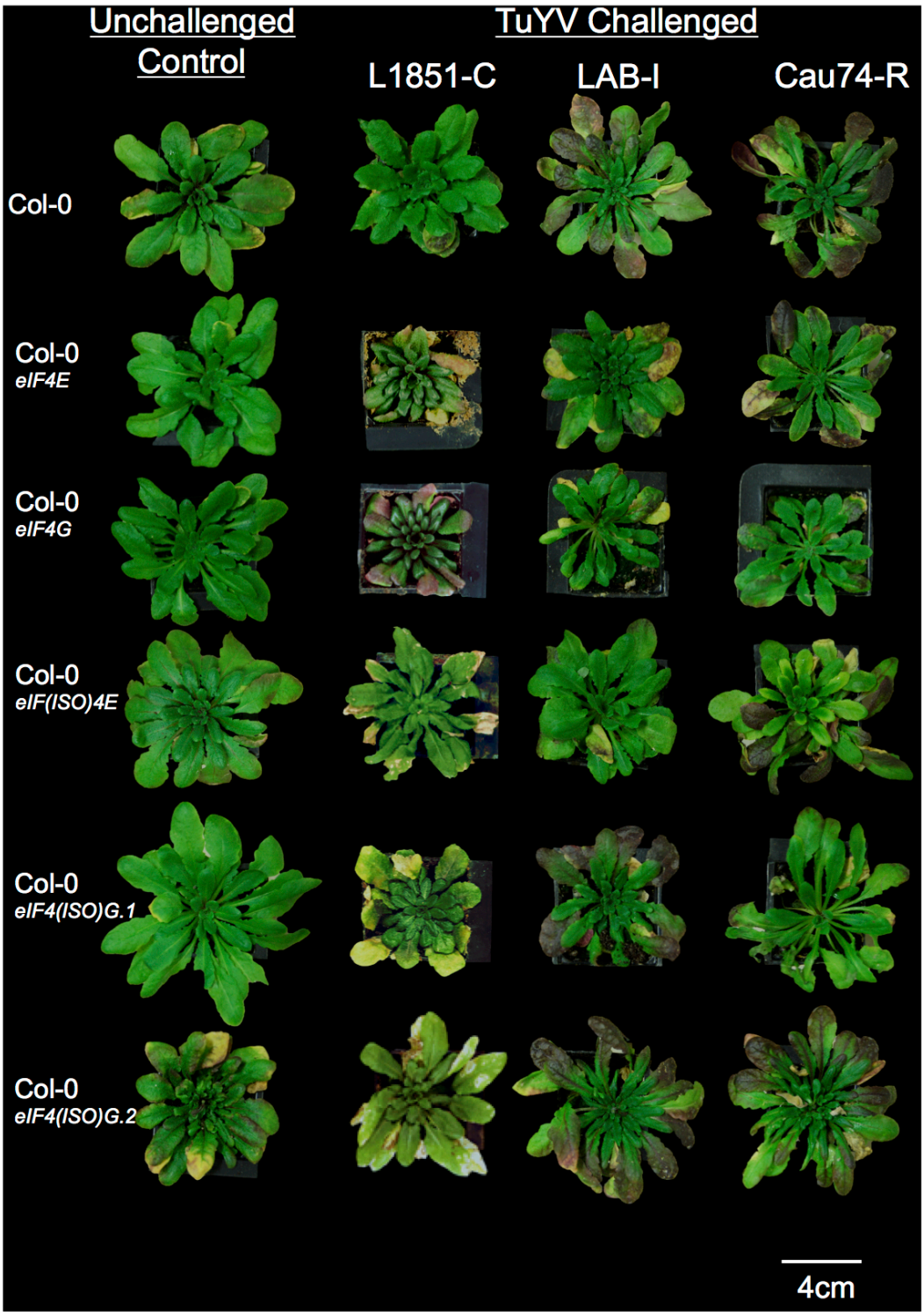

Figure 6.7 – TuYV Challenged *Arabidopsis* Knock-out Lines
Phenotypes of *Arabidopsis* knock-out lines after challenging with three different TuYV isolates (L1851-C, LAB-I, Cau74-R), using *M. persicae* as the transmission vector. 30 days post inoculation (DPI).

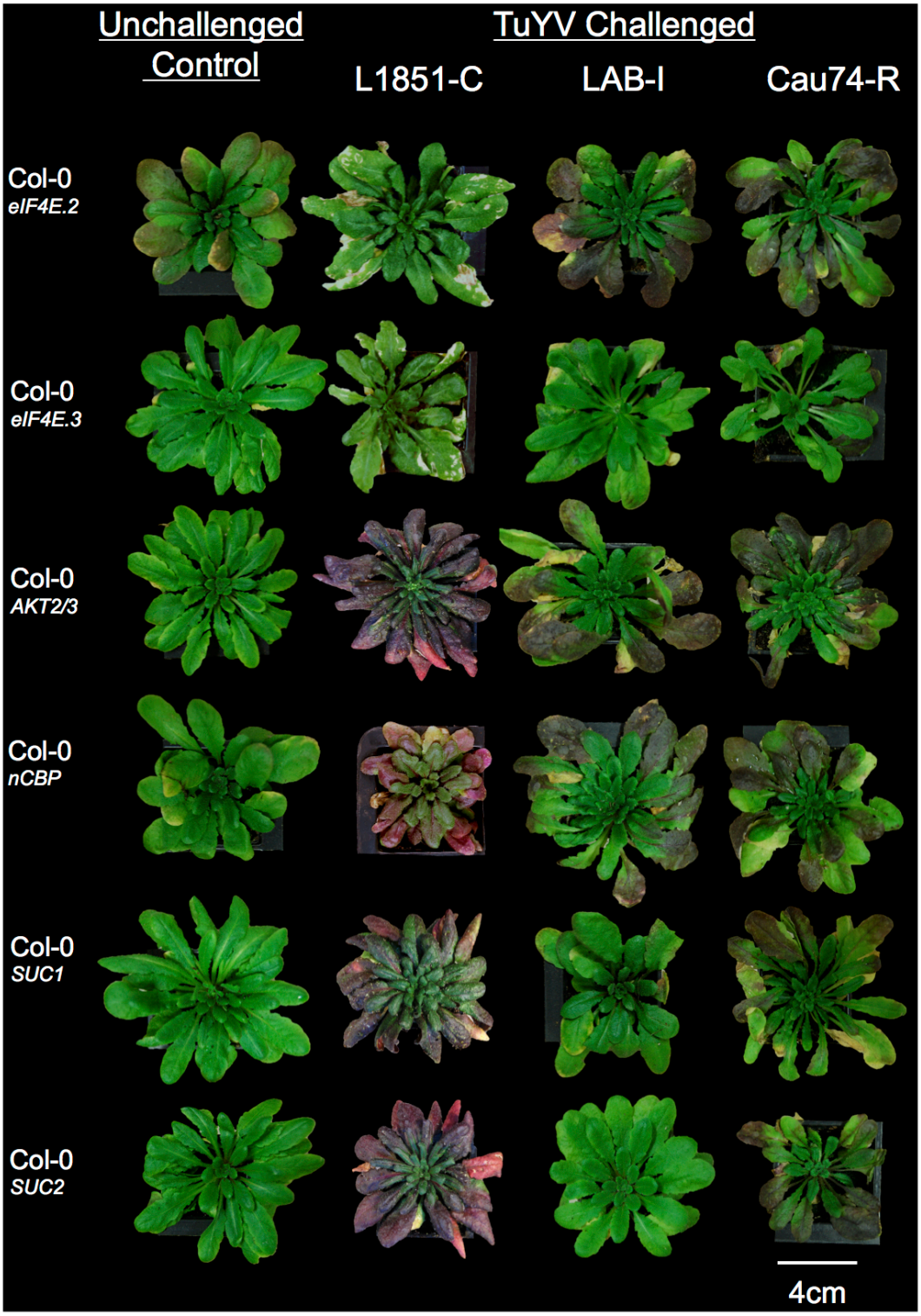

Figure 6.8 – TuYV Challenged *Arabidopsis* Knock-out Lines
Phenotypes of *Arabidopsis* knock-out lines after challenging with three different TuYV isolates (L1851-C, LAB-I, Cau74-R), using *M. persicae* as the transmission vector. 30 DPI.

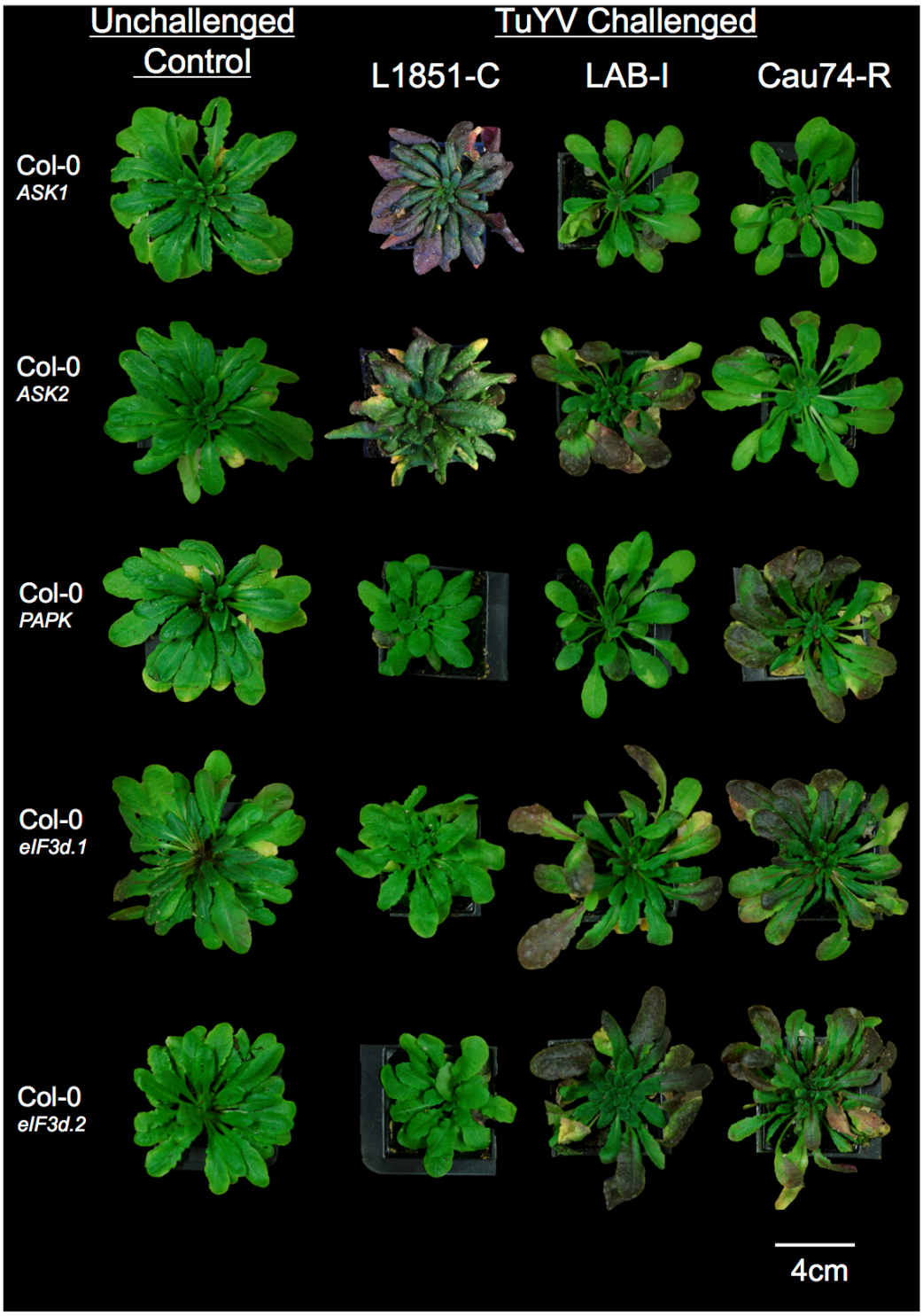

Figure 6.9 – TuYV Challenged *Arabidopsis* Knock-out Lines
Phenotypes of *Arabidopsis* knock-out lines after challenging with three different TuYV isolates (L1851-C, LAB-I, Cau74-R), using *M. persicae* as the transmission vector. 30 DPI.

6.3.3.1 Homozygous lines Phenotypes

Phenotypes of unchallenged control knock-out lines generally were very similar to Col-0, which is the genetic background of each knock-out (Table 6.1). However, *eIF4E* and *eIF4G* mutant lines had poor germination (∼10%) compared to the other lines (∼90-100%). TuYV-challenged Col-0 plants had consistent purpling and yellowing of outer leaves with leaf curling symptoms with all TuYV isolates (Figure 6.7). *eIF4E* knock-out plants exhibited stunting when inoculated with TuYV L1851-C; all challenged *eIF4E* plants consistently had yellowing and purpling symptoms of the outer leaves (Figure 6.7). The TuYV L1851-C challenged *eIF(iso)4E* knock-out line had one plant that exhibited stunting with yellowing of the outer leaves, LAB-I TuYV induced similar stunting symptoms, but with purpling of the outer leaves, Cau74-R TuYV-challenged plants were not stunted but had yellowing and purpling of the outer leaves along with leaf curling (Figure 6.7). All *eIF4E.2* challenged plants had purpling of their outer leaves, L1851-C TuYV-challenged plants also exhibited necrosis of the outer leaves, Cau74-R challenged plants also had leaf curling (Figure 6.8). All *eIF4E.3* plants challenged with TuYV and showing symptoms had yellowing of the outer leaves, both L1851-C TuYV and Cau74-R TuYV-challenged plants were also stunted. L1851-C TuYV-challenged plants also had necrotic areas on the outer leaves, which became less prominent after the removal of aphids (Figure 6.8).

All *eIF4G* plants challenged with TuYV showed stunting along with yellowing and purpling of the outer leaves (Figure 6.7). The *eIF(iso)G.1* plants challenged with TuYV L1851-C showed stunting and yellowing of their outer leaves, LAB-I TuYV-challenged plants had stunting and purpling of the outer leaves, with Cau74-R TuYV-challenged plants having no stunting but yellowing of the outer leaves and leaf curling (Figure 6.7). The *eIF(iso)G.2* plants challenged with L1851-C TuYV had areas of necrosis on the outer leaves along with leaf curling, LAB-I TuYV-challenged plants also had leaf curling but with purpling of the outer leaves, Cau74-R TuYV-challenged plants had purpling of the outer leaves (Figure 6.7).

AKT2/3 challenged plants all exhibited leaf purpling, yellowing and curling (Figure 6.8). Both LAB-I and Cau74-R challenged *ASK1* plants had yellowing of the outer leaves and stunting, whereas L1851-C TuYV-challenged plants had purpling and curling of the outer leaves (Figure 6.9). All *ASK2* challenged plants were stunted with yellowing and purpling of the outer leaves,

and those challenged with L1851-C TuYV also exhibited leaf curling (Figure 6.9).

All *PAPK* challenged plants were stunted, with L1851-C and Cau74-R inducing yellowing of the outer leaves, LAB-I and Cau74-R TuYV-challenged plants also had yellowing of the outer leaves and some Cau74-R TuYV-challenged plants having leaf curling symptoms (Figure 6.9). All *nCBP* challenged plants exhibited yellowing, purpling and leaf curling of the outer leaves, with those challenged with L1851-C and Cau74-R being stunted (Figure 6.8). All *eIF3d.1* showed leaf curling and yellowing of the outer leaves, LAB-I and Cau74-R TuYV-challenged plants also had leaf purpling of the outer leaves (Figure 6.9).

6.3.3.2 Heterozygous lines Phenotypes

All *SUC1* challenged plants had leaf curling and yellowing of the outer leaves, with those challenged with L1851-C and Cau74-R TuYV showing leaf purpling of the outer leaves symptoms, as well as Cau74-R plants also suffering stunting (Figure 6.8). *SUC2* plants challenged with L1851-C TuYV had purpling of the outer leaves, LAB-I TuYV-challenged plants had yellowing of the outer leaves and those challenged with Cau74-R TuYV were stunted with purpling and yellowing of the outer leaves (Figure 6.8). All *eIF3d.2* challenged plants showed mixed symptoms when challenged with all TuYV isolates, these varied between stunting, leaf purpling, yellowing and curling (Figure 6.9). Both phenotypes seen in *SUC1* and *eIF3d.2* are consistent with their genotypes of uniformly heterozygous and mixed genotype, respectively See Section 6.3.2.2). However *SUC2* also had a mixed genotype but the phenotype of this line infected with each TuYV isolate gave uniform symptoms, this could be due to the higher titre seen in *SUC2* than *eIF3d.2* equating to more consistent symptoms (See Tables 6.3 - 6.5).

6.4 Discussion

This chapter describes an investigation of loss of function (recessive) resistance to TuYV (of *Brassica* host origin), in which several knock-out plant lines were able to reduce the titre of the virus. The results show interesting parallels with a recent paper (Reinbold *et al.*, 2013), where the TuYV-FL isolate was used. Both results show that the *eIF(iso)4G.1* knock-out line supported a statistically reduced virus titre relative to the Col-0 susceptible control, but in this study UK isolates have lowest titre in *eIF4G* mutants rather than *eIF4E* when TuYV-FL was used. However, the results in this study highlight that poleroviruses do not seem to interact with *eIF(iso)4E*, unlike the *Potyviridae* (Duprat *et al.*, 2002; Reinbold *et al.*, 2013). It also appears that BWYV, TuYV and BMYV all utilise eIF factors, but to varying degrees and there is not a reliance on a single plant mechanism/gene for translation (Reinbold *et al.*, 2013). The difference in susceptibility of these eIF knock-out lines might be due to the divergence of *Brassica* UK TuYV isolates from TuYV-FL. It was shown that double knock-out mutants (*eIF(iso)4G.1* and *eIF(iso)4G.2*) produced a stronger resistance to TuYV-FL; this could be a future avenue of work with European TuYV isolates, but this family of genes could potentially causes growth and germination defects, and as such might not be a viable model for crop systems.

It appears there is no single gene in the eukaryotic translation initiation complex which can convey extreme resistance to any TuYV isolate, only *eIF(iso)4G.1* had significant reductions in TuYV titre across the three isolates (Tables 6.3 - 6.5). *ASK1* and *ASK2* knock-out plants also had significant reductions in titre of Cau74-R TuYV (Table 6.5) and *ASK2* to LAB-I (Table 6.4), but they will not be able to provide broad spectrum resistance as they are inherently dependent on the structure of P0, which is reflected in these results. As P0 is highly variable between isolates with high mutation rates, potentially facilitating resistance breaking properties as it rapidly evolves. Additionally, *ASK* genes do not provide extreme resistance, allowing the accumulation of TuYV and the opportunity for TuYV to acquire beneficial mutations. *PAPK* knock-out plants did show a reduced virus titre when challenged with LAB-I TuYV; the reduction was significant in Cau74-R TuYV-challenged plants, this indicates that *PAPK* could have an affect on TuYV movement much like it has on TMV (Citovsky, 1999). However like other knock-out lines this did not give complete resistance. These results suggests that TuYV is adept at utilising multiple genes for each function and/or there are additional plant-virus interactions we are not

yet aware of. As such a multi-gene knock-out approach might be viable to reduce TuYV titre, possibly conveying extreme resistance.

AKT2/3 and *eIF(iso)4E* were the only homozygous knock-out lines that demonstrated no significant reduction in TuYV viral titre (See Tables 6.3 - 6.5). Either TuYV viral factors do not interact with these parts of the plant machinery or these are not singularly necessary.

Genotyped *Arabidopsis* knock-out line *SUC1* individuals were all heterozygous for the T-DNA insertion. This should not occur as naturally segregating populations cannot contain only heterozygous individuals, this could indicate that homozygous plants are lethal or that the sample size was to small to detect other genotypes. Other mixed genotype lines were *SUC2* and *eIF3d.2*, which had mixed genotypes of wild-type, heterozygous and homozygous. *eIF3d.2* might be a candidate for resistance if homozygous lines could be produced in future studies, as homozygous *eIF3d.2* individuals had a very low virus titre. This is the same for *SUC1* and *SUC2* knock-out lines, which had lower ELISA values than the susceptible control with *SUC1* showing a large reduction in viral titre (Table 6.5), this implicates sucrose loading and/or its machinery in the movement of TuYV between plant cells. Future work would be to produce stable homozygous lines of these gene knock-outs.

Phenotypes of each plant after TuYV challenge were stunted with areas of necrosis and leaf purpling, yellowing and curling, but this was possibly due to aphid damage (Figures 6.7 - 6.9). This is due to the fact TuYV does not cause necrosis, only discolouration has been documented. Along with ELISA results not correlating with phenotype, L1851-C TuYV-challenged plants had fewer individuals with symptoms (Table 6.3) but had higher ELISA values than Cau74-R TuYV-challenged plants (Figures 6.4 - 6.6), which had a higher prevalence of individuals with symptoms but with lower ELISA values when compared to susceptible Col-0 TuYV-challenged plants (Table 6.5). As reported in OSR, the symptoms of TuYV in *Arabidopsis* are muted, as the main effect of TuYV infection is reduction in yield rather than effects on plant morphology, which mimics stress responses. However future work looking at tolerance of these lines or others could incorporated rosette size, leaf area, Leaf morphology, root phenotype, stalk height and flowering times to assess TuYV effect in a quantitative manner. None of the *Arabidopsis* knock-out lines showed uniform reductions in TuYV symptoms against the three TuYV isolates.

The possibility of genes becoming unusable to viruses (loss of function) combined with previous work showing how mis-spliced (Keren *et al.*, 2010) versions of eIFs, or single amino acid changes could convey extreme resistance to some viruses (Gallois *et al.*, 2010; Wang and Krishnaswamy, 2012; Liu *et al.*, 2013; Nellist *et al.*, 2014), shows that this can be a valuable source of resistance. This type of resistance could also be extreme and durable, as eIF-induced passive resistance creates an environment where the virus cannot replicate and therefore the virus cannot co-evolve to become suited to the change, as viruses will be unable to gain advantageous mutations. It has also been demonstrated that this type of resistance does not have a fitness cost (Nellist *et al.*, 2014), because of the polygenic nature of the eIFs in some plant species. Unfortunately this was not proved in the knock-out lines investigated as TuYV was still detected (Table 6.1). If the interaction between eIF and TuYV is related to viral movement, another proposed role, this would convey resistance but certain cells would be infected but TuYV would be unable to spread systemic. This could be the case as ELISA values were reduced with eIF knock-out lines, however plants were still infected although with reduce titre, possible due to limited TuYV movement. However the success potential of this form of resistance in crops is hindered by the low germination could just reflect the total loss of *eIF4E* and *eIF4G* as they are important house keeping genes maintaining, thus plants possessing truncated or altered forms of these genes could circumvent this fitness cost, or it could be down to the age of stored seeds from the stock centre reducing their viability. However, TuYV appears to be able to utilise several of these genes, so extreme resistance will most likely lead to even more extreme fitness costs to the plant, leading to the conclusion that this form of resistance utilising eIFs in *Arabidopsis* or crop species might not be possible for TuYV, or at best will only provide partial resistance. A potential way of producing a crop lines with these multi-gene resistance is via genome editing as traditional breeding of so many house-keeping alleles would be too costly (Li *et al.*, 2012; Hartung and Schiemann, 2014). However this technology and if it comes under EU GMO regulations is still problematic and might not be a viable option in the future (Wang *et al.*, 2014).

Although this study uncovered potential virus-plant interactions possibly explaining TuYV movement and translation interactions, no single extreme or broad-spectrum resistance was found. This study was conducted with a 10°C germination regime that could of had an epigenetic effect on RNA silencing, which could explain some of the variation found in ELISA results (See Figures 6.4

- 6.6). Future work should take this into account and have multiple temperatures used to ensure resistance doesn't break down (Zhong *et al.*, 2013; Baulcombe and Dean, 2014). Further work on possible plant tolerance indicated by TuYV titre reductions, production of homozygous, double knock-out lines and investigations of new potential sources needs to be undertaken to find a novel source of extreme broad spectrum resistance to TuYV in *Arabidopsis* and beyond.

Chapter 7

General Discussion

7.1 Summary of Findings

The exploitation of naturally occurring resistance to plant pathogens in crops and close relatives is the best approach to disease control, as this is the only current way of introducing resistance into crops within Europe (Gaskell *et al.*, 1999). Plant varieties with resistance to viruses are considered the most cost-effective and reliable approach to control, as they should produce equivalent or greater yields, possibly with reduced inputs such as pesticides relative to susceptible crops (Kang *et al.*, 2005). This is also the only approach permitted in several areas of the world, including Europe, as genetically modified crops are not allowed to be grown commercially (Romeis *et al.*, 2008; Devos *et al.*, 2009). However, monogenic approaches in particular are often rapidly overcome by less common, or mutated/recombinant viral isolates (Stuthman *et al.*, 2007). Turnip yellows virus (TuYV) is emerging as a major cause of yield loss in oilseed rape (OSR) and other *Brassica* crops such as cabbage and Brussels sprouts. The losses have been shown to be up to 46% for OSR (Australian Government, 2008), 65% for Brussels sprouts (Walsh, 2011) and 36% for cabbage (Walsh, 2008). These losses result in large economic losses (estimated at £65 million for the UK OSR per annum), making OSR less popular as a break crop, which could have a detrimental effect on yields of other crops such as wheat (Angus *et al.*, 1991). To keep OSR profitable, increase food security and to try and achieve the target yield of 6.5 t/ha (Berry and Spink, 2006) introduction of natural resistance to TuYV into commercial varieties is desirable, particularly as other defences such as use of pesticides are becoming increasingly inadequate (IRAG-UK, 2011; The European Commission, 2013). To help in this endeavour

as the part of a larger Crop Improvement Research Club (CIRC funded by Biotechnological and Biological Sciences Research Council (BBSRC)) project, I investigated the genetic diversity as well as other biological properties of TuYV to provide a basis on which to investigate the spectrum of plant resistance to diverse isolates of TuYV. "If you know the enemy and know yourself, you need not fear the result of a hundred battles. If you know yourself but not the enemy, for every victory gained you will also suffer a defeat. If you know neither the enemy nor yourself, you will succumb in every battle" (Tzu, 2012).

This study has shown that:

1. TuYV is widespread in Europe as it was detected in all counties in the UK sampled as well as all of the other countries that were part of this study.
2. There are three distinct genotypes within the UK, two of which were also detected within mainland Europe based on TuYV gene P5. These genotypes could be interpreted as different species due to the genetic divergence from one another in TuYV genes P1, P3, P4 and P5 (Chapter 3).
3. TuYV gene P5, the minor coat protein, is a good candidate for genetic classification of TuYV isolates, as both amino acid and nucleotide phylogenetic analyses were able to distinguish between geographical and host origins (Chapter 3).
4. These species are also divergent from all other published *Luteoviridae* including the first sequenced TuYV isolate describing the species, TuYV-FL (accession number X13063, Veidt *et al.* (1988)) (Chapter 3).
5. There were two very strongly supported self-homologous recombination breakpoints in the TuYV genome at 3488nt (P3a and P3) and 4823nt (P5), only isolates with a single recombination point were detected. There were 27 isolates with recombination within P3 and 62 isolates with recombination in P5 (Chapter 3).
6. Many weed species can harbour TuYV that can go on to infect OSR, this includes plant families previously not reported as hosts including; *Apiaceae*, *Caprifoliaceae*, and *Resedaceae*, *Geraniaceae* and *Scrophulariaceae*, with the species: weld, wild geranium, dock, spear thistle, verbascum, teasel and cow parsley (Chapter 4).
7. All crops in this study showed susceptibility to TuYV (carrot, lettuce, sugar beet and field bean). This confirms that members of the plant family *Api-*

aceae are potential hosts of TuYV, as well as sugar beet (*Beta vulgaris*) which was previously thought not to be a host. The inability of TuYV to infect sugar beet was one of the criteria cited in the re-classification of TuYV away from Beet western yellows virus (BWYV) (Chapter 4).

8. Production of an infectious clone of a UK *Brassica* originating TuYV was achieved using a gateway cloning system capable of infecting OSR and *Nicotiana benthamiana*. This was also aphid transmissible (Chapter 5).

9. An *Arabidopsis thaliana* knock-out study revealed that no single eIF gene could convey extreme resistance to TuYV, thus the virus either uses multiple eIF genes for translation, or some other not yet understood mechanism (Chapter 6).

10. The *Arabidopsis thaliana* knock-out study also showed statistical evidence that limiting the movement of TuYV within the plant could offer a source of resistance with removal of potassium channels, sodium symporter channels or plasmodesmal-associated protein kinase which makes post-transcriptional modifications to plant virus movement proteins. Again however this did not offer broad-spectrum or extreme resistance and three lines were heterozygous so need further testing (Chapter 6).

7.1.1 TuYV Incidence

As a result of investigating the genetic diversity of TuYV, incidence data was also gained from all over Europe. This revealed that TuYV has far a higher incidence in the main OSR growing countries in mainland Europe when compared to UK incidence. France, Germany and Poland consistently had >90% incidence year on year, whereas the UK had between 0-92% with the highest average incidence in 2012 of 59.3%, indicating incidence in the UK is highly linked with warm autumns causing larger flights of the aphid vector *M. persicae*. Colder regions had lower incidences e.g Ukraine and Denmark had some OSR crops with 0% incidence. It has also been shown in this study that there does seem to a consistent, low level of infection within weeds located within and around OSR fields in the UK, acting as a source of inoculum of crops. With the lack of effective aphid control measures and Europe seemingly under more aphid pressure due to the consistently high incidence of TuYV even in insecticide-treated crops, TuYV is widespread in Europe and at very high levels. TuYV is only going to become a more serious problem with increased temperatures (extending vector area of

influence), insecticide resistance and banning of active ingredients. This study has significantly improved the understanding of the incidence and prevalence for TuYV within OSR crops. With results similar to other *Luteoviridae*, which are also phloem limited, TuYV has gone unnoticed due to its lack of symptoms and chronic nature (Latch, 1977; Gray *et al.*, 1996). With such high incidences addition of a extremely pathogenic inducing satellite could cause even larger problems for *Brassica* as well as other crops yields and their variability as crops (Xu and Roossinck, 2000).

7.1.2 TuYV Genetic Diversity

The ability to amplify and sequence whole TuYV genomes has helped to understand the diversity of European isolates of TuYV, which seems to be genetically distinct from the originally sequenced and studied TuYV-FL. Instead, in many cases the sequenced TuYV European isolates genotypes were less genetically distant to Brassica yellows virus (BrYV) than TuYV-FL. This full genome approach will be invaluable in amplifying, sequencing and in analysing TuYV isolates in future. This has been assisted by 5′ RACE on one common and one intermediate isolate with the addition of sequence information of the 3′ UTR from multiple UK isolates permitting RT-PCR primer design in conserved sites. This information along with all of the full genome sequence data gathered during this project will provide valuable information for the future. It has already been used to develop a real-time PCR method to detect TuYV in aphids by Rothamsted Research. Conserved single nucleotide polymorphisms which distinguished BMYV from TuYV sequenced in this study help create an effective assay for detecting which strains are present within UK *M. persicae*.

European isolates of TuYV are highly divergent from the original TuYV-FL and all other *Luteoviridae* viruses according to the genetic analysis carried out (Table 3.4 and Figures 3.10 and 3.22), therefore there has been speciation from other viruses for which sequence is available (Figure 3.27). Certain isolates might need further classification due to low amino acid and nucleotide identity in certain genes (Table 3.9). Three distinct genotypes were found when phylogenetic analysis investigated sequence variation of P5. The common and uncommon groups were found both in the UK and Europe, as well as the third "weed-like" group consisting mainly of UK Suffolk weed isolates and one OSR isolate from Cornwall UK. However this "weed-like" group did not consist of all TuYV

isolates sequenced from weeds, the majority of weed isolates were common or uncommon genotypes. Many of these weed isolates were shown to be capable of infecting OSR (Table 4.4).

Due to multiple phylogenetic analyses being able to discern the three European genotypes from each other and other published closely related species including TuYV-FL, as well as the genetic distance of these groups I propose that there needs to be further classification of these *Polerovirus* species. This is further supported by the host range of European TuYV isolates includes sugar beet and having differing reliance on eIF gene members providing biological difference between the European isolates and the from TuYV-FL. I propose that viruses closely related to TuYV-FL maintain the TuYV classification, but the remainder of the European isolates are reclassified as BrYV viruses as they have closer genetic links to the Chinese *Brassica* infecting isolates. The naming of each species should be: BrYV-EC designation for the common European genotype, the uncommon European genotype having the BrYV-EU designation, with the "weed-like" European genotype having the designation of BrYV-EW and Chinese isolates to be classified as BrYV-A as they originate from Asia and this allows further genotype classification of Asian isolates in the future.

The future of TuYV OSR and other crop resistance breeding will need to be assessed against the genotypes highlighted in this study and possibly the recombinant forms as there a large number (89) of recombinant isolates detected, suggesting it is beneficial for the virus by increasing host range, strain competitiveness or aphid transmission efficiency. Otherwise, resistance-breaking strains could quickly establish and become the prominent genotype. This is a possibility due to variation that was found in the host range and pathogenicity of the three different TuYV species investigated in this study, hence fully tested broad-spectrum resistance will be necessary for future strategies for controlling TuYV.

7.1.3 Host Range and Disease Management

TuYV is not seed-borne so it needs to be constantly maintained in a plant host or aphid, hence it needs alternative hosts to survive through the entire year. Therefore, it is necessary to know the wild host range of TuYV to have the possibility of better management strategies by their removal by rouging, which is the pro-

cess of scalping off established weed ecosystems to generate new growth (Zitter and Simons, 1980; Chan and Jeger, 1994). However, these management strategies work better with non-persistent viruses, as host eradication and barrier plants can cause vectors to lose their transient virus by the time they start to feed on crops (Hooks and Fereres, 2006). Poleroviruses, including TuYV (Stevens *et al.*, 2008b) are known to have wide host ranges. The known host range of TuYV is growing as further studies are carried out, with the addition of new crop hosts as well as wild species that are ubiquitous within the environment. Effective management of the virus will be difficult as many plant species are active reservoirs along with the large number of aphid species, which are said to transmit TuYV. This suggests that plant resistance to TuYV will be important in reducing losses and increasing yields, as the logistics and inputs of conventional management will be too expensive.

7.1.4 Production of a TuYV Infectious Clone

The successful production of an infectious clone of TuYV that was achieved during this study and the resulting pipeline to create it, designed within this study, will allow further more in-depth investigation of TuYV for example host range determinates. As the clones can systemically infect plants and be transmitted by aphids, it has at this time all the properties of a naturally-occurring TuYV isolate. The inability of the infectious clone to infect *Nicotiana benthamiana* with Agro-inoculation might be due to human error or the application was inadequate for the differing vascular systems of these species, further refinement will be required. Altering the genome in any way will allow interpretation of its effect on function, pathogenicity or host range. It will also allow further investigation of TuYV host range as it will allow a standard protocol of inoculation which will stop false negative results occurring due to aphids not feeding on the challenged plants. This will help assess plant resistance in the future by producing infectious TuYV isolates from samples accrued and stored during this study, which will allow all genetic groups found, to be investigated in regard to their potential ability to break resistance and alternative phenotypes.

7.1.5 Novel Sources of Resistance

TuYV isolates from three TuYV genotypes were used during the *Arabidopsis* knock-out study (common, uncommon and a recombinant isolate), which demon-

strated there were differences between isolates and their reliance on different plant genes. During this study no single gene knock-out could convey broad-spectrum resistance to TuYV. The only eIF gene to cause consistent significance reductions in TuYV titre across the different isolates was *eIF(iso)4G.1* knock-out lines, however TuYV was still at detectable levels. As resistance was incomplete the investigation of double knock-outs of eIF genes would be required to try and establish complete resistance, however this would make the practical application of this gene family in crops less likely, as they control major housekeeping functions and could have a negative effect on plant fitness (Reinbold *et al.*, 2013). Although no eIF gene conveyed extreme resistance, *eIF3D.2* knock-out plants did offer results that might warrant further investigation as the heterozygous population had large reductions in viral titre, hence isolation of a homozygous population line could provide a source of resistance to TuYV. However, *eIF3D.2* as well as *SUC1* and *SUC2* did not have uniform homozygous plants for the knock-out, so this caveat must be taken into account and will need addressing in future work. eIF-based resistance has been accomplished before against *Potyviridae* (Le Gall *et al.*, 2011) and seems to have no detrimental effect on the plant (Duprat *et al.*, 2002), but this does not seem likely to be successful with TuYV at this moment. This is due to either TuYV being able to use multiple eIF gene members, or a mechanism involved with translation/movement, which are not yet understood. Limiting TuYV movement in plants was another potential source of resistance and knock-out of sodium symporters, or plasmodesmal-associated protein kinase resulted in large and significant TuYV titre reductions in *Arabidopsis*, but not extreme resistance. Viral movement limiting resistance might not prove as durable, as it could allow viral evolution but could be additive to other resistance sources increasing durability (Lecoq *et al.*, 2004).

7.2 Future Work

Future work will be the further study and understanding of TuYV including: cell to cell movement, nature of infection, and these differences compared to other poleroviruses, as well as between the three European TuYV species. This could be started with the production of homozygous knock-out lines of *eIF3D.2* and *SUC1* and *SUC2*, as the heterozygous lines provided large reductions in TuYV titre within *Arabidopsis*. Homozygous mutants may result in improved resistance and possibly broad-spectrum extreme resistance. One of the outputs of this PhD was the production of an infectious clone and the development of a pipeline to

produce further clones. The clone can be used in the future to maintain representatives of the three distinct European species, plus recombinant isolates for future study. The clones can then be used to investigate host range determinates, pathogenicity and other factors of the genetically distinct TuYV species. It would also be interesting to see whether diseases in other crops are attributable to TuYV, such as potato plants with Potato leaf-roll virus-like symptoms, and assess if TuYV has equal or larger impact on yield on these crops than it has on *Brassica* crops. There were ELISA-positive weed and OSR samples where TuYV could not be effectively amplified for sequencing and with knowledge of antisera cross-reactivity it might be revealing to further investigate those samples for closely related or novel viruses. TuYV is an unusual plant virus due to its expansive host range, incidence and vascular tropism which are of yet not fully understood and there are many areas needing further exploration in the future to alleviate this knowledge vacuum around TuYV. Along side this gene identification in resistant crops species would help future prevention of TuYV impact and help understand plant host interactions. These will be the necessary areas of future work to combat and understand TuYV.

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Appendix A

GenBank Accession Details

Table A.1 – Details of Sequences Used from GenBank.

Origin	Gene	Sequence Details	GenBank Accession	Reference
TuYV-FL	Whole genome	cDNA	X13063	Veidt *et al.* (1988)
BrYV-ABJ	Whole genome	cDNA	HQ388348	Xiang *et al.* (2011)
BrYV-BBJ	Whole genome	cDNA	HQ388349	Xiang *et al.* (2011)
BrYV-AJS	Whole genome	cDNA	HQ388350	Xiang *et al.* (2011)
BrYV-BJS	Whole genome	cDNA	HQ388351	Xiang *et al.* (2011)
BMYV	Whole genome	cDNA	NC003491	Guilley *et al.* (1995)
BWYV	Whole genome	cDNA	NC004756	Su *et al.* (1999)
BChV	Whole genome	cDNA	NC002766	Hauser *et al.* (2002)
CYDV-RPV	Whole genome	cDNA	NC002198	Direct submission
CtRLV	Whole genome	cDNA	NC006265	Huang *et al.* (2005)
PLRV	Whole genome	cDNA	NC001747	Prüfer *et al.* (1992)
TVDV	Whole genome	cDNA	EF529624	Mo *et al.* (2010)
SCYLV	Whole genome	cDNA	NC000874	Moonan *et al.* (2000)
CpCSV	Whole genome	cDNA	NC008249	Guilley *et al.* (1994)
CABYV	Whole genome	cDNA	NC003688	Abraham *et al.* (2006)
MABYV	Whole genome	cDNA	NC010809	Xiang *et al.* (2008)
LABYV	Whole genome	cDNA	NC027703	Knierim *et al.* (2015)
BWYV-3′ RNA	3′ proximal half	cDNA	X13062	Veidt *et al.* (1988)