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Brassicaceae – *Turnip yellows virus* interactions

Elvis Asare-Bediako
(BSc, MPhil)

A thesis presented for the degree of Doctor of Philosophy in Plant and
Environmental Sciences

University of Warwick

School of Life Sciences

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TABLE OF CONTENTS

Table of contents	i
List of tables	vi
List of figures	x
Acknowledgements	xiii
Declaration	xiv
Abstract	xv
List of abbreviations	xvi
Chapter 1: Introduction	1
1.1 Brassicaceae	1
1.1.1 Oilseed rape	2
1.1.2. <i>Arabidopsis thaliana</i>	4
1.2 The <i>Polerovirus</i> genus	5
1.2.1 Genome structure and organisation	8
1.2.2 Functions of proteins	9
1.2.3 Virus infection and replication	11
1.2.4 <i>Polerovirus</i> transmission	12
1.3 <i>Turnip yellows virus</i> (TuYV)	13
1.3.1 History of <i>Turnip yellows virus</i> (TuYV)	13
1.3.2 Genetic variation of <i>Turnip yellows virus</i> (TuYV)	14
1.3.3 Incidence of <i>Turnip yellows virus</i> (TuYV) infection in oilseed rape crops	16
1.3.4 Effect of <i>Turnip yellows virus</i> (TuYV) on the yield of oilseed rape	18
1.3.5 Management of <i>Turnip yellows virus</i> (TuYV)	19
1.4 Host resistance	23
1.4.1 Types of resistance	24
1.4.2 Models of resistance	25
1.4.3 Genetic basis of resistances in <i>Poleroviruses</i>	27
1.4.4 Genetic basis of resistance to <i>Turnip yellows virus</i> (TuYV) in <i>Brassica napus</i>	29

1.4.5 Pathogen derived resistance (Genetic engineering)	30
1.5 Aims and objectives	33
Chapter 2: Incidence of <i>Turnip yellows virus</i> (TuYV) infecting winter oilseed rape (<i>Brassica napus</i>) in England	34
2.1 Introduction	34
2.2 Materials and methods	36
2.2.1 Surveys of winter oilseed rape crops	36
2.2.2 Detection of <i>Turnip yellows virus</i> (TuYV) in the sample	40
2.2.3 Data analysis	41
2.3 Results	43
2.3.1 Field observations	43
2.3.2 Incidence of <i>Turnip yellows virus</i> (TuYV) in winter oilseed rape crops	43
2.3.3 Within field distribution	48
2.3.4 Number of <i>Myzus persicae</i> caught in the suction traps closest to sampled fields	50
2.3.5 Relationship between <i>Myzus persicae</i> and <i>Turnip yellows virus</i> (TuYV) incidence	51
2.4 Discussion	53
Chapter 3: Genetic diversity, evolution and genetic structure of <i>Turnip yellows virus</i> (TuYV) infecting oilseed rape crops in England	59
3.1 Introduction	59
3.2 Materials and methods	62
3.2.1 Virus isolates	62
3.2.2 Preparation of total plant RNA	66
3.2.3 Primer design	66
3.2.4 First strand cDNA synthesis	68
3.2.5 Amplification of cDNA (Polymerase chain reaction)	69
3.2.6 Gel purification	69
3.2.7 Cloning and sequencing	69
3.2.8 Sequence comparisons and phylogenetics analyses	71

3.2.9	Detection of recombination	72
3.2.10	Genetic diversity	72
3.2.11	Determination of genetic distance and selection pressure	73
3.2.12	Determination of substitution rate	74
3.2.13	Neutrality test	74
3.2.14	Analysis of population differentiation and variation	75
3.3	Results	76
3.3.1	Sequence analysis	76
3.3.2	Phylogenetic analysis	77
3.3.3	Mixed genotypes	88
3.3.4	Alignments of P0 and P3 amino acid sequences	88
3.3.5	Genetic diversity within P0 and P3 genes and different geographical regions	90
3.3.6	Recombination in the <i>Turnip yellows virus</i> (TuYV) isolates	93
3.3.7	Nucleotide substitution rates in the <i>Turnip yellows virus</i> (TuYV isolates)	95
3.3.8	Analyses of genetic distance and natural selection within P0 and P3 of the <i>Turnip yellows virus</i> (TuYV) isolates	97
3.3.9	Neutrality test	98
3.3.10	Analyses of genetic structure of TuYV sub-population	98
3.3.11	Analyses of molecular variation (AMOVA)	102
3.4	Discussion	104
Chapter 4: Isolating and differentiating genotypes of <i>Turnip yellows virus</i> (TuYV) infecting oilseed rape in England using group specific primers and polymerase chain reaction		114
4.1	Introduction	114
4.2	Materials and methods	116
4.2.1	Field survey for <i>Turnip yellows virus</i> (TuYV) isolates	116
4.2.2	Detection of <i>Turnip yellows virus</i> (TuYV) in the field samples using ELISA	116
4.2.3	RNA extraction	117
4.2.4	Primer design	117

4.2.5	Optimisation of RT-PCR conditions for the genotype specific primers	120
4.2.6	Detecting and differentiating <i>Turnip yellows virus</i> (TuYV) isolates with different genotypes from field samples	120
4.2.7	Transmission of the <i>Turnip yellows virus</i> (TuYV) isolates with different genotypes to oilseed rape plants	121
4.2.8	Verification of the genotypes of <i>Turnip yellows virus</i> (TuYV) isolates from field samples	121
4.2.9	Sequence comparisons of P0 gene of the <i>Turnip yellows virus</i> (TuYV) isolates maintained in oilseed rape cv. Mikado	122
4.2.10	Sequence comparisons and phylogenetic analyses	123
4.3	Results	124
4.3.1	Incidence of <i>Turnip yellows virus</i> (TuYV) in oilseed rape	124
4.3.2	Cumulative numbers of <i>Myzus persicae</i> caught in Rothamsted insect survey suction trap	124
4.3.3	RT-PCR assay design	125
4.3.4	Genotype discrimination of different <i>Turnip yellows virus</i> (TuYV) isolates	126
4.3.5	<i>Turnip yellows virus</i> (TuYV) isolates propagated in oilseed rape plants	127
4.3.6	Genetic purity of <i>Turnip yellows virus</i> (TuYV) obtained from field plants in Lincolnshire	128
4.3.7	Phylogenetic analysis of <i>Turnip yellows virus</i> (TuYV) isolates	129
4.3.8	Amino acid sequence alignment	131
4.4	Discussion	133
Chapter 5: Interaction between <i>Brassicaceae</i> and <i>Turnip yellows virus</i>		138
5.1	Interaction between a <i>Brassica napus</i> diversity fixed foundation set (DFFS) and <i>Turnip yellows virus</i> (TuYV)	138
5.1.1	Introduction	138
5.1.2	Materials and methods	140
5.1.3	Results	144
5.1.4	Discussion	149

5.2 The effects of <i>Turnip yellows virus</i> (TuYV) on the growth and yield of of oilseed rape	152
5.2.1 Introduction	152
5.2.2 Materials and methods	153
5.2.3 Results	157
5.2.4 Discussion	164
5.3 The interaction between <i>Arabidopsis thaliana</i> accessions and <i>Turnip yellows virus</i> (TuYV)	169
5.3.1 Introduction	169
5.3.2 Materials and methods	170
5.3.3 Results	174
5.3.4 Discussion	184
 Chapter 6: A quantitative trait locus (QTL) analysis of partial resistance of oilseed rape (<i>Brassica napus</i>) to <i>Turnip yellows virus</i> (TuYV) and assessment of the spectrum of resistance	187
6.1 A quantitative trait locus analysis of partial resistance of oilseed rape cv Yudal against <i>Turnip yellows virus</i> (TuYV)	187
6.1.1 Introduction	187
6.1.2 Materials and methods	189
6.1.3 Results	192
6.1.4 Discussion	200
6.2 Resistance of oilseed rape cv Yudal to <i>Turnip yellows virus</i> (TuYV) <i>isolates</i> of different genotypes	204
6.2.1 Introduction	204
6.2.3 Materials and methods	205
6.2.4 Results	208
6.2.5 Discussion	210
 Chapter 7: General discussion	213
REFERENCES	225
APPENDICES	249

LIST OF TABLES

Chapter 1

Table 1.1 <i>Luteoviridae</i> family, its genera and their species	7
Table 1.2 The gene-for-gene hypothesis of Flor (1971)	26

Chapter 2

Table 2.1 Field locations, cultivar, seed treatment and foliar sprays used in the fields sampled	38
Table 2.2 Mean autumn percentage incidence of <i>Turnip yellows virus</i> (TuYV) in winter oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire in the 2007- 8, 2008-9 and 2009-10 crop seasons	44
Table 2.3 Mean spring percentage incidence of <i>Turnip yellows virus</i> (TuYV) in winter oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire in the 2007-8, 2008-9 and 2009-10 crop seasons	45
Table 2.4 Incidence and spatial analysis of <i>Turnip yellows virus</i> (TuYV) in oilseed rape crops sampled in Lincolnshire, Warwickshire and Yorkshire in the 2007-8, 2008-9 and 2009-10 crop seasons	47

Chapter 3

Table 3.1 Field isolates of <i>Turnip yellows virus</i> (TuYV) selected for sequencing	63
Table 3.2 <i>Turnip yellows virus</i> (TuYV) isolates from GenBank	65
Table 3.3 Sequence of primers used for RT-PCR amplification	67
Table 3.4 Nucleotides (nt) and amino acid (aa) sequence identities of <i>Turnip yellows virus</i> (TuYV) field isolates and selected published isolates retrieved from GenBank	77
Table 3.5 Nucleotides (nt) and amino acid (aa) sequence identities of <i>Turnip yellows virus</i> (TuYV) isolates within and between clades of Maximum likelihood phylogenetic tree	81
Table 3.6a Genetic variability within ORF0 and ORF3 DNA sequences of <i>Turnip yellows virus</i> (TuYV) isolates	91
Table 3.6b Genetic diversity within the <i>Turnip yellows virus</i> (TuYV) populations in Lincolnshire, Yorkshire and Warwickshire based on 699 bp of the P0 gene of the viral genome	92
Table 3.7 Number of haplotypes of <i>Turnip yellows virus</i> (TuYV) populations shared among the regions based on the P0 gene nucleotide sequences	93
Table 3.8 Recombination within and between P0 and P3 genes	95

Table 3.9 Nucleotide substitution rate and TMRA estimates for ORF0 of <i>Turnip yellows virus</i> (TuYV) isolates from oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire during 2007-10, 2008-9 and 2009-10 crop seasons	96
Table 3.10 Mean pairwise genetic distance and the selective pressures within P0 and P3 genes of the <i>Turnip yellows virus</i> (TuYV) isolates	98
Table 3.11 Neutrality test for OR0 and ORF3 of <i>Turnip yellows virus</i> (TuYV)	98
Table 3.12 Genetic differentiation and gene flow between <i>Turnip yellows virus</i> (TuYV) populations in Lincolnshire, Yorkshire and Warwickshire (Spatial genetic structure of TuYV populations) based on ORF0 nucleotide sequence dataset	99
Table 3.13 Gene flow between <i>Turnip yellows virus</i> (TuYV) populations in Lincolnshire, Yorkshire and Warwickshire based on ORF0 nucleotide sequence dataset	100
Table 3.14 Genetic differentiation between <i>Turnip yellows virus</i> (TuYV) populations in the 2007-8, 2008-9 and 2009-10 crop seasons (Temporal genetic structure) based on ORF0 nucleotide sequence dataset.	101
Table 3.15 Gene flow between <i>Turnip yellows virus</i> (TuYV) populations in the 2007-8, 2008-9 and 2009-10 crop seasons based on the ORF0 nucleotide sequence dataset.	102
Table 3.16 AMOVA of <i>Turnip yellows virus</i> (TuYV) populations at different geographical regions and crop seasons (based on ORF0 nucleotide sequence dataset)	103
 Chapter 4	
Table 4.1 P0 gene primers used in the RT-PCR assay to discriminate three distinct genotypes of <i>Turnip yellows virus</i> (TuYV).	118
Table 4.2 The incidence of <i>Turnip yellows virus</i> (TuYV) in oilseed rape crops in Warwickshire and Lincolnshire during the winter of the 2010-11 crop season.	124
Table 4.3 Cumulative August to November <i>Myzus persicae</i> numbers caught in the Rothamsted Insect Survey suction traps in Lincolnshire (Kirton) and Warwickshire (Wellesbourne) from 2007 to 2010	125
Table 4.4 Detection of <i>Turnip yellows virus</i> (TuYV) isolates with different genotypes in oilseed rape using AB23-AB24, AB17-AB24 and AB17-AB18 assays	127
Table 4.5 Nucleotide and amino acid sequence identities of <i>Turnip yellows virus</i> (TuYV) isolates based on 726 bp fragment of the P0 gene	131

Chapter 5

Table 5.1 Background information on <i>Brassica napus</i> DFFS accessions evaluated for susceptibility to <i>Turnip yellows virus</i> (TuYV).	140
Table 5.2 <i>Turnip yellows virus</i> (TuYV) infection on <i>Brassica napus</i> accessions, the quantity of virus detected and the effect on dry weight	145
Table 5.3 Effect of <i>Turnip yellows virus</i> (TuYV) infection on fresh weight of <i>Brassica napus</i> accessions	147
Table 5.4 ELISA absorbance (virus accumulation) and mean plants heights of oilseed rape cultivars challenged with <i>Turnip yellows virus</i> (TuYV) for six weeks	158
Table 5.5 Mean fresh weight and mean dry weight of oilseed rape cultivars challenged with <i>Turnip yellows virus</i> (TuYV) and mock challenged	160
Table 5.6 Mean number of branches and mean number of pods of oilseed rape plants challenged with <i>Turnip yellows virus</i> (TuYV) and mock challenged	161
Table 5.7 Mean number of seeds per pod and mean seed yield (g) of oilseed rape cultivars challenged with <i>Turnip yellows virus</i> (TuYV) and mock-challenged.	163
Table 5.8 Correlations between the vegetative and economic yield parameters of oilseed rape cultivars	164
Table 5.9 List of the <i>Arabidopsis thaliana</i> accessions used, their stock numbers and their geographical origin	171
Table 5.10 <i>Turnip yellows virus</i> (TuYV) infection on <i>Arabidopsis thaliana</i> accessions, the quantity of virus detected and the effect on plant height	176
Table 5.11 Effect of <i>Turnip yellows virus</i> (TuYV) infection on mean rosette diameter (cm) and mean number of branches of <i>Arabidopsis thaliana</i> accessions	178
Table 5.12 Effect of <i>Turnip yellows virus</i> (TuYV) infection on mean number of pods of <i>Arabidopsis thaliana</i> accessions	180
Table 5.13 Effects of <i>Turnip yellows virus</i> (TuYV) infection on the number of seeds per pod and seed production.	182
Table 5.14 Correlation coefficients	183

Chapter 6

Table 6.1 Distribution of Absorbance values from TAS-ELISA on Darmor-*bzh* x Yudal DH populations (SP1 and SP2) challenged with *Turnip yellows virus* (TuYV) 193

Table 6.2 Details of quantitative trait locus (QTL) for *Turnip yellows virus* (TuYV) resistance detected on linkage group C4 (N14) of *Brassica napus* estimated from mean ELISA absorbance data in sub-populations of 115 – 118 Darmor-*bzh* x Yudal doubled-haploid lines. 198

Table 6.3 Susceptibility of oilseed rape cultivars Darmor-*bzh* and Yudal to different isolates of *Turnip yellows virus* (TuYV) 210

LIST OF FIGURES

Chapter 1

- Figure 1.1 Organisation of *Polerovirus* genome 9
- Figure 1.2 Symptoms of *Turnip yellows virus* infection 17

Chapter 2

- Figure 2.1 Field 1 sampled in Lincolnshire in December (autumn) during 2008-9 crop 43
- Figure 2.2 Field 1 sampled in Lincolnshire in April (spring) during 2008-9 crop 43
- Figure 2.3 Correlation between autumn and spring percentage incidences of *Turnip yellows virus* (TuYV) 48
- Figure 2.4 Spatial distribution of *Turnip yellows virus* (TuYV) infected – plants in oilseed rape fields in Lincolnshire in autumn of the the 2007-8 crop 49
- Figure 2.5 Spatial distribution of *Turnip yellows virus* (TuYV) infected - plants in oilseed rape fields in Lincolnshire in spring of the 2007-8 crop 50
- Figure 2.6 Rothamsted Insect Survey catches of *Myzus persicae* in suction traps located in Lincolnshire (Kirtton), Warwickshire (Wellesbourne) and Yorkshire (Askham Bryan) 51
- Figure 2.7 Relationship between cumulative numbers of *Myzus persicae* caught in the Rothamsted Insect Survey suction traps located in Lincolnshire, Warwickshire and Yorkshire between August and November in 2007, 2008 and 2009 and mean percentage *Turnip yellows virus* (TuYV) incidence in oilseed rape crops in the autumn of each year in the three regions 52
- ### Chapter 3
- Figure 3.1 A diagram showing the distribution of *Turnip yellows virus* (TuYV) infected plants in an oilseed rape field in Lincolnshire indicated by black oblongs 62
- Figure 3.2 Diagram showing the primers and their positions in (a) ORF0 and (b) ORF3 region of *Turnip yellows virus* (TuYV) genome (not to scale). 68
- Figure 3.3 Maximum-likelihood phylogenetic tree of nucleotide sequences of P0 gene of *Turnip yellows virus* isolates (n=226) sampled in Lincolnshire, Warwickshire and Yorkshire from 2007-2009. 79
- Figure 3.4 Maximum likelihood tree (abridged) of P0 gene nucleotide sequence of *Turnip yellows virus* (TuYV) isolates from Lincolnshire (prefixed L), Warwickshire (prefixed W) and Yorkshire (prefixed Y) in England 80

Figure 3.5 Maximum likelihood phylogenetic tree of coat protein gene nucleotide sequences of <i>Turnip yellows virus</i> (TuYV) isolates sampled in Lincolnshire, Warwickshire, and Yorkshire in England between 2007 and 2009.	83
Figure 3.6 Maximum likelihood phylogenetic tree (abridged) of coat protein gene nucleotide sequences of <i>Turnip yellows virus</i> (TuYV) isolates from Lincolnshire (prefixed L), Warwickshire (prefixed W) and Yorkshire (prefixed Y) in England.	84
Figure 3.7 Maximum likelihood phylogenetic tree (abridged) of P0 amino acid sequences of <i>Turnip yellows virus</i> (TuYV) isolates from Lincolnshire (prefixed L), Warwickshire (prefixed W) and Yorkshire (prefixed Y) in England.	86
Figure 3.8 Maximum likelihood phylogenetic tree (abridged) of P3 amino acid sequences of <i>Turnip yellows virus</i> isolates (TuYV) from Lincolnshire (prefixed L), Warwickshire (prefixed W) and Yorkshire (prefixed Y) in England.	87
Figure 3.9 Alignment of P0 amino acid sequences of <i>Turnip yellows virus</i> (TuYV) isolates representing the three genetic groups (clades).	89
Figure 3.10 Alignment of P3 amino acid sequences of <i>Turnip yellows virus</i> (TuYV) isolates representing the two genetic groups (clades).	90
 Chapter 4	
Figure 4.1 Design of the RT-PCR assay of the P0 gene of <i>Turnip yellows virus</i> (TuYV).	119
Figure 4.2 RT-PCR amplification products (420 bp and 421 bp) using <i>Turnip yellows virus</i> (TuYV) genotype specific primers AB23-AB24, AB17-AB24 and AB17-AB18	126
Figure 4.3 ELISA absorbance values of oilseed rape plants (Mikado) infected with six isolates of <i>Turnip yellows virus</i> (TuYV) and LAB control	128
Figure 4.4 RT-PCR amplification products using <i>Turnip yellows virus</i> (TuYV) genotype-specific P0 gene primers.	129
Figure 4.5 Maximum likelihood tree of nucleotide sequences of 726 bp of the P0 gene of <i>Turnip yellows virus</i> (TuYV) isolates showing two genetic groups	130
Figure 4.6 Amino acid sequences of 726 nt P0 gene of <i>Turnip yellows virus</i> (TuYV) genotypes 1 and 3	132

Chapter 5

Figure 5.1 Correlation between mean ELISA absorbance values and mean dry weights of *B. napus* accessions 148

Figure 5.2 Correlation between mean absorbance values and mean fresh weights of *B. napus* accessions 149

Chapter 6

Figure 6.1 Frequency distribution of the Darmor-*bzh* x Yudal doubled haploid (DH) lines for partial resistance to *Turnip yellows virus* (TuYV) infection 194

Figure 6.2 Correlations of the virus titre levels (A_{405}) between two DH lines subpopulations (SP1 and SP2) 195

Figure 6.3 Detection of QTL for *Turnip yellows virus* (TuYV) resistance gene on chromosome 14 (i.e. N14) of *Brassica napus* in sub-population SP1 196

Figure 6.4 Detection of QTL for *Turnip yellows virus* (TuYV) resistance on chromosome C4 (i.e. N14) of *Brassica napus* in sub-population SP2 197

Figure 6.5 Linkage group C4 (N14) of *Brassica napus* (from the map DY0703b) showing markers and linked QTL involved in *Turnip yellows virus* (TuYV) resistance 199

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This thesis is dedicated to Yaa Gyapomaa, Abena Serwaa, Ama Afriyie and Kwasi Opoku-Asiama.

DECLARATION

I declare that the work presented in this thesis was conducted by me under direct supervision of Dr John Walsh. None of the work presented has been previously submitted for any other degree.

Elvis Asare-Bediako

ABSTRACT

Turnip yellows virus (TuYV) is the most common and important virus infecting oilseed rape (*Brassica napus*) in the UK. It causes reductions in growth and seed yield in oilseed rape. Between 2007 and 2010, the prevalence of TuYV in oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire was determined; incidences of infection ranged from 0 and 100%. The highest levels of infection were detected in Lincolnshire and the lowest in Yorkshire. Highest incidences were recorded during 2009-10 and the lowest in 2008-9. Incidences of TuYV were closely related to the flight activities *Myzus persicae* vector. Most fields showed slightly aggregated pattern of infection during autumn but spring sampling revealed more random patterns. Phylogenetic analysis of both nucleotide and amino acid sequences of the P0 and P3 genes of TuYV revealed three and two genetic groups of TuYV respectively, infecting oilseed rape in Lincolnshire, Warwickshire and Yorkshire. The P0 gene was more variable than the P3 gene and both were under purifying selection. TuYV populations in the three regions were highly structured with limited gene flow between them. Analysis of molecular variance (AMOVA) indicated 96-97% of the observed variation was due to the variation between isolates within fields. Three RT-PCR assays were developed to differentiate the three genotypes. They successfully detected and discriminated isolates of the two major genotypes from oilseed rape in Lincolnshire. Twenty seven accessions of a *B. napus* Diversity Fixed Foundation Set (DFFS) screened for resistance against TuYV infections varied in their susceptibility to the virus. An accession Yudal had partial resistance to some but not all the isolates of the two major genetic groups tested. TuYV caused yield losses of up to 44.7% in a glasshouse experiment. A major QTL for the partial TuYV resistance was detected on chromosome C4 (N14), explaining up to 50.5% of the observed resistance.

LIST OF ABBREVIATIONS

VIRUS ABBREVIATIONS

BChV	<i>Beet chlorosis virus</i>
BMV	<i>Beet mild yellows virus</i>
BNYV	<i>Broccoli necrotic yellow virus</i>
BWYV	<i>Beet western yellows virus</i>
BYD	<i>Beet yellows virus</i>
BYDV	<i>Barley yellow dwarf virus</i>
CABYV	<i>Curcubit aphid-borne yellows virus</i>
CaMV	<i>Cauliflower mosaic virus</i>
CMV	<i>Cucumber mosaic virus</i>
CYDV	<i>Cereal yellow dwarf virus</i>
PEMV	<i>Pea enation mosaic virus</i>
TMV	<i>Tobacco mosaic virus</i>
TuMV	<i>Turnip mosaic virus</i>
TuYMV	<i>Turnip yellow mosaic virus</i>
TuYV	<i>Turnip yellows virus</i>

COMMON ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
BEAST	Bayesian evolutionary analysis by sampling trees
bp	base pairs
cM	centimorgan
cDNA	complimentary deoxyribonucleic acid
cv	cultivar
CP	coat protein
DFFS	Diversity fixed foundation set
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ELISA	Enzyme linked immunosorbent assay
EM algorithm	Expectation-Maximisation algorithm

gRNA	genomic RNA
HPD	highest probability density
kbp	kilobase pair
kDa	kilodaltons
LSD	Least significant difference
M	Molarity (moles per litre)
min	minutes
mM	millimolar
nt	nucleotide
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PTGS	post transcriptional gene silencing
pmol	picomole (10^{-12} mole)
QTL	quantitative trait locus
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
SKP	S-phase kinase related protein
sgRNA	subgenomic RNA
ssRNA	single stranded RNA
SSR	simple sequence repeats
STS markers	sequence-tagged sites markers
TMRCA	time to the most recent common ancestor
tRNA	transfer RNA
UV	ultraviolet
°C	degree centigrade
μl	microlitre (10^{-3} ml)
μg	microgram (10^{-3} g)
μM	micromolar (10^{-3} M)

CHAPTER 1:

GENERAL INTRODUCTION

1.1 Brassicaceae

The Brassicaceae (formerly Cruciferae) are morphologically diverse and economically valuable. The family currently contains 338 genera and 3709 species (Warwick *et al.*, 2006) of which about 300 species have been recorded in Britain and Ireland but only 50 are native (Rich, 1991). It consists of herbaceous plants with annual, biennial or perennial life spans. They have flowers which are in the form of a cross hence the previous family name Cruciferae, with four petals, usually white, yellow, lavender or pink and an equal number of sepals. There are four long and two short stamens and a two-chambered ovary positioned above the other flower parts. The seeds are produced in pod like fruits and often have a mucilaginous coating that swells when wetted.

Brassica crops worldwide provide the greatest diversity of products used by man (Dixon, 2007). They may be eaten as vegetables (e.g. cabbages, swedes), salads (e.g. “mustard and cress”), and as condiments (e.g. mustard). Brassicas can also be grown for oil-seed (e.g. oilseed rape), fodder or forage for domesticated animals (e.g. turnips) (Rich, 1991; Dixon, 2007) and as soil conditioners as green manuring and compost crops. The family also contains *Arabidopsis thaliana*, the model plant (Dixon, 2007).

1.1.1 Oilseed rape

Oilseed rape (*Brassica napus* L), also known as rapeseed, or canola, is a bright yellow flowering member of the *Brassicaceae* family. *Brassica napus* is an amphidiploid (n=19) evolved through natural hybridisation between wild parental diploids *B. rapa* (syn. *campestris*; genome AA, n = 10) and *B. oleracea* (CC, n = 9) (U, 1935). Such spontaneous interspecific hybridisations may have occurred several times, suggesting oilseed rape is of polyphyletic origin (Olsson, 1960; Song and Osborn, 1992). Oilseed rape has low genetic diversity because it is of recent origin and extensive cultivation and breeding of the crop started not more than 50 years ago. The genetic diversity of oilseed rape can be increased by its artificial resynthesis from two parental species (Gland, 1980). Most efforts so far to use resynthesised oilseed rape have been aimed at introducing one or more genes to improve specific traits, e.g. resistance to *Turnip yellows virus* (TuYV) (Graichen and Peterka, 1999), fungus *Plasmodiophora brassicae* (Diedrichsen and Sacristan, 1991), improvement in meal quality (Gland *et al.*, 1981) or photoperiodic response (Akbar, 1989).

Oilseed rape is grown for the production of animal feed, vegetable oil for human consumption and biodiesel. Oilseed rape is the third largest source of vegetable oil in the world after oil palm and soybean (United States Department of Agriculture, 2011). The main producers of oilseed rape are China, the EU, Canada and India. World production of oilseed rape is growing rapidly; it increased from 36 million tonnes in the 2004 to an estimated 58.4 million tonnes in the 2010-2011 crop season (United States Department of Agriculture, 2011). China, the leading producer, accounts for 12.2 million metric tons, whilst UK, which ranked ninth accounts for 1.9 million metric tons of the global production (Bayer CropScience, 2007a).

Oilseed rape is the third most important crop in the UK after barley and wheat with about 681,000 ha under cultivation (UK Agriculture, 2010). Apart from the cooking oil, the crop is now being increasingly processed for use as a biodiesel. Following crushing of the seeds for biodiesel, the by-product ‘rapeseed meal’ is used as a livestock feed. The crop also has a useful soil-improving role that aids the performance of following crops particularly wheat, hence it is known as a ‘break crop’ (UK Agriculture, 2010).

Despite the economic importance of oilseed rape to the UK economy, yield and production is low. The current average yield of 3.5 tonnes per hectare (Department for Environment Food and Rural Affairs, 2010), is still far below the yield potential of current varieties, estimated at 6.5 t/ha (Berry and Spink, 2006). This could be due to the risk of major fungal and viral diseases build up resulting from an intense cultivation of the crop, due to an increasing demand of oilseed rape for biodiesel, oleochemicals and for healthy vegetable oil (UK Agriculture, 2010).

Major fungal diseases affecting oilseed rape production in the UK include phoma stem canker (black leg disease, caused by *Leptosphaeria maculans*), light leaf spot (*Pyrenopeziza brassicae*), Clubroot (*Plasmodiophora brassicae*), downy mildew (*Peronospora parasitica*), grey mould (*Botrytis cinerea*), sclerotinia (*Sclerotinia sclerotiorum*), dark leaf spot (*Alternaria brassicae*), damping off (*Pythium* spp., *Rhizoctonia* spp.) and verticillium wilt (*Verticillium longisporum*) (Gladders, 2009; Hardwick *et al.*, 1989; Lacey *et al.*, 1987; Rawlinson and Muthyalu, 1979; Scottish Agricultural College, 2009).

Viral diseases are a major contributing factor to low yield and production in oilseed rape in the UK and elsewhere. *Turnip yellows virus* (TuYV, syn. *Beet western yellows virus*), *Cauliflower mosaic virus* (CaMV), *Turnip mosaic virus* (TuMV) and *Broccoli necrotic yellow virus* (BNYV) have been reported infecting oilseed rape in the UK (Walsh and Tomlinson, 1985; Walsh, 1986; Hardwick *et al.*, 1994). Of these, TuYV is the most common with infection levels of up to 100% (Smith and Hinckes, 1985; Walsh, 1986; Stevens *et al.*, 2008). Annual losses to UK oilseed rape industry from blackleg, light leaf spot, sclerotinia and TuYV infections have been estimated to be £36.4 million, £30.3 million, £8.4 million and £17.5 million respectively (Clarke *et al.*, 2009). The annual expenditure at farm level on fungicides for the control of fungal diseases in oilseed rape in the UK in 2008 was estimated at £30 million (Knight and Turner, 2009).

1.1.2 *Arabidopsis thaliana*.

Arabidopsis thaliana is a member of *Brassicaceae* family and is closely related to the genus *Brassica* (Meyerowitz and Pruitt, 1985). *A. thaliana* is a model plant for the genetic study of *Brassica* species; it is small, with a rapid (short) growth cycle producing relatively large amounts of seed (up to 10,000 seeds per plant) and has the benefit of a compacted genome with a low content of repeated sequences; it has perfect flowers (self-fertile) and can also be genetically engineered (Dixon, 2007).

The combined advantages listed above have made *A. thaliana* a useful model for studying plant-pathogen interactions. Stevens *et al.* (2005) identified *A. thaliana* as a host for TuYV. This provided a valuable model pathosystem with which to study aphid-TuYV-oilseed rape (vector-virus-host) interactions such as gene silencing

phenomena and gene regulation. *A. thaliana* has been used in studies on TuMV (Hughes, 2001; Martinez-Herrera *et al.*, 1999) and *Tomato etch virus* (TEV) (Mahajan *et al.*, 1998). In studying the interactions between six wild populations of *A. thaliana* and *Cucumber mosaic virus* (CMV), TuMV, *Turnip yellow mosaic virus* (TYMV) *Turnip crinkle virus* (TCV) and CaMV, Pagan *et al.* (2010) identified *A. thaliana* as a model for the study of plant-virus co-evolution. The roles of polerovirus protein P0 as a suppressor of posttranscriptional gene silencing (Pazhouhandeh *et al.*, 2006) and the TuMV-encoded RNA-silencing suppressor, P1/HC-Pro (Kasschau *et al.*, 2003) have been studied in *A. thaliana*. *A. thaliana* has also been used in studies on a number of other plant pathogens such as *Pseudomonas syringae* (Grant *et al.*, 1995; Kover and Schaal, 2002; Kover *et al.*, 2005) and *Peronospora parasitica* (Bittner-Eddy *et al.*, 2000; Parker *et al.*, 1997).

1.2 The *Polerovirus* genus

Polerovirus, one of the three genera of the family *Luteoviridae*, is an important genus of plant viruses that can infect a wide range of hosts causing significant yield losses. The first symptoms of a polerovirus were observed as leafroll in potato in Europe during the second half of the 18th century. This attracted the attention of scientists due to the economic importance of potato. The virus was subsequently named as *Potato leaf roll virus* (PLRV) from which the genus name *Polerovirus* was derived. The leaf yellowing diseases in sugar beet with resultant yield losses also led to the discovery of two other poleroviruses, *Beet western yellows virus* (BWYV) and *Beet mild yellows virus* (BMV) (Watson, 1952). Other poleroviruses have since been discovered from a wide variety of hosts, for example *Barley yellows dwarf*

virus (BYDV) and the genus is now recognised as one of the most economically important taxa of plant viruses (Smith and Baker, 1999).

The genus *Polerovirus* is believed to have evolved from a recombination event between a sobemovirus and an ancestor that provides the 3' properties (Gibbs, 1995; Mayo and Ziegler-Graff, 1996). The poleroviruses were first classified in a subgroup in the *Luteovirus* genus based on serological relationships, physiological properties of the virus particles and biological relationships such as tissue location and vector relations. Molecular analysis of their nucleotide sequences however discriminated polerovirus from luteoviruses. Subsequently in 1999, the International Committee on Taxonomy of Viruses (ICTV) used genome organisation to define three distinct genera in a new *Luteoviridae* family: *Luteovirus* (type species *Barley yellow-dwarf virus*-PAV), *Polerovirus* (type species PLRV) and *Enamovirus* with the type species *Pea enation mosaic virus*-1 (PEMV-1) (Mayo, 1999). There are also several viruses of the family that have not yet been formally classified into genera (Table 1.1).

Table 1.1 *Luteoviridae* family, its genera and their species (ICTVdB Management, 2006)

Genus	Virus species
Luteovirus	<i>Barley yellow dwarf virus-MAV</i> (BYDV-MAV) <i>Barley yellow dwarf virus-PAV</i> (BYDV-PAV) <i>Barley yellow dwarf virus-PAS</i> (BYDV-PAS) <i>Barley yellow dwarf virus-RGV</i> (BYDV-RGV)
Polerovirus	<i>Potato leafroll virus</i> (PLRV) <i>Cucurbit aphid-borne yellows virus</i> (CABYV) <i>Cereal yellow dwarf virus-RPV</i> (CYDV-RPV) <i>Turnip yellows virus</i> (TuYV, BWYV-FL1; rape and lettuce isolates respectively) <i>Beet mild yellowing virus</i> (BMYV) <i>Beet western yellows virus-USA</i> (BWYV) <i>Beet poleroviruses</i> <i>Beet chlorosis virus</i> (BChV) <i>Carrot red leaf virus</i> (CtRLV) <i>Sugar cane yellow leaf virus</i> (SCYLV)
Isolates that were found similar to known viruses:	Tobacco yellow top virus (synonym of PLRV) Capsicum yellows virus (synonym of PLRV) Tomato yellow top virus (synonym of PLRV) Potato phloem necrosis virus (synonym of PLRV) Solanum yellows virus (synonym of PLRV) Malva yellows virus (synonym of BWYV) Turnip mild yellows virus (synonym of BWYV) Pea leafroll virus (synonym of BWYV) Raddish yellow virus (synonym of BWYV)
Enamovirus	<i>Pea enation mosaic virus</i> RNA-1 (PEMV-1)
Unassigned viruses in this family	<i>Barley yellow dwarf virus-ORV</i> (<i>Oat red-leaf virus</i>) <i>Barley yellow dwarf virus-GPV</i> (<i>S. graminum</i> and <i>R. padi</i>) <i>Barley yellow dwarf virus-SGV</i> <i>Barley yellow dwarf virus-RMV</i> <i>Bean leafroll virus</i> (BLRV) <i>Carrot red leaf virus</i> <i>Chickpea chlorotic stunt virus</i> (CpCSV) <i>Groundnut rosette assistor virus</i> (GRAV) <i>Soyabean dwarf virus</i> (SbDV) <i>Sweet potato leaf speckling virus</i> (SPLSV) <i>Tobacco necrotic dwarf virus</i> (TNDV) <i>Ryegrass chlorotic streak virus</i> (synonym of BYDV)

1.2.1 Genome Structure and Organisation

Viruses of the genus *Polyomavirus* all share the same basic genome structure and it is presumed that the expression strategy and gene function identified for one species will apply to all members (Stevens *et al.*, 2005). All polyomaviruses have isometric (icosahedral) virions, not enveloped, 24-30 nm in diameter, with 32 capsomeres per nucleocapsid (Miller *et al.*, 1995).

The *Polyomavirus* genome consists of a single-stranded plus sense RNA molecule of 5300-5700 nucleotides long. The 5' terminus has a genome-linked protein (VPg) but the 3' terminus has neither a polyA tail nor a tRNA-like structure. The genome encodes six open reading frames (ORFs) numbered from 0 to 5. Proteins translated from these ORFs are referred as P0 to P5. Coding sequences are in two blocks separated by a 200 nucleotides non-coding sequence (D'Arcy and Domier, 2005) (Figure 1.1).

Gene expression is complex and uses different mechanisms such as subgenomic RNA, frame shift, leaky ribosome scanning, termination suppression and polyprotein (D'Arcy and Domier, 2005). The three 5'-proximal ORFs (P0, P1, P2) are expressed from the genomic RNA (gRNA). The initiation of translation of ORF0 begins after a short leader sequence at the first AUG codon of the genome. Leaky scanning of this codon allows some ribosomes to bypass it and initiate translation at the start codon of ORF1 (Miller *et al.*, 1995; Stevens *et al.*, 2005). There is extensive overlapping between the first three ORFs. The translation of ORF2 is achieved when ribosomes undergo a frameshift from ORF1 to ORF2 to produce a fusion protein P1-P2.

The cluster of three genes of the 3'-proximal block (ORFs 3, 4 and 5) are translated from subgenomic RNA which is thought to depend on the initiation of the viral RNA- dependent RNA- polymerase (RdRp) at internal promoter sites on the minus strand synthesised during gRNA replication (Stevens *et al.*, 2005). In the subgenomic RNA, ORF3 codes for coat protein (major capsid protein) and ORF4 which is embedded in ORF3 but in another reading frame, codes for a putative movement protein by a leaky scanning mechanism, as in ORF1 (Mayo and Ziegler-Graff, 1996). ORF5 is translated by in-frame readthrough of the ORF3 stop codon and P5 is therefore found only as a minor fusion protein (P3+P5). The resulting ~75kDa P3-P5 fusion protein is generally referred to as readthrough (RT) protein (Mayo and Ziegler-Graff, 1996).

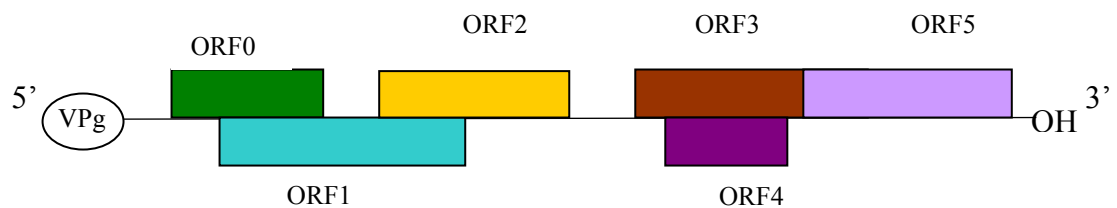


Figure 1.1: Organisation of *Polerovirus* genome (D’Arcy and Domier, 2005)

ORF0, symptoms, host range, suppressor of gene silencing
 ORF1/2, replication; ORF3, coat protein; ORF4, transport protein;
 ORF5, virus accumulation and persistence within the vector.

1.2.2 Functions of Proteins

1.2.2.1 P0

In working with TuYV, Pfeffer *et al.* (2002) strongly implicated P0 as a suppressor of post-transcriptional gene silencing (PTGS) that enabled the virus to overcome

host resistance to infection. P0 acts as an F-box protein, recruiting the post-transcriptional modification system to overcome the post-transcriptional gene silencing system (Bortolamiol *et al.*, 2007; Pazhouhandeh *al.*, 2006). P0 interacts with SKP (S-phase kinase related protein) to constitute a SKP-cullin-F box-P0 (SCF-P0) complex, which presumably targets ARGONAUTE 1 (AGO1) for ubiquitination and degradation by 26S proteasome. P0 is thus said to provoke degradation of AGO1, the slicer protein in the RNA-induced silencing complex (RISC) (Pazhouhandeh, 2007). It is believed that P0 is also involved in symptom development and responsible for determination of the host range of the virus (Pfeffer *et al.*, 2002).

1.2.2.2 P1 and P2

Both P1 and P2 contain sequences strongly indicative of a role in replication (Mayo and Ziegler-Graff, 1996). P1 is known to contain protease motifs and also carries the amino acid sequence shown to be part of VPg, which is found covalently associated with the 5' end of the virus genome (van der Wilk *et al.*, 1997), while P2 carries RdRp, harbouring the consensus core GDD motif (Mayo and Ziegler-Graff, 1996).

1.2.2.3 P3

P3 is the major coat protein (CP) responsible for the formation of viral particles and is required for infection of whole plants, efficient systemic spread and long-distance movement within the phloem (Brault *et al.*, 2005). The CP also participates in various steps of the viral life cycle, such as virion assembly, stability, systemic infection and transmission (Torres *et al.*, 2005). The protein is critical to 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 (50-90% identity) based upon the phylogenetic relationships of their complete nucleotide sequences (Mayo and Ziegler-Graff, 1996).

1.2.2.4 P4

P4 expression is required for the systemic spread of virus infection in whole plants (Stevens *et al.*, 2005) and may fulfil the role a putatively phloem-specific movement protein (MP) function based on its biochemical properties and subcellular localisation. ORF4 is present and highly conserved (42-90% identity) in poleroviruses and luteoviruses but it is not found in enamoviruses.

1.2.2.5 P5 Read-through domain (RTD)

The P5 is involved in symptom induction, virus accumulation and potentially, in systemic spread (Brault *et al.*, 2005). It also plays a key role in transmission efficiency and specificity, as well as in virus persistence within the aphid vector (van den Heuvel *et al.*, 1999).

1.2.3 Virus infection and replication

Virus infection of plants begins with the delivery of the virus into the host cell. This is followed by the uncoating and release of the viral genomic RNA into the host cytoplasm. The viral RNA ORF1 and ORF2 are translated to produce RdRp fusion protein. A negative-sense complementary ssRNA is synthesised using the genomic RNA as template. New genomic RNA is synthesised using the negative-sense RNA as template. The negative-sense complementary ssRNA also serves as template for the synthesis of 3' co-terminal subgenomic RNAs (sgRNAs). Translation of these

sgRNAs yields the capsid (and extended CP) and movement proteins. New viral particles are then assembled (D'Arcy and Domier, 2005).

1.2.4 Polerovirus transmission

Poleroviruses are restricted to the phloem tissue of host plants and their aphid vectors strictly transmit them from plant to plant in a persistent circulative, non-propagative manner (Gildow, 1999; Gray and Gildow, 2003). The green peach aphid, *Myzus persicae* (Sulzer), efficiently transmit all beet poleroviruses, whereas *Macrosiphum euphorbiae* (Thomas) and *Brevicoryne brassicae* are less efficient vectors of BMV, BWYV-USA and TuYV (Stevens *et al.*, 2005; Bayer CropScience, 2007b; Schliephake *et al.*, 2000). For successful transmission to take place, virions have to be endocytosed and exocytosed across two epithelial barriers (alimentary canal and accessory salivary glands) in the vector's body (Reinbold *et al.* 2003). Virus particles first enter the aphid's body via ingestion of infected sap, then are transported across the gut wall to the haemocoel and finally accumulate in the accessory salivary gland. The particles are then injected into the plant during penetration of the aphid's stylet during feeding (Stevens *et al.*, 2008).

Efficient transmission of most poleroviruses requires an acquisition and an inoculation access period each of 24 hours (Waterhouse *et al.*, 1998). The virus acquisition access period is determined by the time taken for the aphid's stylet to reach the phloem of the infected source plant and this can be as short as 15 minutes. The latent period (i.e. the time taken between an aphid acquiring the virus before being able to transmit the particles to new host) is usually 24 hours and can be as long as four days (Stevens *et al.*, 2008).

Poleroviruses have also been transmitted successfully by *Agrobacterium tumefaciens*-inoculation (Leisner *et al.*, 1992) and grafting (Barker and Harrison, 1985). Biolistic inoculation allowed the mechanical transmission of BWYV (syn TuYV) and PLRV for the first time with resultant 30-50% systemic infection in *Nicotiana occidentalis* and 15-30% infection in *Nicotiana clevelandii* plants (Hoffmann *et al.*, 2001).

1.3 *Turnip yellows virus* (TuYV)

1.3.1 History of *Turnip yellows virus* (TuYV)

TuYV (syn BWYV), is the most important viral disease infecting oilseed rape in the UK (Walsh, 1986). The virus name has gone through several changes. This problem began when BWYV was identified in the USA as an important virus causing stunting and chlorosis in a wide range of plants resulting in yield losses in crops such as sugar beet, spinach, lettuce and turnip (Duffus, 1961). This was just after a similar virus BMYV had been characterised from sugar beet in the UK (Russell, 1958). The American strains were initially called ‘Radish yellows’ but were subsequently re-named BWYV (Duffus, 1961). In the UK, a BWYV-like virus was subsequently found on hosts, which had previously been reported as immune to BMYV, such as lettuce (Duffus and Russell, 1970). The BWYV-like virus was biologically and serologically similar to BWYV from USA but differences exist in their host ranges. The European strains of BWYV did not infect sugar beet unlike the USA strain (Duffus and Russell, 1970).

In discriminating between the European and USA isolates of BWYV, names such as TuYV (Schubert *et al.*, 1998), *Brassica yellows virus* (Hauser *et al.*, 2000a) and

Brassica yellowing virus (Hauser *et al.*, 2000b) have been proposed for the non-beet infecting isolates. Consequently, the International Committee for the Taxonomy of Viruses (ICTV) approved the proposal to re-classify the non-sugar beet infecting strain of BWYV as an independent virus in the genus *Polerovirus*, family *Luteoviridae*; the name *Turnip yellows virus* (Mayo, 2002).

1.3.2 Genetic variation of *Turnip yellows virus* (TuYV)

Plant RNA viruses (including TuYV) are characterised by the potential for high degree of genetic variation due to short generation times and error prone replication since no proofreading correction mechanism is associated with RdRp (Domingo and Holland, 1994) leading to populations known as quasispecies (Eigen *et al.*, 1988). Studies of genetic structure and diversity in viruses have a practical significance in developing strategies for the control of viral diseases, and in the case of RNA plant viruses, knowledge of genetic diversity is important to efforts in breeding for host resistance (Janssen *et al.*, 2007).

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 (Hauser *et al.*, 2000a; de Miranda *et al.*, 1995; Schubert *et al.*, 1998).

Amino acid sequence comparison of the CP (P3 gene) showed that the lettuce isolate TuYV-FL1, the only fully sequenced isolate of TuYV (Veidt *et al.*, 1988), falls in a distinct group of TuYV isolates from rape, sprouts, cauliflower, broccoli and calabrese (de Miranda *et al.*, 1995).

Similarly, TuYV-FL1 appears to be a particular strain differing within the P0 amino acid sequence by 15-20% from other TuYV isolates and is able to infect fodder beet at a low rate when using *M. persicae* (Hauser *et al.*, 2000b). P0 amino acid sequence comparison of TuYV isolates resulted in three clusters. The first comprised isolates LP2-8, BN5 (from oilseed rape, Germany), GB1 (from oilseed rape, U.K.), K 7526 (from ornamental cabbage, Germany), BRA 753 (red cabbage, Germany); to the second F97 (oilseed rape, France), Raph-Ma (raddish, Germany) and HL-VT (heartsease, Germany). The third cluster was formed solely by FL1 (lettuce, France) (Schubert *et al.*, 1998). This means that TuYV isolates are divergent (Schubert *et al.*, 1998)

The CP (P3) amino acid sequences are highly conserved (more than 90% homology), whereas the P0 sequences are variable (about 30% homology) between species TuYV and BMV (Hauser *et al.*, 2000a). The variability of CP amino acid sequences within members of TuYV is greater than within BMV members (Hauser *et al.*, 2000a). This probably reflects the adaptation of isolates to different plant species and vectors, resulting in a broader host range of the TuYV species (Hauser *et al.*, 2000a). The variability in the CP sequence may also explain the difficulties in raising a common monoclonal antibody (MAb), which would cross-react with all TuYV isolates (Hauser *et al.*, 2000b). For example MAb G4C10 (Rabenstein *et al.*, 1995) raised against BWYV, reacted only with the German isolate (TuYV-BN5) and U.K. isolate (TuYV-GB) but not with France isolates (TuYV-FL, BWYV-Col, and BWYV-Fev) (Hauser *et al.*, 2000a).

Information on genetic diversity of TuYV infecting oilseed rape or for that matter other crops in the UK is however not available. Knowledge of variation in TuYV infecting oilseed rape in the UK is vital in breeding for resistant varieties.

1.3.3 Incidence of *Turnip yellows virus* (TuYV) infection in oilseed rape crops

TuYV infection of oilseed rape crops in England was first reported in 1980 (Gilligan, 1980) but the widespread incidence of the virus in the UK was first reported by Smith and Hinckes (1985). Results from a survey conducted in 1983, covering 80 autumn-sown oilseed rape crops from Aberdeenshire to Essex showed that 97% were extensively infected with TuYV (Smith and Hinckes, 1985). Varying levels of incidence of TuYV infection in oilseed rape crops have since been reported in the UK, ranging from less than 10% to 100% (Hardwick *et al.*, 1994; Hill *et al.*, 1989; Jay *et al.*, 1999; Walsh *et al.*, 1989). In a series of nationwide field surveys carried out by Bayer CropScience in 2006-07, the incidence of TuYV in unprotected oilseed rape crops ranged between 30% and 100% (Bayer CropScience, 2007b). More recently, a survey conducted in 2009 covering oilseed rape crops on 80 farms from the south coast of England to Scotland showed TuYV infection of up to 70% (Home-Grown Cereals Authority, 2009). Further, a plot experiment conducted by Dewar *et al.* (2011) in 2010 showed that TuYV infection in untreated oilseed crop cv. Castille ranged between 43.3% and 80% and that in insecticide treated crop ranged between 11.7% and 76.7%.

Infection of oilseed rape crops by TuYV have also been reported in some other countries including Germany (Schroder, 1994), France (Kerlan, 1991), Austria (Graichen *et al.*, 2000), Czech Republic (Polak and Majkova, 1992), Serbia (Jasnic

and Bagi, 2007), Iran (Shahraeen *et al.*, 2003) and Australia (Coutts and Jones, 2000).

TuYV infection is largely symptomless but symptoms can include interveinal yellowing or reddening / purpling which may be accompanied by dwarfing (Bayer CropScience, 2007b; Stevens *et al.*, 2008) (Figure 1.2). The first symptom of the TuYV infection in oilseed rape can appear as anthocyanous and / or red discolourations at the margins and tips of lower leaves and later show conspicuous discolouration of the whole leaf (Graichen and Peterka, 1999).



Figure 1.2: Symptoms of *Turnip yellows virus* infection (Bayer CropScience, 2007b)

The incidence of TuYV in oilseed rape crops is related to the flight activity of the aphid vectors whilst the spread of the virus depends on the abundance and movement of the vectors within the crop (Walsh and Tomlinson, 1985). In Germany, high levels of TuYV infection were detected in winter oilseed rape crops during 1995-96

season following high levels of flight activity of aphids during the autumn of 1995 (Graichen and Schliephake, 1999).

1.3.4 Effect of *Turnip yellows virus* (TuYV) on the yield of oilseed rape

TuYV infection is thought to be one of the major reasons why oilseed rape crops do not attain their full potential yield in England (Stevens *et al.*, 2008), estimated at 6.5 t/ha (Berry and Spink, 2006) compared to the current yield of 3.5 t/ha (Department for Environment, 2010). TuYV infection seriously affects all components of yield including number of pods per plant, number of seeds per pod, and the oil content per seed (Bayer CropScience, 2007; Hardwick *et al.*, 1994). Plants infected with TuYV also have reduced leaf area per plant and produce fewer primary branches (Jay *et al.*, 1999). The effect of TuYV on the yield of oilseed rape depend on the incidence of virus infection and the crop variety (Walsh *et al.*, 1989). In a field trial there was no significant difference between infected and uninfected plants; however, in a glass house experiment BWYV infection resulted in yield loss of 9.45% in cultivar Jet Neuf and 20.5% yield loss in the cultivar Mikado (Walsh *et al.*, 1989). One estimate of yield losses due to TuYV infection is up to 30% (Home-Grown Cereals Authority, 2009). Smith and Hinckes (1985) reported that experimental plots of oilseed rape with 100% TuYV- infection yielded approximately 10% less seed and 13.4 % less oil than plots with 18% virus infection. In plot experiments in the U.K., the oilseed rape crops with insecticide treatment against *M. persicae* vectors had yield responses which ranged between 1.6% and 14.6% over the unprotected crops (Hill *et al.*, 1989). In plot experiments in Australia, a site with 96% infection suffered yield losses of up to 46% (Jones *et al.*, 2007) and in Germany, plots of winter oilseed with 90-100% TuYV infection yielded between 12% and 34% lower than plots that were

almost virus free (Graichen and Schliephake, 1999). When oilseed rape plants were co-infected with a mix of TuYV, CaMV and TuMV, the yields of plants with severe virus symptoms were reduced by an estimated 70-79% (Hardwick *et al.*, 1994).

1.3.5 Management of *Turnip yellows virus* (TuYV)

Estimates of yield losses have shown that at an individual crop level, control of TuYV could increase average yields from 3.3 t/ha to between 4.4 t/ha and 6.0 t/ha; and if only half of those losses (10-15%) could be prevented by controlling TuYV infection, the value of the yield improvement would be in the range of £100 and £150 per hectare (Stevens *et al.*, 2008). This is equivalent to £60-90 million per year for UK oilseed rape growers (Stevens *et al.*, 2008).

1.3.5.1 Control of TuYV by chemical control of the aphid vectors

Chemical control of the insect vectors is a common approach used worldwide and a key strategy to reduce the impact of TuYV on yield (Stevens *et al.*, 2008). Pyrethroids, carbamate and organophosphate insecticides have been used in the UK for the control of insect pests in crops (Gibson, 1983; Hill *et al.*, 1989; Sassen, 1983). Walsh *et al.* (1989) showed that granular carbamate insecticide carbofuran did not control TuYV, whilst foliar sprays of the pyrethroid, lambda-cyhalothrin were able to reduce the incidence of TuYV by 86% and 72% in trials conducted in 1985/1986 and 1986/1987 cropping seasons respectively. These levels of control were similar to those reported by Smith and Hinckes (1985) and Nagarajan *et al.* (1987). Read and Hewson (1988) also demonstrated that deltamethrin applied at 6.25 g a.i./ha is very effective in controlling the aphid vector, and thus reducing the incidence of TuYV in oilseed rape.

Control of *M. persicae* with insecticides however, has not been entirely effective due to the evolution of clones of the insect which are resistant to these insecticides. At present, three different resistance mechanisms are known in *M. persicae*. These are esterase resistance, modified acetylcholinesterase (MACE) and knockdown resistance (kdr). In esterase-resistant aphids, there is overproduction of carboxyl-esterase enzyme which detoxifies some insecticides before they reach the target sites. This provides broad-spectrum resistance to organophosphates (OPs), whilst carbamates and pyrethroids are also affected to a lesser extent (Foster *et al.*, 2007; Insecticide Resistance Action Group-UK, 2008). Organophosphate and carbamate insecticides attack acetylcholinesterase, the enzyme that regulates the flow of a chemical messenger across the gap (synapse) between nerve cells; this disruption kills the insect (Insecticide Resistance Action Group-UK, 2008). In MACE resistance, the enzyme becomes insensitive to the dimethyl carbamate pirimicarb insecticide, rendering the *M. persicae* immune to this insecticide (Insecticide Resistance Action Group-UK, 2008). In the UK, MACE continues to cause sporadic control problems as a result of the protection it confers to the insecticides, pirimicarb and triazamate (Foster *et al.*, 2007). *M. persicae* with kdr resistance also has a modified target site, insensitive specifically to pyrethroids. So far, the three types of resistance have tended to co-exist, making the *M. persicae*, virtually immune to many of the aphicides applied in the UK (Foster *et al.*, 2007; Insecticide Resistance Action Group-UK, 2008). Stevens *et al.* (2008) have reported that due to the high levels of MACE and kdr resistance in *M. persicae* clones, chemicals will not control up to 80% of current aphid populations.

Neonicotinoid seed treatments, such as Chinook (beta-cyfluthrin + imidacloprid; Bayer, Cambridge, UK) was introduced to control insecticide-resistant *M. persicae* in oilseed rape for several weeks following emergence (Bayer CropScience, 2007). However, this has not been very effective in the UK in controlling the aphid vectors. A second generation neonicotinoid seed treatment, Modesto (beta-cyfluthrin + clothianidin; Bayer, Cambridge, UK) and Cruiser OSR (thiamethoxam + fludioxinil + metalaxyl-M; Syngenta, Cambridge, UK) which are supposed to offer broader spectrum control and longer lasting than Chinook, have therefore been introduced for aphid control in oilseed rape (Bayer CropScience, 2010; Syngenta, 2010). Results of field trials conducted by Dewar *et al.* (2011) revealed that Modesto and Cruiser OSR seed treatments gave significant control of *M. persicae* for up to 10 weeks after sowing, compared to Chinook which was significantly poorer and less persistent. They also reduced secondary spread of TuYV in winter oilseed rape (Dewar *et al.*, 2011). However, it has been observed that where TuYV infections were higher than 85%, control was often poor with Modesto, but where inoculum pressure was lower, both Modesto and Cruiser OSR could provide 50-75% control (Abram, 2010).

Impey (2010) reported that sowing date and seed treatment are important factors when it comes to using seed treatments to reduce virus levels. He said that crops sown at lower seed rates are more at risk from TuYV. In these situations, a better seed treatment is required, as the lower seed rate increases the pressure.

Typical August or September sowings of oilseed rape result in an emerged crop that coincides with the autumn aphid migrations (Stevens *et al.* 2008), providing a

suitable overwintering habitat for the vectors of TuYV (Walsh and Tomlinson, 1985). The effect of sowing date on the incidence of TuYV appears to vary by year as in some years, late sown oilseed rape crops have lower virus incidence (Njuguna *et al.*, 1986), whilst in other years there was no difference in virus incidence regardless of sowing date (Nagarajan *et al.*, 1987).

1.3.5.2 Breeding and growing of resistant varieties

Control of TuYV using insecticides is not completely effective since in addition to oilseed rape and other crucifers, a great number of common weeds and wild species are hosts to TuYV (Graichen and Rabenstein, 1996), *M. persicae* vectors have developed resistance to the insecticides cleared for autumn use on oilseed rape (Collier, 2009; Stevens, 2010) and at high infection pressure newer insecticides are not effective (Dewar *et al.*, 2011).

The most effective control of TuYV may be achieved by using host resistance in oilseed rape breeding programmes to breed TuYV-resistant crops. Graichen and Peterka (1999) successfully transferred the TuYV-resistance identified in a resynthesized oilseed rape line, R54, into a modern oilseed rape material in Germany. The results from their work indicate that there is potential for the use of genetic resistance in the control of TuYV in oilseed rape. To use host resistance effectively, the genetic basis of resistance has to be elucidated (Hughes, 2001).

Further screening of worldwide oilseed rape germplasm may be required to identify different sources of resistance to TuYV to maintain a diverse gene pool for plant breeding.

1.4 Host resistance

The use of crop plants that are resistant to viruses is likely to be the most promising control approach (Hull, 2009). Thus for many years plant breeders have been attempting to produce virus-resistant varieties. There are two sources of resistance genes: natural ones from sexually compatible species and non-conventional ones from genetic modification (Hull, 2009) as described in section 1.4.5. Where suitable genes can be introduced to agriculturally satisfactory cultivars, breeding for resistance to a virus provides one of the best solutions to the problem of virus disease (Hull, 2002; 2009). There are however two major problems associated with such conventional approach. Firstly, the difficulty in finding resistance genes in species that are sexually compatible with the crop species. For example, when Luterbacher *et al.* (2004) screened 600 accessions of closely related wild and cultivated *Beta* species for resistance to foliar diseases, they found out that greater than 62% of the Section *Corollinae* were highly resistant to BMV and *Beet yellows virus* (BYV). However, sexual incompatibility between this section and sugar beet make utilisation of this resource impractical using conventional breeding methods. Secondly, the durability of the resistance gene (how long can the gene be deployed successfully before a resistance breaking (virulent) strain of the virus emerges?). Of the 87 host-virus combinations from which resistance genes have been found (Fraser, 1992), more than 75% of those tested were overcome by virulent virus isolates (Hull, 2002; 2009), implying that they were not durable. Durable resistance refers to resistance that remains effective during its prolonged and widespread use in environments favourable to the pathogen or disease spread (Johnson, 1981).

1.4.1 Types of resistance

Plant resistance to virus infections can be divided into three basic groups, operating at different levels within plant species (Fraser, 1990; Hull, 2009).

1.4.1.1 Immunity (Non-host resistance)

Non-host resistance operate at the species level and is when most plant species are resistant to most viruses (Dawson and Hilf, 1992; Hull, 2002; Maule *et al.*, 2007). Here, virus does not replicate in protoplasts, nor in cells of the intact plant, even in inoculated cells. Inoculum virus may be uncoated (and the genomic RNA or DNA released), but no progeny viral genomes are produced (Hull, 2002). Non-host resistance is durable and therefore is valuable for exploitation in the context of virus-resistant plants; however, it is not tractable by classical genetics and therefore remains very poorly understood (Maule *et al.*, 2007).

1.4.1.2 Acquired resistance

Acquired or induced resistance operates at individual level and is conferred by methods such as cross protection (where a plant is deliberately inoculated with a mild strain of a virus to protect it against a more severe strain of the same virus (Gal-On and Shibolet, 2006; Walkey, 1985), chemical application (Tally *et al.*, 1999) or plant transformation (i.e. pathogen derived resistance). These inducible forms of resistance may be localised or systemic in their response (Kessman *et al.*, 1994).

1.4.1.3 Cultivar resistance

Cultivar resistance describes the situation where one or more cultivars or breeding lines within a species show resistance, whereas others do not (Hull, 2009). This type of resistance operates at the individual level and is heritable. Cultivar resistance often involves a “gene-for-gene” type interaction (see section 1.4.2) and has been most widely used in plant breeding (Crute and Pink, 1996).

1.4.2 Models of Resistance

Flor (1971) proposed the “gene-for-gene” model to explain results of studies on the inheritance of the resistance of flax (*Linum usitatissimum*) to the flax rust fungus (*Melampsora lini*) in which the host resistance gene (*R*) interacts with the pathogen’s avirulence gene (*Avr*).

The model proposes that for resistance to occur, complementary pairs of dominant genes, one in the host and the other in the pathogen, are required. A loss or alteration in the host *R* gene or in the pathogen’s *Avr* gene leads to disease or compatibility (Table 2; Hull, 2009).

Table 1.2. The gene-for-gene hypothesis of Flor (1971)*

Host phenotype	Pathogen phenotype		
	Avirulent	Avirulent	Virulent
	AA	Aa	aa
Susceptible rr	+	+	+
Resistant Rr	-	-	-
Resistant RR	-	-	+

* Adapted from Hull (2009)

The reaction of the host with dominant, R, or recessive, r, gene to the pathogen with either dominant avirulence, AA, or recessive avirulence, Aa, gene is indicated as: +, susceptible; -, resistant.

Basically, the interaction between the *R* and *Avr* genes leads to both a local and systemic signal cascade; the local signalling cascade triggers a host response that contains the pathogen infection to the primary site, whilst the systemic cascade primes defence systems in other parts of plants (Hull, 2009).

Gene-for-gene type interactions have also been proposed for a number of recessive genes conferring resistance against plant viruses. These include recessive allele *sbn-1* with resistance to specific pathotypes of *Pea seed-borne mosaic virus* (Keller *et al.*, 1998), the recessive *bc* alleles with strain specific resistance to *Bean common mosaic virus* (Donovan, 2000; Drijfhout, 1978), recessive allele *retr01* with resistance to TuYV (Rusholme *et al.*, 2007) and recessive allele *bwjv* resistance to BWYV in lettuce (Pink *et al.*, 1991). Most recessive resistance genes interfere with virus replicating cycle, preventing the expression or replication of the viral genome (Hull, 2009).

1.4.3 Genetic basis of resistance to *Poleroviruses*

The first step in the study of genetics of viral resistance is to determine whether the resistance response is inherited, and if so, the number of genes involved and their mode of inheritance (Kang *et al.*, 2005). To date, hundreds of naturally occurring genes for resistance to plant viruses have been reported from studies of both monocot and dicot crops, their wild relatives and the model plant, *Arabidopsis* (Kang *et al.*, 2005). Genetics of resistance has been described in a number of polerovirus-host plant pathosystems.

Several independently inherited components of resistance to BYV and BMV have been identified in sugar beet (Russell, 1972). These include resistance to aphid vectors of the viruses, resistance to virus inoculation and virus tolerance. The expression of resistance to aphids and resistance to virus inoculation in sugar beet can be altered by several factors including the concentrations of major nutrients or trace elements in the soil and factors which affect the concentrations of sugars and amino acids in the leaves (Russell, 1972). Resistance of sugar-beet plants to aphids and to virus inoculation is increased by placing them in darkness or in low light intensity after being infested with viruliferous *M. persicae* (Russell, 1969). A preliminary result from resistance work on sugar beet conducted by Russell (1966) suggested that resistance to BMV may be controlled by recessive genes which occur widely in sugar-beet cultivars.

Grimmer *et al.* (2008) successfully transferred resistance to BMV from garden beet, fodder beet and leaf beet accessions to progeny populations in initial crosses with sugar beet. BMV resistance was successfully inherited in BC₁ and BC₂

generations, suggesting that the resistance could potentially be introgressed from these sources into elite sugar beet lines (Grimmer *et al.*, 2008).

Marczewski *et al.* (2001) reported that resistance to PLRV is controlled by genetic factors that limit plant infection by viruliferous aphids or virus accumulation. Quantitative trait loci (QTL) analysis of virus accumulation revealed one major QTL, PLRV.1, mapped to potato chromosome XI in a resistance hotspot containing several genes for qualitative and quantitative resistance to viruses and other potato pathogens. This QTL explained between 50 and 60% of the phenotypic variance. Marczewski *et al.* (2001) also reported of two additional minor QTL controlling resistance to PLRV accumulation in potato, mapped to chromosomes V and VI. Major gene inheritance of resistance to PLRV was also demonstrated in a parthenogenic population derived from a highly resistant tetraploid andigena landrace, LOP-868 (Velasquez *et al.*, 2007). This major gene or chromosome region seems to control a single mechanism for resistance to infection and virus accumulation in this source (Velasquez *et al.*, 2007).

Wheat substitution line P29, whose 7D chromosome was replaced with wheatgrass (*Thinopyrum intermedium*) chromosome 7E, was completely resistant to *Cereal yellow dwarf virus*, CYDV (Crasta *et al.*, 2000; Sharma *et al.*, 1997). The data from Wiangjun and Anderson (2004) suggest that *T. intermedium*-derived resistance to CYDV is primarily dosage dependent and could be developmentally regulated if the amount of inoculum was large enough (Wiangjun and Anderson, 2004).

Studies in the genetic control of resistance to *Cucurbit aphid borne yellows virus* in *Cucumis melo* (CABYV) reported that the resistance of an Indian melon line PI 124112 to CABYV was conferred by two independent complementary recessive genes, *cab-1* and *cab-2* (Dogimont *et al.*, 1997).

Studies on the genetics of resistance to BWYV revealed that the resistance of lettuce cultivars Burse 17 and Crystal Heart to BWYV was controlled by a single recessive gene designated by *bwyv* (Pink *et al.*, 1991). An ELISA showed that the resistant plants were not immune to infection by BWYV, and may develop some symptoms. However, the concentration of the virus found in the resistant plants was less than in susceptible one (Pink *et al.*, 1991).

1.4.4 Genetic basis of resistance to *Turnip yellows virus* (TuYV) in *Brassica napus*

Resistance against TuYV was first detected in 1992 in the resynthesised oilseed rape line ‘R54’ (Graichen, 1994). Progeny of R54 was used to transfer the resistance into modern oilseed rape breeding material, Caletta (Graichen and Peterka, 1999). Data are not available on other sources of TuYV resistance in *Brassica* spp. including oilseed rape or in *A. thaliana*. Studies on the genetics of TuYV resistance are all based on that derived from R54.

Dreyer *et al.* (2001) identified a single QTL on *B. napus* chromosome N04 (MS17), explaining up to 50% of the phenotypic variation for the TuYV resistance derived from R54. An RFLP marker *wg6f10.H1* linking this QTL to the linkage group N04 has been described. Recently, Juergens *et al.* (2010) also identified a major QTL on

B. napus chromosome N04 (A04) for TuYV resistance derived from R54. Two simple sequence repeats (SSR) markers, three amplified fragment length polymorphism (AFLP) markers and two sequence-tagged sites (STS) markers linking this QTL to the linkage group N04 (A04) have been described.

Studies on the inheritance of TuYV resistance derived from the resynthesised oilseed rape, R54, showed that TuYV resistance is controlled by a single major gene along with additional contributing factors (Graichen, 1998; Dreyer *et al.*, 2001; Juergens *et al.*, 2010). For example, Dreyer *et al.* (2001) and Juergens *et al.* (2010) observed a breakdown in the TuYV resistance during growth periods in the glasshouse and the fields respectively, resulting in higher virus titre values. This breakdown in resistance was attributable to higher temperatures during growth period. They therefore concluded that the TuYV resistance is incomplete but influenced by environmental factors, particularly temperature. An influence of temperature and other environmental factors on virus resistance have also been reported in BWYV of lettuce (Walkey and Pink, 1990) and PLRV of potato (Barker and Harrison, 1984). It is important to point out that there is no available data on the genetics of resistance to TuYV in other brassica crops apart from oilseed rape described above.

1.4.5 Pathogen derived resistance (Engineered virus resistance).

Conventional breeding strategies can involve lengthy backcross breeding programmes to eliminate deleterious genes (Pink and Puddephat, 1999). The deployment of disease resistance genes may be achieved through plant transformation and disease resistance genes introduced directly into elite germplasm without the introduction of deleterious genes (Hughes, 2001).

According to Hull (2009), the idea leading to the concept of pathogen derived resistance for plant viruses are encapsulated as a general concept in a paper by Sanford and Johnston (1985). They suggested that the transgenic expression of pathogen sequences might interfere with the pathogen itself, terming the concept parasite-derived resistance. Several names have since been used to describe this method including non-conventional protection, transgenic resistance and engineered virus resistance but the generally accepted term is now pathogen-derived resistance (PDR) (Hull, 2009). The most commonly used sequences for protecting plants are viral sequences either coding for viral protein which interferes with replication cycle of the target virus, or non-coding sequence, which primes the RNA silencing defence system (Hull, 2009).

The commercial deployment of PDR has involved sequences encoding CP (Birch, 1997; Fuchs and Gonsalves, 1995) where over-expression of the viral CP gene, incorporated in the plant genome, prevents establishment of viral infection (Hughes, 2001). Transgenic approaches to plant virus resistance have been widely explored since the earliest experiments where transgenic tobacco plants expressing *Tobacco mosaic virus* (TMV) CP were challenged with TMV and shown to be resistant (Beachy *et al.*, 1986; Bevan *et al.*, 1985; Powell-Abdel *et al.*, 1986). The sequences encoding viral CPs are the most widely used for conferring protection in plants because this gene was used in the first example of this approach and because CP genes are relatively easy to identify and clone (Hull, 2009). The level of protection conferred by CP gene transgenic plants varies from immunity to delay and attenuation of symptoms (Prins *et al.*, 2008). In some cases protection is broad and effective against several strains of the virus from which CP gene is derived, or even

against closely related virus species (Beachy *et al.*, 1990). However, several possible hazards have been suggested for CP-mediated resistance; transencapsidation, recombination and synergism (Robinson, 1996), all of which results in the evolution of new viruses.

It is now possible to introduce almost any foreign gene into a plant and obtain expression of that gene (Hull, 2009). Koev *et al.* (1998) transformed oats with 5' half BYDV-PAV genome which encodes polymerase genes and found the enhanced resistance to BYDV-PAV and BYDV-MAV in the transformants. Wang *et al.* (2000) also transformed barley with hpRNA of the 3' half of the viral genome, and as a result low virus titre and enhanced resistance were found in the transformant. Judging from the above protection of oats and barley against BYDV using pathogen derived resistance approach, there could be some prospects in protecting brassica crops including oilseed rape against TuYV, also a member of the same family, *Luteoviridae*.

1.5 AIMS AND OBJECTIVES

The main aim of the project was to study the importance and diversity of *Turnip yellows virus* (TuYV) in oilseed rape (*B. napus*) in England, investigate interactions between the two and the suitability / potential of *A. thaliana* as a model to investigate TuYV- Brassicaceae interactions.

Specific objectives of the project were:

1. To determine the incidence and spatial distribution of TuYV in winter oilseed rape crops in three regions of the UK in autumn and spring over three growing seasons.
2. To study the genetic diversity in TuYV within and between crops and regions based on sequence comparison of the ORF0 (P0 gene) and ORF3 (P3 gene) regions of the viral genome.
3. To investigate the interactions between:
 - A *B. napus* diversity fixed foundation set and TuYV.
 - *A. thaliana* ecotypes and TuYV.
4. To determine the effect of TuYV on growth and yield of oilseed rape in the glasshouse.

CHAPTER 2:

INCIDENCE OF *TURNIP YELLOW VIRUS* (TuYV) INFECTING WINTER OILSEED RAPE (*BRASSICA NAPUS*) IN ENGLAND

2.1 Introduction

Oilseed rape production in the U.K. has been increasing due to an increasing demand for rapeseed oil both as healthy edible oil and as a renewable source of biodiesel. For example, the area of oilseed rape production in the U.K. increased from 570,000 ha in 2009 to 642,000 ha in 2010; the corresponding production during these periods also increased from 1.912 million tonnes to 2.23 million tonnes (Department for Environment Food and Rural Affairs, 2010). This increased production has brought about a high disease pressure for important oilseed rape fungal pathogens and for major viral pathogens particularly TuYV.

TuYV is an economically important disease of oilseed rape which can reduce yields up to 45% in the U.K. (Stevens and Clark, 2009). It affects all constituents of yield including number of pods per plant, number of seeds per pod, seed yield per plant, oil content per seed (Hardwick *et al.*, 1994; Jay *et al.*, 1999; Bayer CropScience, 2007b) and increases glucosinolates (Jay *et al.*, 1999; Blake, 2009). TuYV infection is thought to be one of the major reasons why oilseed rape crops do not attain their yield potential (Stevens *et al.*, 2008). The current average yield of oilseed rape estimated at 3.5 t/ha (Department for Environment Food and Rural Affairs, 2010) is far below the yield potential of current varieties estimated at 6.5 t/ha and the ultimate yield potential estimated at 9.2 t/ha (Berry and Spink, 2006). Effective control of TuYV in oilseed rape is therefore necessary to improve yields. Information on the relative importance of TuYV infection of oilseed rape crops at

various locations, in different years and the type of disease pattern and spread in the field are important prerequisites for developing effective strategies for controlling the disease in oilseed rape crops. Information on the flight activities of the aphid vectors in different regions and growing seasons is also important for developing disease control strategies.

This chapter described the incidence and distribution of TuYV in fields of oilseed rape in three regions of England in the autumn and spring of three crop seasons. The number of *M. persicae* caught in the suction traps located nearest to the sampling sites were compared with TuYV incidences in order to determine whether there was any relationship between the two.

2.2 Materials and Methods

2.2.1 Surveys of winter oilseed rape crops

Using a line transect sampling method (Buckland *et al.*, 2001), the leaves of 100 plants were sampled from three crop fields in each of three oilseed rape-growing regions of England, Eastern (Lincolnshire), Northern (Yorkshire) and Midlands (Warwickshire) in autumn (November-December) and the following spring (April) during the 2007-8, 2008-9 and 2009-10 crop seasons. The Lincolnshire fields were near Long Sutton, the Warwickshire fields were near Gaydon, and the Yorkshire sites were near Allerton, Little Ouseburn, Aberford, Green Hammerton and Whixley. The locations of the fields were determined using a Global Positioning System (Garmin E-Trex GPS Receiver, Garmin Corporation Olathe, KS, USA) (Table 2.1).

The sampling procedure involved estimating the length and breadth of each field in order to divide the field into ten equally-spaced transects with ten equally-spaced-samples collected per transect. Where fields were exceptionally large, only a proportion of the fields were sampled. The mean field size, where available, was 20.8 ha (ranged from 4.3 to 40.0 ha).

2.2.1.1 Sampling sites

Farms sampled rotate oilseed rape crops every 3-6 years with crops such as wheat, potato, sugar beet, peas and may also practice land fallowing. Winter oilseed rape is normally sown in England between late August and early September, overwinters, flowers in the spring (April-May) and is harvested in July/August. Seed sowing in field three in Warwickshire during the 2008-9 crop season was however, delayed by the prevailing weather conditions until late September. Where available, information

on oilseed rape cultivars, date of planting, seed treatments, foliar sprays and their dates of application are presented in Table 2.1. The seed treatments were mainly Chinook (beta-cyfluthrin and imidacloprid, Bayer Crop Science, Cambridge, U.K.) and Modesto (beta-cyfluthrin and chlothianidin, Bayer Crop Science, Cambridge, U.K.) (Table 2.1). The sampled winter oilseed rape fields bordered fields of cereals, sugar beet, oilseed rape, or woodland.

Table 2.1 Field locations, cultivar, seed treatment and foliar sprays used in the fields sampled

Region \ (Crop year)	Location field	Latitude \ longitude	Cultivar	Seed treatment	Planting date	Foliar spray (Date)
Lincs (2007-8)	1	52.87 ⁰ N 0.25 ⁰ E	Castille Ovation	Chinook	31.08.2007	Cypermethrin ^a (10.2007)
	2	52.80 ⁰ N 0.23 ⁰ E	Astrid	Chinook	28.08.2007 29.08.2007	Cypermethrin (10.2007)
	3	52.79 ⁰ N 0.21 ⁰ E	Ovation	Chinook	02.08.2007	Cypermethrin (10.2007)
Warks (2007-8)	1	52.11 ⁰ N 1.45 ⁰ W	Lioness	Chinook	23.08.2007	Cypermethrin 10 ^b (29.09.2007; 12.10.2007)
	2	51.74 ⁰ N 1.46 ⁰ W	Lioness	Chinook	24.08.2007	Cypermethrin 10 (29.09.2007; 12.10.2007)
	3	51.90 ⁰ N 1.47 ⁰ W	Lioness	Chinook	24.08.2007	Cypermethrin 10 (29.09.2007; 12.10.2007)
Yorks (2007-8)	1	54.04 ⁰ N 1.33 ⁰ W	Lioness	Chinook	09.09.2007	Permasect ^c (30.09.2007) Starion Flo ^d (30.09.2007) Hallmark Zeon ^e (30.09.2007)
	2	53.84 ⁰ N 1.35 ⁰ W	NK Bravour	Chinook	08.09.2007	Permasect (30.09.2007 Starion Flo (30.09.2007 Hallmark Zeon (30.09.2007
	3	54.01 ⁰ N 1.36 ⁰ W	Astrid	Chinook	06.09.2007	Permasect (30.09.2007 Starion Flo (30.09.2007 Hallmark Zeon (30.09.2007
Lincs (2008-9)	1	52.80 ⁰ N 0.22 ⁰ E	Astrid, Castille	Modesto	29.08.2008	N/A ^f
	2	52.79 ⁰ N 0.23 ⁰ E	Astrid	Modesto	28.08.2008	N/A
	3	52.87 ⁰ N 0.22 ⁰ E	Astrid	Modesto	27.08.2008	NA
Warks (2008-9)	1	52.18 ⁰ N 1.45 ⁰ W	Astrid	Chinook	26.08.2008	Cypermethrin 10 (26.09.2008)
	2	52.17 ⁰ N 1.45 ⁰ W	Astrid	Chinook	27.08.2008	Cypermethrin 10 (26.09.2008)
	3	52.20 ⁰ N 1.44 ⁰ W	Astrid	Chinook	27.08.2008	N/A

Table 2.1 continued

Region \	Location		Cultivar	Seed treatment	Planting date	Foliar spray (Date)
(Crop year)	field	Latitude \ longitude				
Yorks (2008-9)	1	54.04 ⁰ N 1.33 ⁰ W	N/A	Chinook	N/A	Cypermethrin N/A
	2	54.03 ⁰ N 1.36 ⁰ W	N/A	N/A	N/A	N/A
	3	53.85 ⁰ N 1.35 ⁰ W	N/A	N/A	N/A	N/A
Lincs (2009-10)	1	52.81 ⁰ N 0.21 ⁰ E	Ovation	Chinook	24.08.2009	Permasect (01.10.2009)
	2	52.79 ⁰ N 0.24 ⁰ E	PR46W21	Chinook	26.08.2009	Permasect (01.10.2009)
	3	52.80 ⁰ N 0.22 ⁰ E	Cabernet	Modesto	25.08.2009	Permasect (01.10.2009)
Warks (2009-10)	1	52.20 ⁰ N 1.44 ⁰ W	Astrid	Chinook	11.08.2009	Cypermethrin 10 ^f (24.09.2009)
	2	52.20 ⁰ N 1.43 ⁰ W	Astrid	Chinook	11.08.2009	Cypermethrin 10 (24.09.2009)
	3	52.2 ⁰ N 1.45 ⁰ W	Astrid	Chinook	25.08.2009	Cypermethrin 10 (24.09.2009)
Yorks (2009-10)	1	54.01 ⁰ N 1.29 ⁰ W	N/A	N/A	N/A	N/A
	2	54.04 ⁰ N 1.32 ⁰ W	N/A	N/A	N/A	Delcis ^g (24.09.2009) Starion Flo (22.10.2009)
	3	53.84 ⁰ N 1.35 ⁰ W	Astrid	N/A	N/A	Permasect (28.08.2009)

^a Product (trade) name not supplied by the farmer. The insecticide was applied in late October 2007.

^b Cypermethrin 10 is a product name for cypermethrin (Greenriver Industry Co., Guangdong, China).

^c Permasect is a product name for cypermethrin (Nufarm UK. Ltd, Bradford, U.K.).

^d Starion Flo is a product name for bifenthrin (Belchim Crop Protection, St Neots, U.K.).

^e Hallmark Zeon = 100g/l lambda-cyhalomethrin +1, 2-benzisothiazolin-3-one (Syngenta, Cambridge, U.K.)

^f Information not available.

^g Delcis is a product name for deltamethrin (Bayer CropScience, Cambridge, U.K.).

2.2.2 Detection of *Turnip yellows virus* (TuYV) in the samples

The presence of TuYV was tested by standard triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) using paired wells in microtitre plates (96-well Nunc Maxisorp; Nunc, Roskilde, Denmark) based on the method described by D'Arcy *et al.* (1989). The ELISA was carried out essentially as described by Hunter *et al.* (2002) with minor modifications as described below. The leaf samples were macerated singly or in groups of 2 leaves by macerating them between a pair of steel rollers (Meku–Pollahne, Wennigsen, Germany) and the sap collected in separate microfuge tubes. The primary antibody was rabbit IgG (AS-0049, DSMZ, Braunschweig, Germany). Depending upon availability, secondary antisera were, the rat monoclonal antibody MAFF 24 (Stevens *et al.*, 1995), the mouse monoclonal antibody (AS-0049/1, DSMZ, Braunschweig, Germany), or the mouse monoclonal antibody (1010-03, Neogen Europe Ltd., Auchincruive, U.K.). Tertiary alkaline phosphatase conjugated antisera were goat anti-rat (A8438, Sigma-Aldrich Ltd., Poole, UK), goat anti-mouse (A3562, Sigma-Aldrich Ltd., Poole, U.K.), or rabbit anti-mouse (RAM-AP, DSMZ, Braunschweig, Germany).

Absorbance values ($A_{405\text{nm}}$) were measured with a Biochrom Anthos 2010 microplate reader (Biochrom Ltd., Cambridge, U.K.). Absorbance values of 10 uninfected leaf samples were also measured as negative controls. A sample was deemed to be positive when its absorbance was greater than the mean absorbance of ten healthy samples on each ELISA plate, plus 2.262 x standard deviation of the mean of the ten healthy samples (where 2.262 = Inverse of Student's t-distribution at 5% probability level with 9 degrees of freedom). The healthy (uninfected) leaf samples used as negative controls were obtained from oilseed rape cv Mikado raised

in an insect-proof glasshouse. Leaf samples from TuYV-infected Mikado plants, raised in an insect rearing unit, were used as positive controls in order to validate the ELISA. Each ELISA plate had both the 10 negative (healthy sample) and two positive (TuYV-infected sample) controls.

2.2.3 Data analysis

TuYV incidence data were analysed using a generalised linear model (GLM) (Nelder and Wedderburn, 1972). Differences between county and crop season means and their interactions were compared using the approximate least significant difference (LSD) calculated from the analyses.

A two-sample binomial test (Armitage and Berry, 1994) was used to determine whether there was a significant change in TuYV incidences in each field between autumn and spring. The overall correlation between autumn and spring incidences of TuYV infections was assessed using the Pearson correlation coefficient. All statistical analyses were carried out using GenStat (GenStat Release version 12.1) (Payne *et al.*, 2009).

The cumulative numbers of *M. persicae* caught monthly in the Rothamsted insect survey suction traps closest to the sampling sites (Kirton in Lincolnshire 52.92N 0.05W, Askham Bryan in Yorkshire 53.92N1.16W and Wellesbourne in Warwickshire 52.20N 1.60W) were determined from the weekly suction trap aphid catches obtained between August (crop sowing) and November for each year. (The sampling sites in Lincolnshire were approximately 34.44 km from the suction trap; Warwickshire sites were approximately 20.76 km from the suction trap and

Yorkshire sites were approximately 20.52 km from the suction trap). A non-linear regression analysis fitting an exponential function was used to explore the relationship between mean TuYV incidences in autumn and the cumulative aphid counts.

A Black-White (BW) join-count statistic (Cliff and Ord, 1969) was calculated to assess spatial autocorrelation within each field on each sampling times, comparing a null hypothesis of random distribution of infected samples with an alternative hypothesis of spatial clustering. The statistic measures the number of neighbour pairs containing both an infected and a healthy plant, where neighbours were defined here to be adjacent samples in vertical or horizontal directions (each non-edge sample has four neighbours, i.e. “rook case”). The significance of the join-count statistic is achieved by computing a standard normal deviate, called a Z-score which is given by the formula:

where σ_{BW} is the standard deviation for BW joins. The expected number of BW neighbours is calculated based on the overall proportion of infected plants and represents the likely pattern under a random distribution.

With the aim of detecting evidence of clustering, a one-sided test for negative values of the BW join-count statistic was appropriate, negative values indicating a positive spatial autocorrelation between infected plants and probability levels of $P \leq 0.05$ indicating significant spatial autocorrelation.

2.3 Results

2.3.1 Field observations

Plants sampled in autumn during the 20007-8 and 2008-9 crops were at 2-4-leaf growth stage (Figure 2.1) but during spring they were advanced in growth, either about to flower or had flowered (Figure 2.2).



Figure 2.1 Field 1 sampled in Lincolnshire in December (autumn) during 2008-9 crop. Plants are young with 3-4 leaves.



Figure 2.2 Field 1 sampled in Lincolnshire in April (spring) during 2008-9 crop. Plants have just flowered

2.3.2 Incidence of *Turnip yellows virus* (TuYV) infection in winter oilseed rape crops

The overall mean TuYV incidences recorded in the autumn of the 2007-8 ($36.67 \pm 4.56\%$) and 2009-10 ($48.67 \pm 3.62\%$) crop seasons were not significantly different

from each other but were significantly higher ($P < 0.001$) than recorded in the autumn of the 2008-9 crop ($6.11 \pm 2.33\%$) (Table 2.2). Highly significant differences ($P < 0.001$) in the mean percentage autumn virus incidences were found between the counties (Table 2.2). Lincolnshire had the highest mean TuYV incidence ($55.00 \pm 3.69\%$), followed by Warwickshire ($23.78 \pm 3.94\%$) whilst Yorkshire had the lowest ($12.67 \pm 3.18\%$). The interaction effects between the counties and crop seasons for autumn virus incidences were significant ($P = 0.034$). The highest incidence ($94.00 \pm 4.08\%$) was recorded in Lincolnshire during the autumn of the 2009-10 crops, whilst the lowest ($2.33 \pm 2.60\%$) was recorded in Yorkshire during the autumn of the 2008-9 crop.

Table 2.2 Mean autumn percentage incidence of *Turnip yellows virus* (TuYV) in winter oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire in the 2007- 8, 2008-9 and 2009-10 crop seasons

Region	Mean TuYV incidence (%) in autumn of crop season			Means ^a
	2007-8	2008-9	2009-10	
Lincolnshire	58.00 ± 8.51 b ^{b,c}	13.00 ± 5.80 d, e	94.00 ± 4.08 a	55.00 ± 3.69 a
Warwickshire	27.33 ± 7.69 c, d	3.00 ± 2.94 e	41.00 ± 8.48 b, c	23.78 ± 3.94 b
Yorkshire	24.67 ± 7.43 c, d	2.33 ± 2.60 e	11.00 ± 5.40 d, e	12.67 ± 3.18 c
Means ^d	36.67 ± 4.56 a	6.11 ± 2.33 b	48.67 ± 3.62 a	

^a Means in the same column followed by different letters are significantly different from each other ($P < 0.001$).

^b Mean \pm standard error of mean

^c Region-crop season incidence interaction means followed by different letters are significantly different from each other ($P = 0.034$).

^d Means in the same row followed by different letters are significantly different from each other ($P < 0.001$).

Analysis of the TuYV incidence data was carried out using a generalised linear model (GLM).

The highest overall mean TuYV incidence in spring was recorded in the 2007-8 crop ($55.67 \pm 6.91\%$), followed by the 2009-10 crop ($53.78 \pm 5.86\%$); the 2008-9 crop

had the lowest ($8.67 \pm 4.11\%$) (Table 2.3). Lincolnshire had the highest overall mean spring incidence ($56.67 \pm 5.17\%$), followed by Warwickshire ($42.00 \pm 6.43\%$) and Yorkshire had the lowest ($19.44 \pm 5.55\%$). The GLM analysis did not indicate a significant interaction between the counties and crop seasons for the spring incidences of TuYV ($P = 0.163$). However, the highest mean incidence ($94.00 \pm 6.04\%$) was recorded in Lincolnshire in spring of the 2009-10 crop, whilst the lowest mean incidence ($3.67 \pm 4.79\%$) was recorded in Yorkshire during 2008-9 crop season (Table 2.3).

Table 2.3 Mean spring percentage incidence of *Turnip yellows virus* (TuYV) in winter oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire in the 2007-8, 2008-9 and 2009-10 crop seasons

Region	Mean TuYV incidence (%) in spring of crop season			Means ^a
	2007-8	2008-9	2009-10	
Lincolnshire	66.00 ± 12.08 ^{b,c}	10.00 ± 7.65	94.00 ± 6.04	56.67 ± 5.17 a
Warwickshire	68.33 ± 11.86	12.33 ± 8.38	45.33 ± 12.69	42.00 ± 6.43 a
Yorkshire	32.67 ± 11.96	3.67 ± 4.79	22.00 ± 10.56	19.44 ± 5.55 b
Means ^d	55.67 ± 6.91 a	8.67 ± 4.11 b	53.78 ± 5.86 a	

^a Means in the same column followed by different letters are significantly different from each other ($P = 0.002$).

^b Mean and standard error (mean \pm SE).

^c The interaction effect between region and crop season is not significant ($P = 0.163$).

^d Means in the same row followed by different letters are significantly different from each other ($P < 0.001$).

Analysis of the TuYV incidence data was carried out using a generalised linear model (GLM).

The comparisons of autumn and spring incidences of TuYV in the individual 27 fields in the three regions over the three crop seasons using the two-sample binomial test are shown in Table 2.4. Large differences in the incidences of TuYV in the various fields, counties and crop seasons surveyed, ranging from 0% (recorded in

autumn and spring in Warwickshire in 2008-9) to 100% (recorded in Lincolnshire in the autumn of 2009) were found. There were significant ($P < 0.05$) changes in the proportions of plants infected with TuYV between autumn and spring in most fields in 2007-8 (all 3 fields in Warwickshire and one each in Lincolnshire and Yorkshire). Whereas in subsequent crops only a few fields showed significant changes, three fields in 2008-9 (two in Lincolnshire and one in Warwickshire) and only one field in 2009-10 (Yorkshire) (Table 2.4).

Overall, there was a significantly high correlation ($r = 0.88$, $P < 0.001$, d.f. = 25) between autumn and spring incidences of TuYV in the oilseed rape crops surveyed (Fig. 2.3).

Table 2.4 Incidence and spatial analysis of *Turnip yellows virus* (TuYV) in oilseed rape crops sampled in Lincolnshire, Warwickshire and Yorkshire in the 2007-8, 2008-9 and 2009-10 crop seasons

Region	Location Field	Crop season	TuYV incidence ^a		Spatial analysis ^b			
			(%)		Autumn		Spring	
			Autumn	Spring	Z-score ^c	P-value	Z-score	P-value
Lincs	1	2007-8	59	89***	-1.922	0.027*	-0.297	0.350
	2	2007-8	53	47	-0.990	0.161	-0.990	0.161
	3	2007-8	62	62	-0.114	0.455	0.531	0.298
Warks	1	2007-8	14	53***	-0.295	0.384	-2.177	0.015*
	2	2007-8	18	64***	-0.874	0.191	0.555	0.290
	3	2007-8	50	88***	-0.596	0.276	-0.132	0.448
Yorks	1	2007-8	42	74***	0.043	0.483	-1.032	0.151
	2	2007-8	8	4	-0.207	0.418	0.220	0.413
	3	2007-8	24	20	-0.823	0.206	-0.073	0.471
Lincs	1	2008-9	7	16*	-0.066	0.474	0.112	0.456
	2	2008-9	10	9	-0.325	0.373	-0.207	0.418
	3	2008-9	21	5***	-0.822	0.206	0.200	0.421
Warks	1	2008-9	7	13	-0.217	0.414	0.291	0.386
	2	2008-9	2	24***	0.268	0.395	-1.193	0.119
	3	2008-9	0	0	n.a.	n.a.	n.a.	n.a.
Yorks	1	2008-9	1	6	0.153	0.439	0.271	0.393
	2	2008-9	4	2	0.033	0.487	0.240	0.406
	3	2008-9	2	3	-0.268	0.395	-0.101	0.460
Lincs	1	2009-10	100	99	n.d.	n.d.	0.153	0.439
	2	2009-10	86	86	0.460	0.323	-0.114	0.454
	3	2009-10	96	97	-0.342	0.367	0.323	0.374
Warks	1	2009-10	50	59	0.447	0.328	-0.728	0.234
	2	2009-10	55	59	-1.192	0.117	0.561	0.288
	3	2009-10	18	18	-0.017	0.494	0.384	0.351
York	1	2009-10	18	22	-0.262	0.397	0.518	0.303
	2	2009-10	5	6	-0.017	0.493	-0.208	0.418
	3	2009-10	10	39***	0.217	0.414	-1.076	0.141

***Significant at $P < 0.001$; *Significant at $P < 0.05$

^aAutumn and spring incidences of TuYV were compared by a two-sample binomial test.

^bRook's connection of Black-White join-count statistic was used to test for the spatial autocorrelation of the TuYV-infected plants in each of the 27 oilseed rape fields, where possible.

^cNegative values for Z-score implies the observed BW joins is less than the expected BW joins, indicating clustering; positive Z-scores implies the observed BW joins is greater than the expected BW joins, indicating randomness of infected plants.

n.a. = not applicable because the virus incidence was zero.

n.d. = not determined because the virus incidence was 100%.

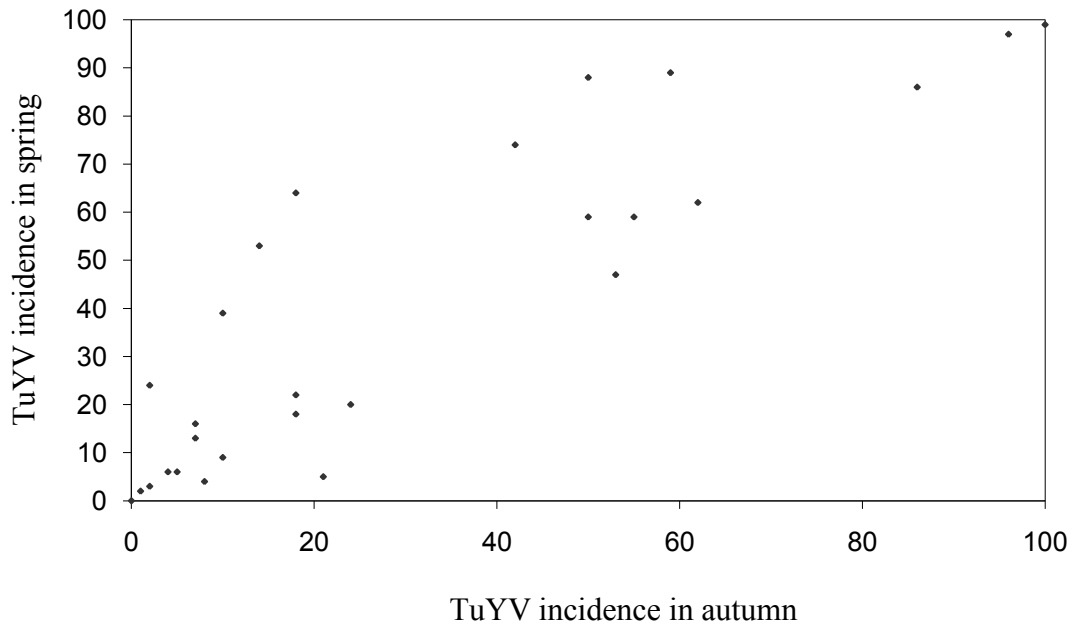


Figure 2.3 Correlation between autumn and spring percentage incidences of *Turnip yellows virus* (TuYV) infection in oilseed rape crops. Correlation coefficient, $r = 0.886$, $P < 0.001$ at 25 d.f.

2.3.3 Within field virus distribution

The results of the analysis of spatial distribution of TuYV-infected plants are given in Table 2.4. Where possible, Black-White join-count statistics were calculated for each of the 27 fields surveyed in the three-crop seasons and then tested as standard normal deviates (Z -scores). Most of the fields (17 of the 25 analysed) showed positive but non-significant spatial autocorrelation (negative Z -scores, $P > 0.05$) when sampled in autumn, indicating that most of the infected plants showed a slightly aggregated pattern of distribution. For a one-sided test at a probability of 0.05, values less than -1.645 (large negative Z -score) indicated that the number of observed join-counts was significantly less than expected, an indication of a significant clustering of the infected plants. Lincolnshire field 1 was the only field showing significant positive autocorrelation ($Z = -1.922$, $P < 0.05$) between the infected plants in autumn (2007), indicating significant clustering (Figure 2.4). Spring sampling revealed a slightly more random pattern with half of the fields (13

of the 26 analysed) showing negative spatial autocorrelation (positive Z-scores) (e.g. Warwickshire field 2 in spring 2010, $Z = 0.561$, $P = 0.288$, Figure 2.5). Warwickshire field 1 showed significant positive autocorrelation ($Z = -2.177$, $P < 0.05$) between the infected plants in spring 2008, indicating significant clustering.

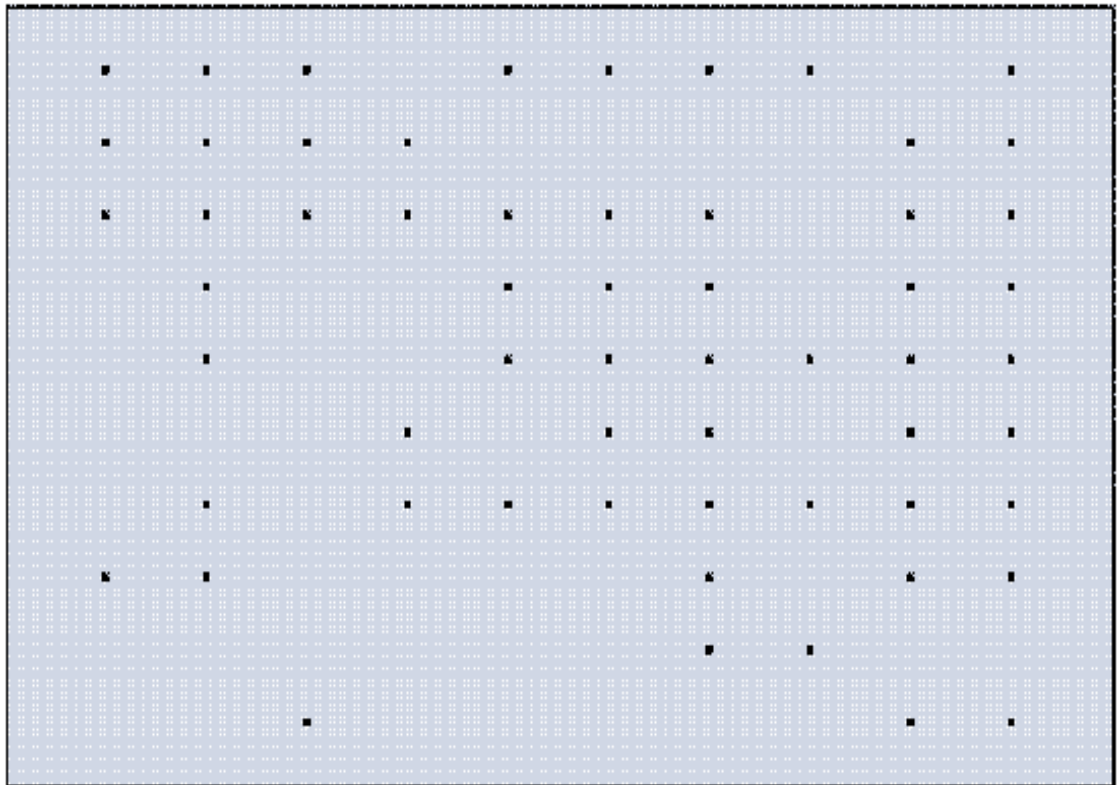


Figure 2.4 Spatial distribution of *Turnip yellows virus* (TuYV)-infected plants in oilseed rape field 1 in Lincolnshire in autumn of the 2007-8 crop season showing significant clustering.

Rook's connection of Black-White join-count statistic was used to test for the spatial autocorrelation of the TuYV-infected plants. In this field $Z = -1.922$, $P < 0.05$).

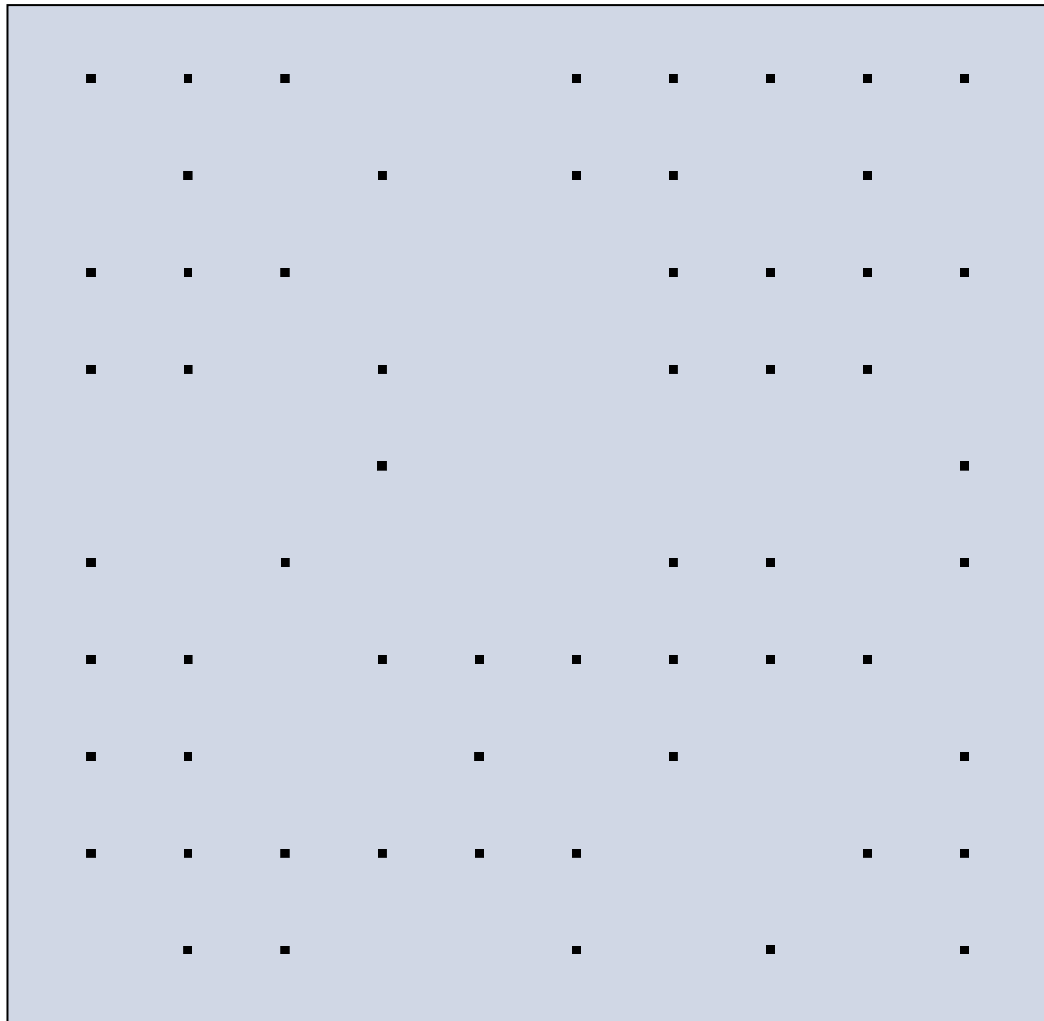


Figure 2.5 Spatial distribution of *Turnip yellows virus* (TuYV) -infected plants in oilseed rape field 2 in Warwickshire in spring of the 2009-10 crop season showing random distribution.

Rook's connection of Black-White join-count statistic was used to test for the spatial autocorrelation of the TuYV-infected plants. In this field $Z = 0.561$, $P = 0.288$.

2.3.4 Numbers of *Myzus persicae* caught in the suction traps closest to sampled fields

There were two peaks of flight activity of *M. persicae* in Lincolnshire, Warwickshire and Yorkshire in most years (2007-2009) (Fig. 2.6). The first peak occurred between June and July and the second occurred between September and November in each year; the latter coincided with the emergence of oilseed rape crops. Where known, crops were sown in August or September. The highest cumulative (August to

November) trap catches of *M. persicae* during the three crop seasons occurred in Lincolnshire and the lowest in Yorkshire; catches in the 2009 were highest and those in 2008 were lowest.

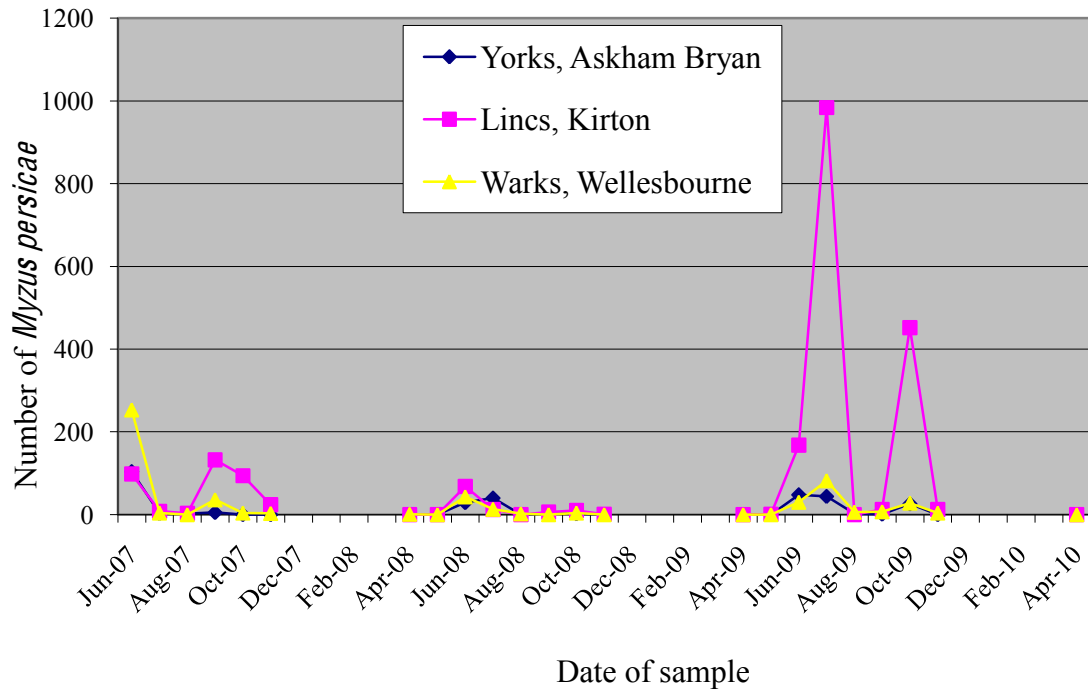


Figure 2.6 Rothamsted Insect Survey catches of *Myzus persicae* in suction traps located in Lincolnshire (Kirton), Warwickshire (Wellesbourne) and Yorkshire (Askham Bryan). (Source: Aphid Bulletin, Rothamsted Insect Survey, Rothamsted Research, U.K.).

2.3.5 Relationship between *Myzus persicae* and *Turnip yellows virus* (TuYV) incidence

Regression analysis revealed a highly significant association between the numbers of *M. persicae* caught in the suction traps between August and November each year and the incidence of TuYV in oilseed rape crops (d.f. = 2, 8; F = 24.2; $P < 0.001$) in autumn of each year (Figure 2.7).

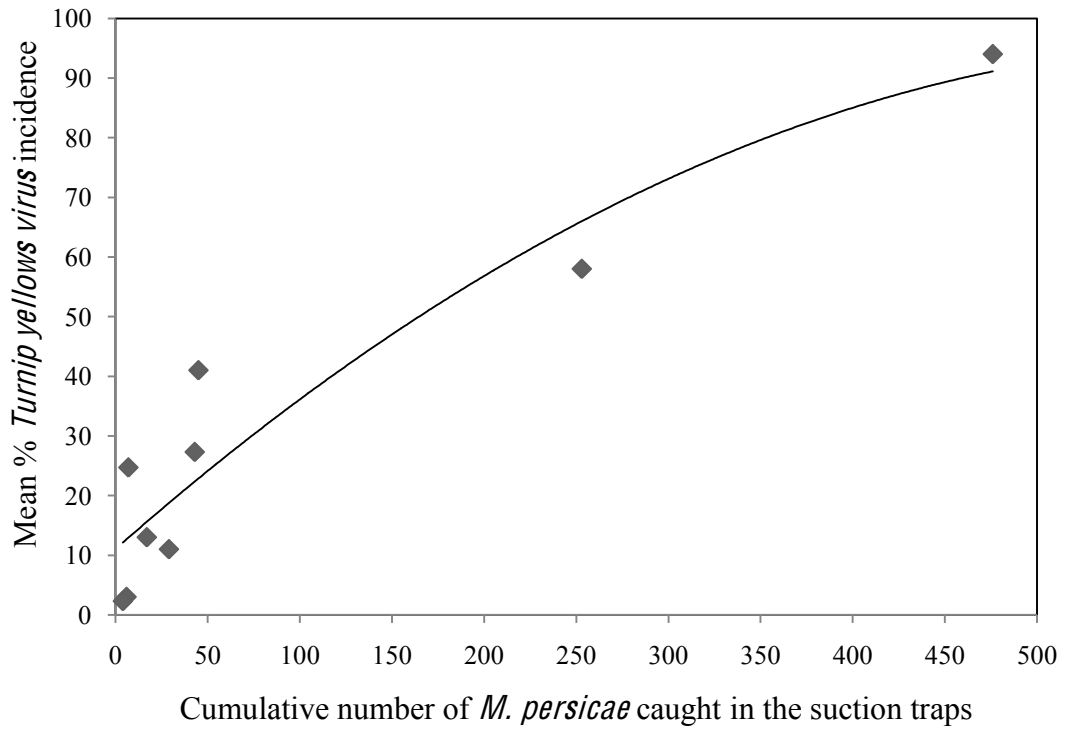


Figure 2.7 Relationship between cumulative numbers of *Myzus persicae* caught in the Rothamsted Insect Survey suction traps located in Lincolnshire, Warwickshire and Yorkshire between August and November in 2007, 2008 and 2009 and mean percentage *Turnip yellows virus* (TuYV) incidence in oilseed rape crops in the autumn of each year in the three regions (d.f. = 2, 8; $F = 24.2$; $P < 0.001$).

2.4 Discussion

This study has shown that TuYV is prevalent in winter oilseed rape fields in three regions of England with incidences ranging from 0-100%. The virus was detected in 26 of the 27 oilseed rape fields sampled from three counties (Lincolnshire, Warwickshire and Yorkshire). This finding corroborates the previous reports of widespread occurrence of TuYV in oilseed rape crops in the UK (Bayer CropScience, 2007b; Hill *et al.*, 1989; Smith and Hinckes, 1985; Stevens *et al.*, 2008; Walsh *et al.*, 1989) where incidences of 0-100% were also reported. The only field where no infection was recorded (Warwickshire field three in the 2008-9 crop season) was sown late (27th September, 2008) relative to the other two Warwickshire fields (sown 26-27th August, 2008) and very few plants had emerged which were very small when sampled on 15th December, 2008.

The analysis of the autumn incidences of TuYV in the oilseed rape crops in the different regions sampled were clearly associated with the cumulative numbers of *M. persicae* caught in the suction traps in these regions between August and November. This indicates that the significantly higher incidences of TuYV recorded in the 2007-8 and 2009-10 crops compared with those in the 2008-9 crops were due to the higher flight activity of *M. persicae* between August and November in 2007 and 2009, relative to 2008 (Figure 2.4). Graichen and Schliephake (1999) also demonstrated that a high incidence of TuYV in winter oilseed rape appeared to be closely related to the flight activity of *M. persicae* vectors in Germany. Clark and Stevens (2009) indicated a close correlation between *M. persicae* numbers caught in the autumn and the TuYV incidence in oilseed rape in the UK. The timing and intensity of the spring and summer *M. persicae* aphid flights in the Columbia basin in the USA were

associated with heat unit accumulation (day degrees) (Thomas *et al.*, 1997). The differences between the accumulated day degrees in the springs (January to May) of 2007, 2008 and 2009 (Collier, 2010) do not appear to account for the abundance of *M. persicae* caught in the local suction trap between August and November in these years and hence the incidence of TuYV. It is possible that the accumulated day degrees later in these years might account for the differences. Mild autumn conditions favour the development of the aphid vectors and encourage TuYV spread (Stevens *et al.*, 2008). The low numbers of aphids in 2008 were said to be due to wet and windy weather and an abundance of natural enemies (Collier, 2008) accounting for the low incidence of TuYV.

In general, the incidence of TuYV within oilseed rape crops is considered to increase from initial autumn infection to a maximum level in the following spring (Stevens *et al.*, 2008). For the most part, this study supports this; significant increases were observed in the incidence of TuYV in eight fields between autumn and spring, no significant change in 18 fields and a significant decrease in only one field. Most of the increases in incidence between autumn and spring occurred in the 2007/8 crop. All the fields in Warwickshire showed a significant increase in TuYV incidence between autumn 2007 and spring 2008 and the cumulative day degrees at our site in Warwickshire (10 km from the Warwickshire fields sampled) were greater between January and May 2007 than for the same months in 2008 and 2009 (Collier, 2010).

The significant regional differences in TuYV incidence, where highest levels of infection were observed in Lincolnshire and lowest in Yorkshire are likely to be attributable to a number of factors. There was much higher flight activity of *M.*

persicae in Lincolnshire in the autumns of 2007 and 2009 relative to Yorkshire and Warwickshire (see Figure 2.6). Also, the large area of vegetable brassicas grown in Lincolnshire is likely to be a reservoir of TuYV and source for aphids. Regional differences in the numbers of aphids caught in suction traps with resistance to insecticides have been observed. Resistance of *M. persicae* to pirimicarb (modified acetylcholinesterase, MACE) has been highest in the Kirton (Lincolnshire) suction trap (Collier, 2009), with 100% of *M. persicae* being MACE in 2008. Between 1986 and 1989, surveys in England and Wales detected lower TuYV incidences in the north and east (Hill *et al.*, 1989). In 1992 and 1993, the incidence of TuYV was higher in Wales, the midlands, western and south western regions of England than in the eastern, south eastern or northern regions of England (Hardwick *et al.*, 1994). My data over three years and that of others (Blake, 2009; Impey, 2010, Clark and Stevens, 2009) suggest that there has been a change in prevalence, in that the highest levels of TuYV have been in Lincolnshire, close to the Wash and on the south coast.

The spatial autocorrelation analysis revealed that TuYV-infected oilseed rape plants showed either random or aggregated pattern of distribution within individual fields, with most fields showing slightly aggregated patterns during autumn. This finding agrees with that of Bourdon (1987) who reported that crop plants infected with viruses can show random or aggregated distributions, with aggregated distributions more common in vector-borne viruses. Aphid-borne viruses usually exhibit an aggregated pattern of distribution because aphids are attracted to small isolated groups of plants rather than continuous swards (A'Brook, 1973). There is very little data on the spatial incidence of TuYV, however Raybould *et al.* (1999) also reported that plants infected by TuYV and other viruses (CaMV, TuMV and *Turnip yellow*

mosaic virus, TuYMV) were distributed randomly, or were very weakly aggregated within populations of wild *Brassica oleracea*. It was observed in this study that most of the oilseed rape crops showed positive autocorrelation (slight aggregated pattern) during autumn but a more random pattern during the following spring. Aggregation of infected plants appears where there is limited spread of the virus from the initial (primary) foci of infection (Eckel, 1993). This suggests that autumn infection (tending towards aggregation) is mostly due to primary infection with some, probably limited, secondary infection. The change observed between the reduction in aggregation between autumn (18 out of 25 had negative Z-scores and only seven had positive Z-scores) and the following spring (13 out of 26 had negative Z-scores and 13 had positive Z-scores) indicates that the infection of plants between the autumn sampling dates and April of the following years was mostly due to secondary spread of the virus within the fields, rather than further primary infection coming from outside the fields. This is consistent with the lack of aphid vectors caught in suction traps during this period (Anon, 2011), the characteristic spatial spread of persistently transmitted viruses (Thresh, 1976) and my data showing increased incidence in some fields in spring relative to the previous autumn.

The results of this study suggest that the insecticide seed treatments of oilseed rape may not control TuYV. Despite all the seed for the crops planted in 2007 were treated with Chinook, high levels of TuYV infection were detected in autumn (up to 62%) and in the following spring (up to 89%) indicating that at the infection / inoculum pressures in this year, the treatment did not give effective control. The crops planted in 2008 that were sampled were planted with seed treated with Modesto, or Chinook and lower TuYV incidences were detected in autumn (up to

21%) and spring (up to 24%). However, this was probably due mostly to the low numbers of aphids in the latter part of 2008, rather than the insecticide treatments. This is also supported by data from the crops planted in 2009 that were sampled, where a field planted with Modesto-treated seed had 96% infection in autumn and fields planted with Chinook-treated seed had up to 100% infection in autumn. Others have stated that Modesto and Cruiser (thiamethoxam, fludioxinil and mefenoxam) seed treatments did not reduce the incidence of TuYV in oilseed rape relative to non-insecticidal seed treatment in the U.K. (Abram, 2010). When infection levels were higher than 85% (presumably in untreated plots / fields), control was often poor with Modesto, however, when infection levels were lower (no incidences given), both Modesto and Cruiser could provide 50-75% control (Abram, 2010). Stevens and Clark (2009) stated that when 72% of winged *M. persicae* carry TuYV, it is extremely difficult to prevent widespread primary virus infection of crops such as oilseed rape, even with extensive seed treatments and / or aphicide sprays. As aphids need to feed on plants that have had Chinook, Modesto, or Cruiser seed treatments in order to come in to contact with the active ingredients, they can transmit TuYV before they are killed. Hence, these treatments are likely to be more effective in reducing secondary spread, rather than primary infection of crops. In this study, of the crops with high levels (>50%) of TuYV infection in autumn for which Z- scores were obtained, five of the six had negative Z-scores, suggesting the infections were mostly due to primary infection. The lack of control of primary TuYV infection of oilseed rape by these seed treatments has been highlighted previously (Stevens, 2010). In plot experiments at two sites in the U.K., Cruiser OSR and Modesto significantly reduced the incidence of TuYV in oilseed rape, relative to untreated plots, even where the untreated plots had 80% incidence of TuYV (Dewar *et al.*,

2011). Chinook also significantly reduced TuYV incidence relative to untreated plots at the site where untreated plots had incidences of 43.3% and 56.7%, but not at the site where untreated plots had 80% incidence (Dewar *et al.*, 2011).

This current work also shows that the autumn insecticide spray treatments (all pyrethroids) that were applied in addition to the insecticide seed treatments were ineffective in controlling TuYV in the fields sampled with high levels of TuYV. Earlier research (Hill *et al.*, 1989; Read and Hewson, 1988; Walsh *et al.*, 1989) showed that autumn pyrethroid insecticide sprays were capable of giving some control of TuYV. Recently, there has been an increase in the number of *M. persicae* carrying kdr (resistance to pyrethroids) and MACE (resistance to pirimicarb) in field, glasshouse and suction trap samples (Collier, 2009). *M. persicae* has high levels of MACE (90%) and 20% carry kdr resistance (Collier, 2009). This probably accounts for the lack of control this work and others (Graichen and Rabenstein, 1996) have observed. With *M. persicae* having resistance to these, the only active ingredients of sprays cleared for autumn use on oilseed rape, they are unlikely to give reliable control of TuYV (Stevens, 2010).

CHAPTER 3:

GENETIC DIVERSITY, EVOLUTION AND GENETIC STRUCTURE OF *TURNIP YELLOW*S VIRUS (TuYV) INFECTING OILSEED RAPE CROPS IN ENGLAND

3.1 Introduction

Turnip yellows virus (TuYV) is a member of the genus *Polerovirus* of the family *Luteoviridae* (D'Arcy and Domier, 2005). It consists of a single-stranded plus sense RNA genome of approximately 6 kb, which is divided into six open reading frames numbered from 0 to 5 (Miller *et al.*, 1995). The 5'-proximal half of the genome (ORF0, ORF1, ORF2) is expressed from the genomic RNA and encodes viral proteins (P0, P1, P3, respectively) necessary for infection (Retenauer *et al.*, 1993). The 3'-terminal ORFs (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, read through domain, RTD) and cell-to-cell movement (P4, transport protein) (Brault *et al.*, 2005; Reinbold *et al.*, 2003). The 3' region is more conserved, whilst the 5'-half is more variable in poleroviruses (Hauser *et al.*, 2000a).

TuYV is the most common virus infecting oilseed rape in the UK (Hardwick *et al.*, 1994; Hill *et al.*, 1989; Jay *et al.*, 1999; Smith and Hinckes, 1985; Walsh *et al.*, 1989) where yield losses of up to 45% due to this virus have been reported (Stevens and Clark, 2009).

Plant viruses with RNA genomes (including TuYV) have high potential for genetic variation due to the error prone nature of their RNA-dependent RNA polymerase

(Domingo and Holland, 1994; Garcia-Arenal *et al.*, 2001). Mutation and recombination are the two main types of errors which bring about this genetic variation (Garcia-Arenal *et al.*, 2003). The genetic variation may be influenced by evolutionary factors such as genetic drift and selection including selection pressures associated with maintenance of functional structures, host plant selection, and virus-vector selection (Garcia-Arenal *et al.*, 2003). Molecular studies of poleroviruses infecting oilseed rape, beet and other crops have revealed high levels of genetic diversity within TuYV isolates (de Miranda *et al.*, 1995; Schubert *et al.*, 1998; Hauser *et al.*, 2000a). Studies based on CP gene nucleotide sequence comparisons have indicated variation amongst TuYV isolates from lettuce, rape, sprouts, cauliflower, broccoli and calabrese (de Miranda *et al.*, 1995). In comparing P0 amino acid sequences of TuYV isolates, Schubert *et al.* (1998) have shown that isolates from oilseed rape were quite different from each other and also different from the lettuce isolate (TuYV-FL).

Despite the number of studies concerning variation of poleroviruses in different host plants including oilseed rape from different countries, very little is known of the genetic diversity and structure of TuYV population in oilseed rape in the U.K. or in the world. Knowledge of the genetic diversity and genetic structure of TuYV infecting oilseed rape in the UK is crucial as variation among the TuYV populations may affect virulence, infectivity, transmission, and symptom severity. It is therefore important to consider this when developing a control strategy. Because TuYV proteins P0 and P3 play an important role in virulence and pathogenesis (Pfeffer *et al.*, 2002; Torres *et al.*, 2005), knowledge of mode of evolution of these genes can provide insights into epidemiological dynamics of TuYV. This may be useful in

predicting the genetic basis and periodicity of future epidemics of TuYV in oilseed rape and for developing effective strategies to control the TuYV disease. According to Garcia-Arenal *et al.* (2001), knowledge of evolution of plant viruses is important for the development of efficient and stable control strategies, as often, there is evolution of resistance breaking pathotypes which renders the control measures ineffective.

The aim of the work described in this chapter was to determine the molecular diversity and genetic structure in TuYV infecting oilseed rape in England. Therefore two genomic regions were selected: ORF0 and ORF3, because ORF0 was considered to code for the most variable portion and ORF3 was the most studied and less variable. The study was based on the samples collected from fields in Lincolnshire, Warwickshire and Yorkshire, over three consecutive years (2007-2009) as described in chapter 2, in order to gain deeper insight into the relationships of TuYV isolates within and between fields and regions of England, variations in different years, their mode of evolution and how they compared with other published isolates.

3.2 Materials and Methods

3.2.1 Virus isolates

Up to ten oilseed rape leaf samples that were positive in the enzyme linked immunosorbent assay (ELISA) were selected from each of the three crop fields from the three regions in England (see chapter 2) using a modification of the W-shaped path sampling method (Basu *et al.*,1977). The distributions of infected plant samples in each field were plotted on a 10 by 10 grid map and the virus isolates selected along a W-shaped path covering the entire grid map. The starting point was at the top left-hand corner of the field map and infected samples located at or near the interception of each of the 10 sampling paths and the W (Figure 3.1) were selected for sequencing. These samples were kept at -80°C until needed. A total of 226 field isolates were analysed (Table 3.1.) together with some published isolates available in the GenBank (Table 3.2).

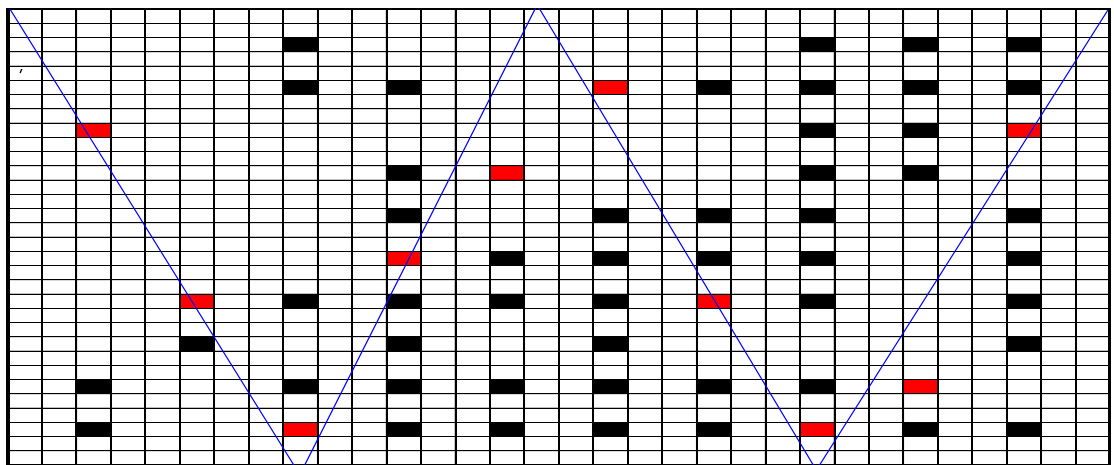


Figure 3.1 A diagram showing the distribution of *Turnip yellows virus* (TuYV) infected plants in an oilseed rape field in Lincolnshire indicated by black oblongs.

The red shaded oblong points represent the isolates selected for sequencing along the W-shaped path.

Table 3.1 Field isolates of *Turnip yellows virus* (TuYV) selected for sequencing

Name of isolates	Date	Field	Plant host /	
			Oilseed cultivar	rape
L9, L14, L30, L44, L66, L71, L89, L98	December 2007	1	Castille, Ovation	
L105, L113, L137, L155, L161, L187, L188, L199	December 2007	2	Astrid	
L203, L214, L230, L235, L243, L256, L266, L273, L288, L300	December 2007	3	Ovation	
W1, W6, W19, W52, W71, W77, W80, W93, W95,	December 2007	1	Lioness	
W108, W123, W149, W151, W154, W160, W175, W179, W184, W195, W196,	December 2007	2	Lioness	
W211, W222, W225, W234, W241, W259, W267, W276, W287, W300	December 2007	3	Lioness	
Y2, Y14, Y29, Y36, Y45, Y59, Y63, Y72, Y88, Y90	December 2007	1	Lioness	
Y102, Y182, Y454, Y459, Y481	December 2007	2	NK Bravour	
Y208, Y213, Y223, Y226, Y228, Y241, Y277, Y297, Y298	December 2007	3	Astrid	
L903, L917, L930, L936, L939, L960, L962, L968, L976, L983	April 2009	1	Astrid, Castille	
L1005, L1010, L1014, L1030, L1047, L1048, L1050, L1068, L1080a, L1080b	April 2009	2	Astrid	
L1129, L1149, L1150, L1155	April 2009	3	Astrid	
W903, W909, W914, W916, W929, W932, W933, W936, W979,	April 2009	1	Astrid	
W1029, W1051, W1052, W1059, W1062, W1079, W1088a, W1088b, W1095	April 2009	2	Astrid	
Y917, Y920, Y926, Y933, Y945, Y990	April 2009	1	N/A	
Y1062, Y1068	April 2009	2	N/A	
Y1103a, Y1103b, Y1109, Y1159	April 2009	3	N/A	

NA = Information not available

Table 3.1 continued

	December 2009	1	N/A
L1201, L1213, L1230, L1241a, L1241b, L1249, L1253, L1266, L1271, L1287, L1296a, L1296b			Ovation
L1309, L1314, L1321, L1340, L1352, L1360, L1365, L1388, L1397,	December 2009	2	PR46W21
L1403, L1408a, L1408b, L1421, L1430, L1435, L1442, L1458, L1465a, L1465b, L1488, L1497	December 2009	3	Cabernet
W1209, W1216, W1226, W1232, W1237, W1247, W1252, W1259, W1267, W1273, W1294, W1296	December 2009	1	Astrid
W1318a, W1318b, W1321, W1328, W1342, W1349, W1354, W1374, W1380, W1393	December 2009	2	Astrid
W1403a, W1403b, W1409, W1422, W1441, W1450, W1462, W1470, W1479, W1483, W1490	December 2009	3	Astrid
Y1208, Y1212, Y1213, Y1240, Y1248, Y1259, Y1266, Y1267, Y1271, Y1288, Y1294	December 2009	1	N/A
Y340, Y1351, Y1355, Y1365, Y1399,	December 2009	2	N/A
Y1407, Y1418, Y1441, Y1443, Y1451, Y1456, Y1476, Y1481a, Y1481b, Y1485, Y1489	December 2009	3	N/A
Laboratory isolate (LAB)	December 2007		Mikado

NA = Information not available

Isolates prefixed by L were from Lincolnshire, W were from Warwickshire, and Y were from Yorkshire.

Same isolates followed by different letters were obtained from the same plant sample.

Table 3.2 *Turnip yellows virus* (TuYV) isolates from GenBank

Name of isolates (GenBank accession number)	Plant host	Geographic region, country	Genomic region	Reference
TuYV-FL1 (X13063)*	Lettuce	Vaucluse, France	whole genome	Veidt <i>et al.</i> (1988)
BWYV-Col (AF168600)	Oilseed rape	Haut-Rhin, France	ORF0	Hauser <i>et al.</i> (2000a)
BWYV-Fev (AF168601)	Field bean	Haut-Rhin, France	ORF0	Hauser <i>et al.</i> (2000a)
TuYV-BN5 (AF168606)	Oilseed rape	Gatersleben, Germany	ORF0	Hauser <i>et al.</i> (2000a)
TuYV-GB (AF168608)	Oilseed rape	Norfolk (Norwich), England	ORF0	Hauser <i>et al.</i> (2000a)
TuYV-Beijing (FJ606451)	undetermined	Beijing, China	ORF0	Han, C. unpublished
Isolate 3a1 strain bwyv-2 (L39968)	Oilseed rape	East Anglia, England	ORF3 (CP)	de Miranda <i>et al.</i> (1995)
Isolate 3a2 strain bwyv-1 (L39969)	Oilseed rape	East Anglia, England	ORF3 (CP)	de Miranda <i>et al.</i> (1995)
Isolate 3b strain bwyv-2 (L39970)	Oilseed rape	East Anglia, England	ORF3 (CP)	de Miranda <i>et al.</i> (1995)
Isolate 4a strain bwyv-1 (L39971)	Oilseed rape	Lorraine, France	ORF3 (CP)	de Miranda <i>et al.</i> (1995)
Isolate 7 strain bwyv-1 (L 39974)	sprout	East Anglia, England	ORF3 (CP)	de Miranda <i>et al.</i> (1995)
Isolate 8 strain bwyv-1 (L39975)	cauliflower	East Anglia, England	ORF3 (CP)	de Miranda <i>et al.</i> (1995)
Isolate 5 strain bwyv-1 (L39986)	calabrese	East Anglia, England	ORF3 (CP)	de Miranda <i>et al.</i> (1995)

3.2.2 Preparation of total plant RNA

Total plant RNA was prepared by using RNeasy Plant Mini Kit (QIAGEN, UK) according to the manufacturer's instructions. The RNA was eluted from the columns by 50 μ l of RNASE-free water. RNA concentration was determined using a NanoDrop[®] ND-1000 spectrophotometer (Thermo Scientific). The purified RNA was stored at -80°C until needed.

3.2.3 Primer design

The nucleotide sequences of TuYV isolates published by Veidt *et al.* (1988), Jones *et al.* (1991), de Miranda *et al.* (1995), Schubert *et al.* (1998), and Hauser *et al.* (2000a) were retrieved from NCBI database. Multiple sequence alignment was performed using the ClustalW software (Thompson *et al.*, 1994) implemented in MEGA 5 software (Tamura *et al.*, 2011). Forward and reverse primers were designed for the target P0 and P3 genes in the most conserved areas within the ORF0 and ORF3 regions respectively of the alignment, with the help of PrimerSelect algorithm of DNASTAR Lasergene 8 software (Burland, 2000). The possible occurrence of homo- and heterodimer formation was assessed for each primer pair by estimating the thermodynamic parameters using DNASTAR Lasergene 8 software (Burland, 2000). The forward and reverse primers designed by Jones *et al.* (1991) which targeted the CP of TuYV were also included in the study. Information about the primers is presented in Table 3.3 and Figure 3.2.

Table 3.3 Sequence of primers used for RT-PCR amplification

Primer name	Sequence, 5' - 3' (direction)	Orientation	Position in the sequence	Target region
AB1	ACCAGGAGGGTATCCTTAGT	Forward	10 – 30	ORF0
AB2	ATGCAATTTGTYGCTCACGAYAACT	Forward	34 – 58	ORF0
AB3	TCATACAAACATTTTCGGTGTAGAC	Reverse	760 – 785	ORF0
AB9 ^a	CAGGYGCTGCCTGGGCTA	Forward	2937 – 2954	ORF3
AB7 ^a	GGAGAGGGAGAAGGCCCT	Reverse	4136 – 4154	ORF3
AB12	GTTGAACTTCTTTACTCGT	Forward	3218 – 3236	ORF3
AB13	AGGGAGAAGGCCCTGGGCT	Reverse	4113 - 4131	ORF3

^a Primers AB9 and AB7 correspond to primers P1 and P2 used by Jones *et al.* (1991) and flank the coat protein and intergenic region between the coat protein and the polymerase gene. The other primer pairs were AB1-AB3, AB2-AB3, AB9-AB7 and AB12-AB13 with corresponding expected products sizes of 775 bp, 751 bp, 1217 bp and 913 bp respectively

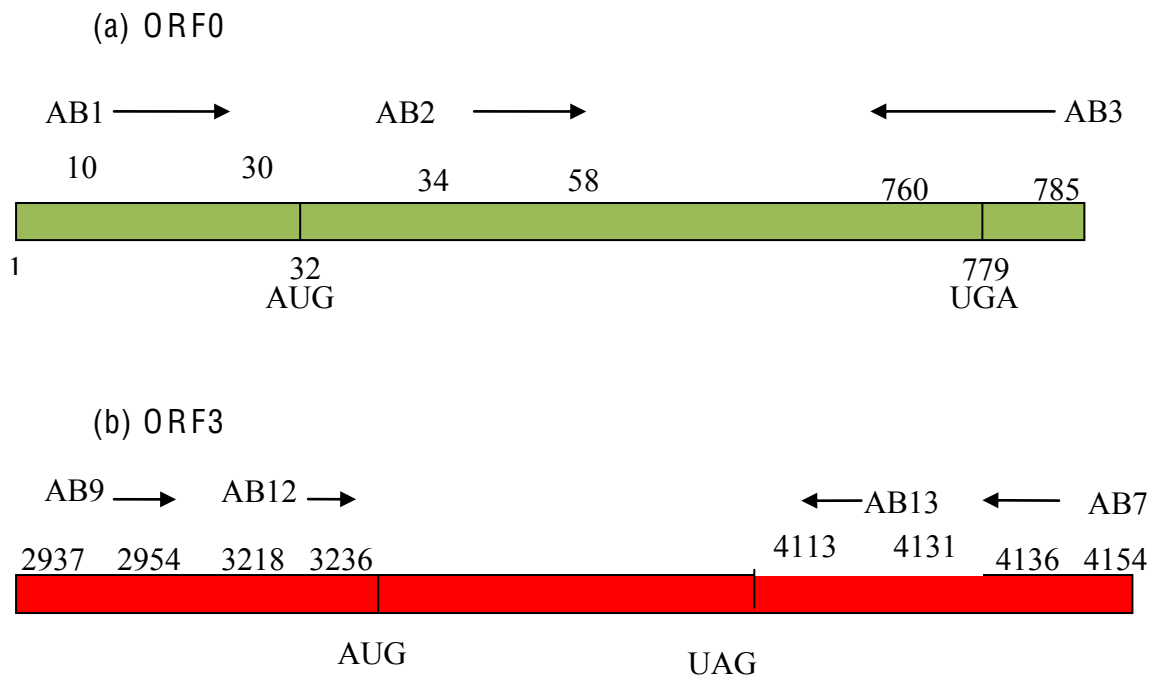


Figure 3.2 Diagram showing the primers and their positions on (a) ORF0 and (b) ORF3 region of *Turnip yellows virus* (TuYV) genome (not to scale). The arrows show the orientation of the primers in the RT-PCR amplification. AUG is the start codon and UAG is the stop codon.

3.2.4 First strand cDNA synthesis

cDNA was synthesised from purified RNA, by a modification of methods described by Jones *et al.* (1991), Schubert *et al.* (1998) and Hauser *et al.* (2000a). For the ORF0 (P0 gene), 0.5 μg of extracted RNA was added to the 16 μl reaction mixture containing 4 μl of 5X First strand buffer (Invitrogen), 50 pmol AB3 (upstream) primer, 1 mM dNTP, 10 mM DTT, and 200U of SuperScriptTM II RT (Invitrogen). For CP gene amplification, 10 pmol each of upstream primers AB7 or AB13 and 100U of SuperScriptTM II RT were used. For reverse transcription, the reaction mixture was transferred to a pre-warmed thermal cycler and incubated for 45 min at 42 $^{\circ}\text{C}$, followed by 5 min at 95 $^{\circ}\text{C}$ to inactivate the reverse transcriptase and to denature the template.

3.2.5 Amplification of cDNA (Polymerase chain reaction)

Five microlitres (5 μ l) of first-strand cDNA was added to 45 μ l PCR reaction mixture containing 5 μ l 10x PCR buffer (Invitrogen), 1.5 mM magnesium chloride, 0.2 mM dNTP, 2 units of Taq-DNA Polymerase (Invitrogen), 10 pmol of each of specific primer AB1/AB2 and AB3 (for P0 gene); AB9, AB7 and AB12, AB13 (for CP gene). The primer sequences are listed in Table 3.3. The PCR reaction mixture was incubated in a pre-warmed thermal cycler under the following conditions: one cycle for 5 mins at 95°C; 30 cycles at 95°C for 30s (denaturation), 50°C for 1 min (annealing) and 72°C for 1 min (extension) and one cycle at 72°C for 10 mins. Annealing temperatures of 43°C and 58°C was used for primer pairs AB12/AB13 and AB9/AB7, respectively. The RT-PCR products were separated by electrophoresis in 1% agarose gels stained with GelRed (Biotium Inc., USA).

3.2.6 Gel purification

Bands corresponding to the expected sizes were excised and gel purified using the QIAquick Gel Extraction Kit Protocol (QIAGEN, UK). The DNA was eluted with 30 μ l elution buffer (10 mM Tris HCl, pH 8.5) and either used for direct sequencing or cloned when mixed infection was suspected.

3.2.7 Cloning and sequencing

The initial strategy was to sequence the purified PCR products directly without cloning. Where sequences indicated mixed genotypes from the same host plant, cloning was used. Mixed genotypes were indicated by multiple peaks (polymorphism) in the chromatogram following alignment of forward and reverse sequences. In such cases the purified reverse transcription (RT)-PCR products were

cloned in either pCR[®]2.1 plasmid from the TA Cloning[®] Kit (Invitrogen, UK), or PJET1.2 vector from CloneJET PCR Cloning Kit (Fermentas, UK) following the manufacturer instructions. As was done by de Miranda *et al.* (1995), three clones from each isolate were sequenced to assess variation within a virus isolate and to ensure consistent and reliable sequence data. Prior to sequencing, the plasmid DNAs were amplified using illustra TempliPhi Amplification Kit (GE Healthcare, UK) according to the manufacturer's instruction. The clones were sequenced with primers M13 reverse and M13 forward, complementary to the vector pCR[®]2.1 from the TA Cloning Kit or primers PJET1.2 forward and PJET1.2 reverse, complementary to vector PJET1.2 from the CloneJET PCR Cloning Kit.

The cDNA products were sequenced in both directions using the dideoxy-mediated chain-termination method. 10 µl sequencing reaction mixtures included 2 µl BigDye, 2 µl 5X sequencing buffer, 20 ng DNA, 5 pmol primer and sterilized distilled water. Sequencing mixtures were transferred to a pre-warmed thermal cycler and incubated at the following conditions: 96 °C for 1 min; 25x at 96 °C for 10 sec, 50 °C for 5 s, 60 °C for 2 min; then followed by 12 °C for 2 min. The sequencing mixtures were then taken to the Genomic Centre of the Wellesbourne campus of the School of Life Sciences, University of Warwick, UK for sequencing by a technician.

DNASTAR Lasergene software (Burland, 2000) was used to visualise the chromatogram, evaluate the quality of each nucleotide in the sequence, detect and evaluate nucleotide changes and construct consensus sequences for each amplicon. Polymorphic sites were confirmed manually by examining the chromatograms of each sequence by eye. Both non-coding and the primer sequences were discarded

from the alignments. Additional sequences published by Veidt *et al.* (1988), Hauser *et al.* (2000a) and de Miranda *et al.* (1995) were retrieved from GenBank, checked and added to the data sets. The consensus sequences developed by Hauser *et al.* (2000a) did not entirely cover ORF3 (CP); and Schubert *et al.* (1998)'s consensus P0 gene sequences also did not cover the region which was analysed. Consequently they were not included in the analyses.

Multiple alignments of the sequences were done using the ClustalW programme (Thompson *et al.*, 1994) implemented in MEGA version 5.2 (Tamura *et al.*, 2011). When nearly identical nucleotide sequences were obtained for two or more clones from the same plant sample, only one of them was selected for subsequent multiple sequence alignment and phylogenetic analysis (Ala-Poikela *et al.*, 2005). Alignments were also adjusted manually to ensure correct reading frames. There were a total of 232 P0 nucleotide sequences and 233 coat protein nucleotide sequences included in the analyses.

3.2.8 Sequence comparisons and phylogenetic analyses

Nucleotide and the deduced amino acid sequence identities were determined for both P0 and P3 genes sequence datasets using BioEdit v7.0.5 (Hall, 2005). For both P0 and P3 gene sequence alignments, the most appropriate nucleotide substitution model was selected by MODELTEST (Posada and Crandall, 1998) implemented in MEGA version 5 programme (Tamura *et al.*, 2011), using the Akaike Information Criterion (Akaike, 1974), Bayesian Information Criterion (Schwarz, 1978) and the hierarchical likelihood ratio test (Pol, 2004). The best fit nucleotide substitution model was then used for phylogenetic analyses using the maximum-likelihood

method implemented in MEGA 5 and the resultant phylogenetic trees visualised using FigTree v1.3.1 software (Rambaut and Drummond, 2010). To verify the statistical validity of the clusters obtained, bootstrap analysis was done on 1000 trials. The neighbour-joining method also implemented in MEGA 5 was used for comparison.

3.2.9 Detection of recombination

The occurrence of recombination within and between the P0 and P3 genes was determined for each sequence dataset. In this case the ClustalW-aligned sequences of P0 and P3 genes belonging to the same isolates were concatenated and recombination analysis was performed by seven different methods available in the Recombination Detection Programme v.3.4.4 i.e. RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMAERA, SISCAN and 3SEQ employing the defaults parameters (Martin *et al.*, 2010). For a more reliable results, only recombination signals detected by five of the methods and showing significant support with Bonferroni-corrected P -value cut off of 0.05 were considered, similar to the strategies adopted by Pagan and Holmes (2010).

3.2.10 Genetic diversity

Using DnaSP V.5.0 programme (Librado and Rozas, 2009) the following indices of genetic diversity were measured for all samples of each gene (P0 and P3) and for each geographic region (Lincolnshire, Warwickshire and Yorkshire): haplotype diversity (h), nucleotide diversity (π), number of segregating sites (S) and total number of mutations (Eta).

3.2.11 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 and between P0 and P3 genes nucleotide sequence datasets were estimated using Maximum likelihood model (Tamura *et al.*, 2004) with gamma rate of variation among sites (shape parameter $r = 6.0$). Standard error estimates were obtained by bootstrap procedure (1000 replicates). The analyses were conducted in MEGA 5.

The Maximum Likelihood analysis of natural selection codon-by-codon method via HyPhy package (Kosakovsky *et al.*, 2005) implemented in MEGA 5 (Tamura *et al.*, 2011) was used to estimate the numbers of inferred synonymous substitutions per synonymous site (d_S) and the numbers of non-synonymous substitutions per non-synonymous site (d_N). These estimates were produced using the joint Maximum Likelihood reconstructions of ancestral states under the defaults Muse-Gaut model (Muse and Gaut, 1994) and General Time Reversible model (Nei and Kumar, 2000). The test statistic d_N-d_S was used for detecting codons that have undergone positive selection. A positive value for the test statistic indicates an overabundance of nonsynonymous substitutions. In this case, the probability of rejecting the null hypothesis of neutral evolution (P -value) was calculated using the methods of Kosakovsky and Frost (2005) and Suzuki and Gojobori (1999). Values of P less than 0.05 are considered significant at a 5% level. The overall ratio d_N/d_S was also calculated from the mean values of d_N and d_S to compare the selection pressures acting on the P0 and P3 genes of TuYV. The gene is under positive (or diversifying) selection when the d_N/d_S ratio is > 1 , negative (or purifying) selection when the d_N/d_S ratio < 1 , and neutral selection when d_N/d_S ratio = 1.

3.2.12 Determination of substitution rate

For each of the P0 and P3 gene nucleotide sequence datasets, rates of nucleotide substitution per site and the time to the most recent common ancestor (TMRCA) were estimated using the Bayesian Markov chain Monte Carlo (MCMC) approach available in the BEAST package v1.6.1 (<http://beast.bio.ed.ac.uk>) (Drummond and Rambaut, 2007). The analysis was run using the best fit substitution model, among the default models, i.e. Hasegawa-Kishino-Yano (HKY) model with invariant sites and gamma distribution among sites rate variation with six rate categories (Hasegawa *et al.*, 1985). Using uncorrelated lognormal relax clock model and 20 million steps of MCMC which was sampled after 10,000 states, constant population size and exponential population growth dynamic models were tested. Statistical uncertainty in the data was reflected by the 95 % highest probability density (HPD) values. Results were examined using the TRACER v1.6 programme implemented in the BEAST package (Drummond and Rambaut, 2007). Convergence was assessed with ESS (effective sample size) values after a burn-in of 2 million steps.

3.2.13 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, 1983). 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 (Kimura, 1983). The significance of each test statistic was estimated by 10,000 permutations.

3.2.14 Analysis of population differentiation and variation

The genetic differentiation of populations within and between sites was determined by the likelihood ratio test (χ^2) (Workman and Niswander, 1970) and four permutation based statistical tests, K_s^* , Z , H_s and S_{nn} (nearest neighbour statistic) which represent the most powerful sequence-based statistical tests for genetic differentiation (Hudson, 2000; Hudson *et al.*, 1992). The extent of genetic differentiation or the level of gene flow between populations was estimated by the statistics N_{st} (estimates of gene flow between populations) (Lynch and Crease, 1990) and F_{st} (the interpopulational component of genetic variation or the standardised variance in allele frequencies across populations) (Hudson *et al.*, 1992). The absolute values of F_{st} ranges between 0 and 1 for undifferentiated to fully differentiated populations respectively. Normally, an absolute value of $F_{st} > 0.33$ suggests infrequent gene flow, and an absolute value of $F_{st} < 0.33$ suggests frequent gene flow (Rozas *et al.*, 2003; Wei *et al.*, 2009). N_{st} is the ratio of the average genetic distance between genes from different populations relative to that among genes in the population at large. Extreme N_{st} estimates of 0 and 1 indicates no and complete population subdivision, respectively (Lynch and Crease, 1990). The statistical tests for genetic differentiation and gene flow estimates were performed with DnaSP version 5.0. (Librado and Rozas, 2009).

The geographical and spatial patterns of genetic differentiation were evaluated by performing analysis of molecular variance (AMOVA) using Arlequin version 3.0 (Excoffier *et al.*, 2005). The significance of each genetic differentiation statistic was estimated by 1000 permutations.

3.3 Results

3.3.1 Sequence analysis

Two distinct parts of the genome were sequenced to estimate the genetic variability of TuYV isolates. They were ORF0, which encodes a protein that is involved in post transcriptional gene silencing, host range specificity and symptom expression, and ORF3 which encodes the coat protein (CP). After editing, the final sequences analysed were the full sequence (609 nt) of CP (P3) gene and partial sequences (699 nt) of the P0 gene (The full P0 sequence comprises 750 nt). The sequences obtained in this work were analysed together with those retrieved from the GenBank.

The field isolates analysed shared nucleotide identities ranging from 94 to 100% for P3 and from 91.7 to 100% for P0. The deduced amino acid sequences of the sequenced isolates also ranged from 92.5 to 100% for the P3 and 86.2 to 100% for the P0 (Table 3.3), indicating higher variability within the P0 than the P3 (i.e. the P3 nucleotide sequences are more closely related than that of P0).

The P0 sequences of the sequenced isolates shared 86.9 to 98.8% nucleotide identities and 79.8 to 100% deduced amino acid identities with that of published isolates from GenBank (Table 3.4). The ORF3 nucleotide sequence identities between the sequenced isolates and the published isolates ranged between 93.5% and 99.8%, and the identities of the corresponding predicted amino acid sequences ranged from 91 to 100% (Table 3.4).

Table 3.4 Nucleotides (nt) and amino acid (aa) sequence identities of *Turnip yellows virus* (TuYV) field isolates and selected published isolates retrieved from GenBank.

Sequences	Sequence identities (%)	
	Nucleotide	Amino acid
(a) P0 gene		
Between sequenced isolates	91.7 - 100	86.2 - 100
Between sequenced isolates and published isolates	86.9 - 98.8	79.8 - 100
(b) P3 gene		
Between sequenced isolates	94 - 100	92.5 - 100
Between sequenced isolates and published isolates	93.5 - 99.8	91 - 100

3.3.2 Phylogenetic analyses

The maximum likelihood tree for the partial ORF0 nucleotide sequence data revealed that the 226 TuYV isolates sequenced in this study from oilseed rape formed three main genetic groups corresponding to three clades supported by bootstrap values greater than 85% for all isolates (Figures 3.3) and greater than 70% for the abridged analysis (Figure 3.4). TuYV isolates collected from the three geographical regions and at different years were spread throughout the three clades (i.e. the isolates did not cluster according to geographical regions or years of collection). Clade 1 containing the majority of the sequenced isolates (192 isolates), clustered with the published isolate BWYV-Col (accession number AF168600) with which they shared nucleotide sequence identities ranging between 92.9% to 99.1% and deduced amino acid sequence identities of 89.2 to 97.8% (Table 3.5). Seven isolates (L188, L1014, L1271, W1088b, Y917, Y1266 and Y1485), clustered in

clade 2 with published isolate BWYV-Fev (AF168601) from field bean in France, sharing a nucleotide sequence identity of 93.9 to 97.1%, and deduced amino acid sequence identities of 91.4 to 97.4%. Isolates TuYV-BN5 (accession number AF168606 from oilseed rape in France, TuYV-GB (accession number AF168608) from oilseed rape in Norfolk and the LAB isolate from oilseed rape in Suffolk, clustered with 31 field isolates in clade 3, sharing nucleotide and amino acid sequence identities of 92.1 - 99.4% and 88.4 - 100% respectively (Table 3.4). TuYV-FL1 (accession number X13063) did not cluster with any of the isolates sequenced from oilseed rape or other published isolates, indicating long evolutionary distance separating from the other isolates.

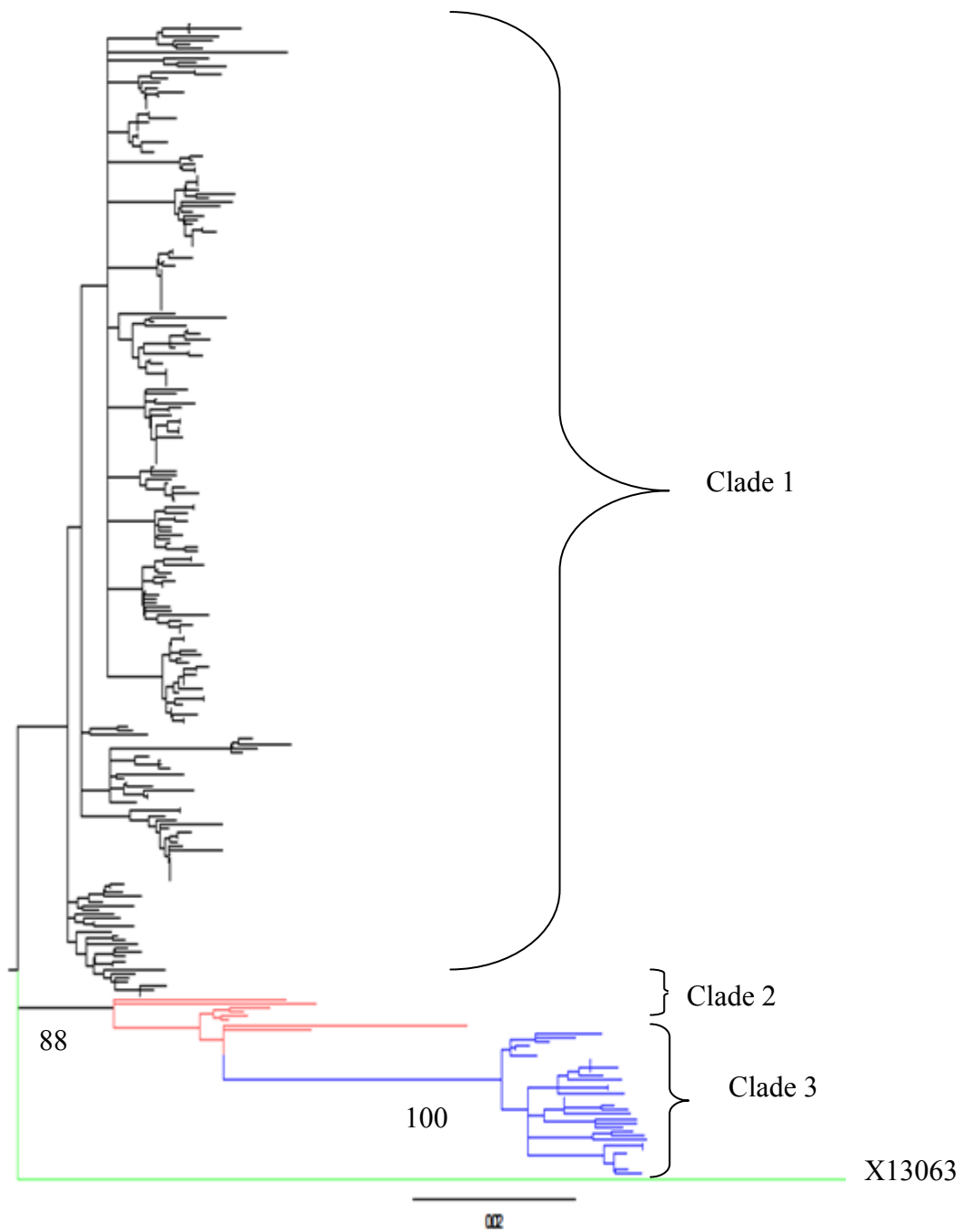


Figure 3.3 Maximum-likelihood phylogenetic tree of nucleotide sequences of P0 gene of *Turnip yellows virus* (TuYV) isolates (n=226) sampled in Lincolnshire, Warwickshire and Yorkshire from 2007-2009.

The isolates formed three clades, denoted: 1 (black branches), 2 (red branches) and 3 (blue branches). The green branch is the published isolate TuYV-FL (accession number X13063) (Veidt *et al.*, 1988). The tree is midpoint rooted, and significant bootstrap support values ($\geq 80\%$; 1000 bootstrap replicates) indicated at major nodes. The scale bar signifies a genetic distance of 0.02 nucleotide substitutions per site. Identities of the isolates are recorded in Table 3.3. See Figure 3.4 for an abridged form of this phylogenetic tree.

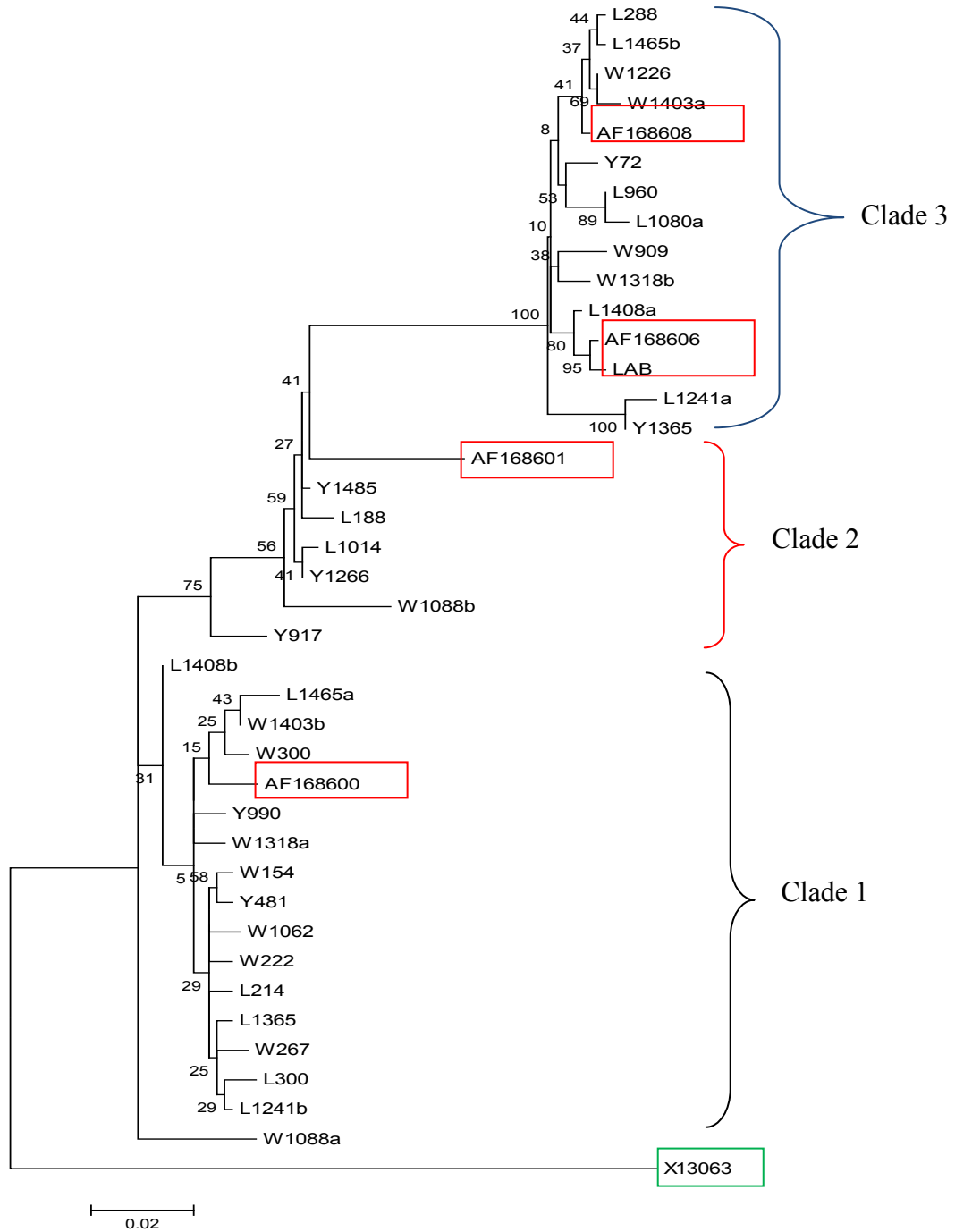


Figure 3.4 Maximum likelihood tree (abridged) of PO gene nucleotide sequence of *Turnip yellows virus* (TuYV) isolates from Lincolnshire (prefixed L), Warwickshire (prefixed W) and Yorkshire (prefixed Y) in England.

The isolates in red boxes are GenBank accession numbers of isolates published by Hauser *et al.* (2000a), and isolate X13063 in the green box is the GenBank accession number of isolate TuYV-FL published by Veidt *et al.* (1988) and LAB isolate. The scale bar signifies a genetic distance of 0.02 nucleotide substitutions per site.

Table 3.5 Nucleotides (nt) and amino acid (aa) sequence identities of *Turnip yellows virus* (TuYV) isolates within and between clades of Maximum likelihood phylogenetic tree

Clade	P0 sequence identity		Clade	P3 sequence identity	
	(%)			(%)	
	nt	aa		nt	aa
Within clade 1	96.2 - 100	94.4 - 100	Within clade 1	97.7 - 100	96.5 - 100
Within clade 2	97.2 - 99.2	97.1 - 99.1	Within clade 2	98.3 - 100	98.0 - 100
Within clade 3	97.4 - 100	96.1 - 100	Between clades 1 and 2	94.0 - 96.0	92.5 - 95.0
Between clades 1 and 2	94.4 - 96.1	91.4 - 96.1	-	-	-
Between clades 1 and 3	91.7 - 94.1	86.6 - 89.2	-	-	-
Between clades 2 and 3	94.1 - 95.7	88.4 - 92.7	-	-	-
Between clade 1 and AF168600	92.9 - 99.1	89.2 - 97.8	Between clade 1 and L39968, L39969, L39970	93.9 - 99.1	97- 100
Between clade 2 and AF168601	93.9 - 97.1	91.4 - 97.4	Between clade 2 and L39971, L39974, L39975, L39986	94.7 - 99.6	92 - 100
Between clade 3 and AF168606 and AF168608	92.1 - 99.4	88.4 - 100	-	-	-

Phylogenetic analysis of CP gene nucleotide sequences clustered the TuYV isolates into only two genetic groups (clades), supported with bootstrap values of greater than 80% for all isolates (Figures 3.5) and greater than 75% for the abridged analysis (Figure 3.6). Group 1 which had the largest number of isolates clustered with isolates 3a1 strain bwyv-2 (accession number L39968), isolates 3a2 strain bwyv-1 (accession number L39969), isolate 3b strain bwyv-2 (accession number L39970) all from oilseed rape in England, published by de Miranda *et al.* (2005). Group 2 field isolates clustered with isolate 4a strain bwyv-1 (accession number L39971), isolate 7

strain bwyv-1 (accession number L39974), isolate 8 strain bwyv-1 (accession number L39975), and isolate 5 strain bwyv-1 (accession number L39986) all from oilseed rape or vegetable brassicas in England also published by de Miranda *et al.* (2005). The tree produced by the neighbour joining method had similar topology as that of the Maximum likelihood tree (results not shown).

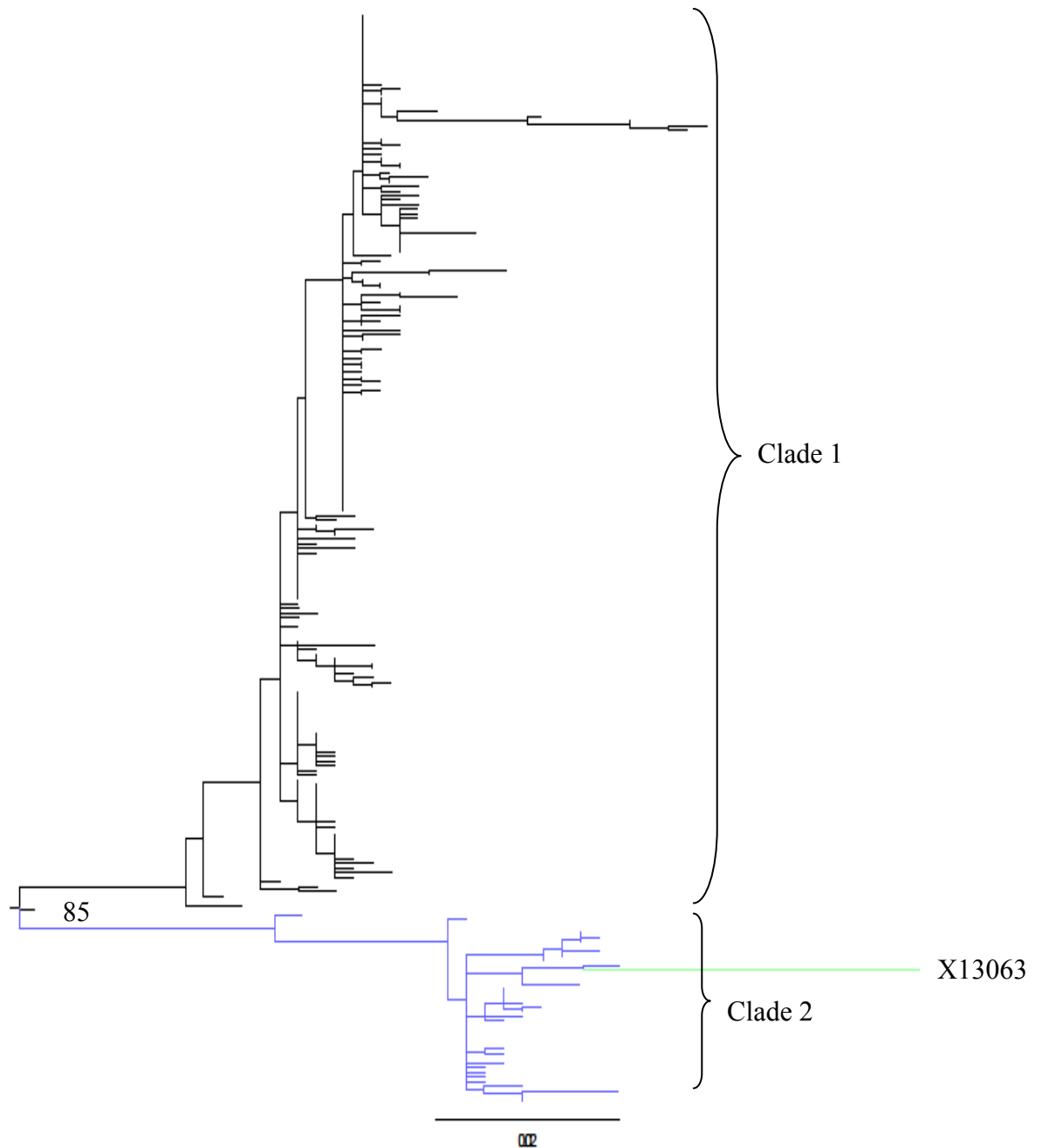


Figure 3.5 Maximum likelihood phylogenetic tree of coat protein gene nucleotide sequences of *Turnip yellows virus* (TuYV) isolates sampled from Lincolnshire, Warwickshire, and Yorkshire in England between 2007 and 2009. The isolates formed two clades, denoted: 1 (black branches), 2 (blue branches). The green branch is the published isolate TuYV-FL (accession number X13063) (Veidt *et al.*, 1988) which is close to, but has a different phylogenetic history to clade 1 isolates. The tree is midpoint rooted and significant bootstrap support values ($\geq 70\%$; 1000 bootstrap replicates) indicated at major nodes. The scale bar signifies a genetic distance of 0.02 nucleotide substitutions per site. See Figure 3.6 for an abridged form of this phylogenetic tree.

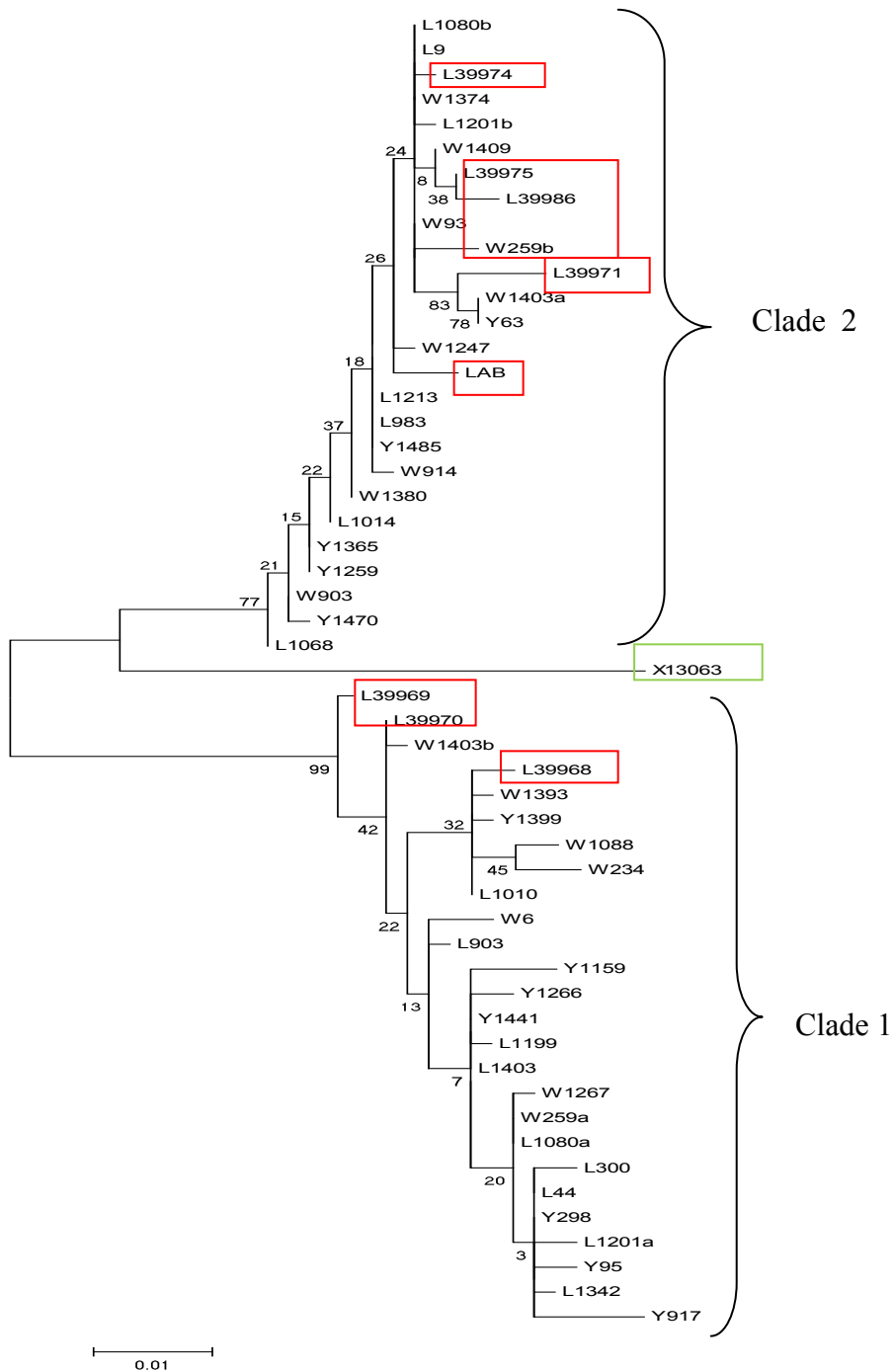


Figure 3.6 Maximum likelihood phylogenetic tree (abridged) of coat protein gene nucleotide sequences of *Turnip yellows virus* (TuYV) isolates from Lincolnshire (prefixed L), Warwickshire (prefixed W) and Yorkshire (prefixed Y) in England.

Isolates in red boxes are GenBank accession numbers published by de Miranda *et al.* (1995) and the LAB isolate. X13063 in the green box is the GenBank accession number of isolate TuYV-FL published by Veidt *et al.* (1988). The scale bar signifies a genetic distance of 0.01 nucleotide substitution per site.

The maximum likelihood phylogenetic tree for the P0 amino acid sequences had similar topology (Figure 3.7, appendix 3.1) as that of nucleotide sequence, with three clades (genetic groups). Similarly, the maximum likelihood tree for the P3 amino acid sequences had similar topology (Figure 3.8, appendix 3.2) as that of the nucleotide sequence, with two clades (genetic groups).

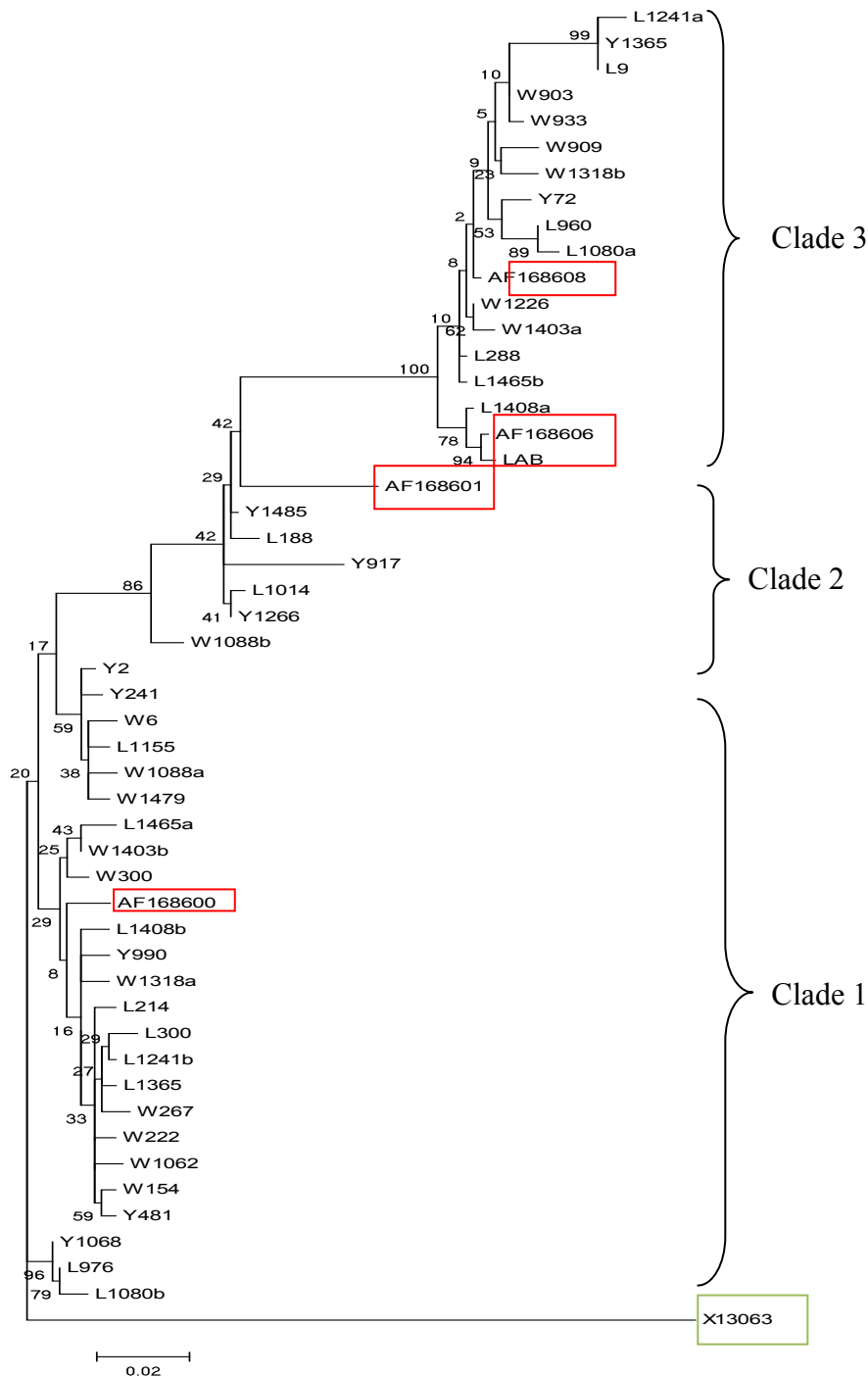


Figure 3.7 Maximum likelihood phylogenetic tree (abridged) of PO amino acid sequences of *Turnip yellows virus* (TuYV) isolates from Lincolnshire (prefixed L), Warwickshire (prefixed W) and Yorkshire (prefixed Y) in England. Isolates in red boxes are GenBank accession numbers published by Hauser *et al.* (2000a) and the LAB isolate. X13063 in the green box is the GenBank accession number of isolate TuYV-FL published by Veidt *et al.* (1988). The scale bar signifies a genetic distance of 0.02 amino acid substitution per site.

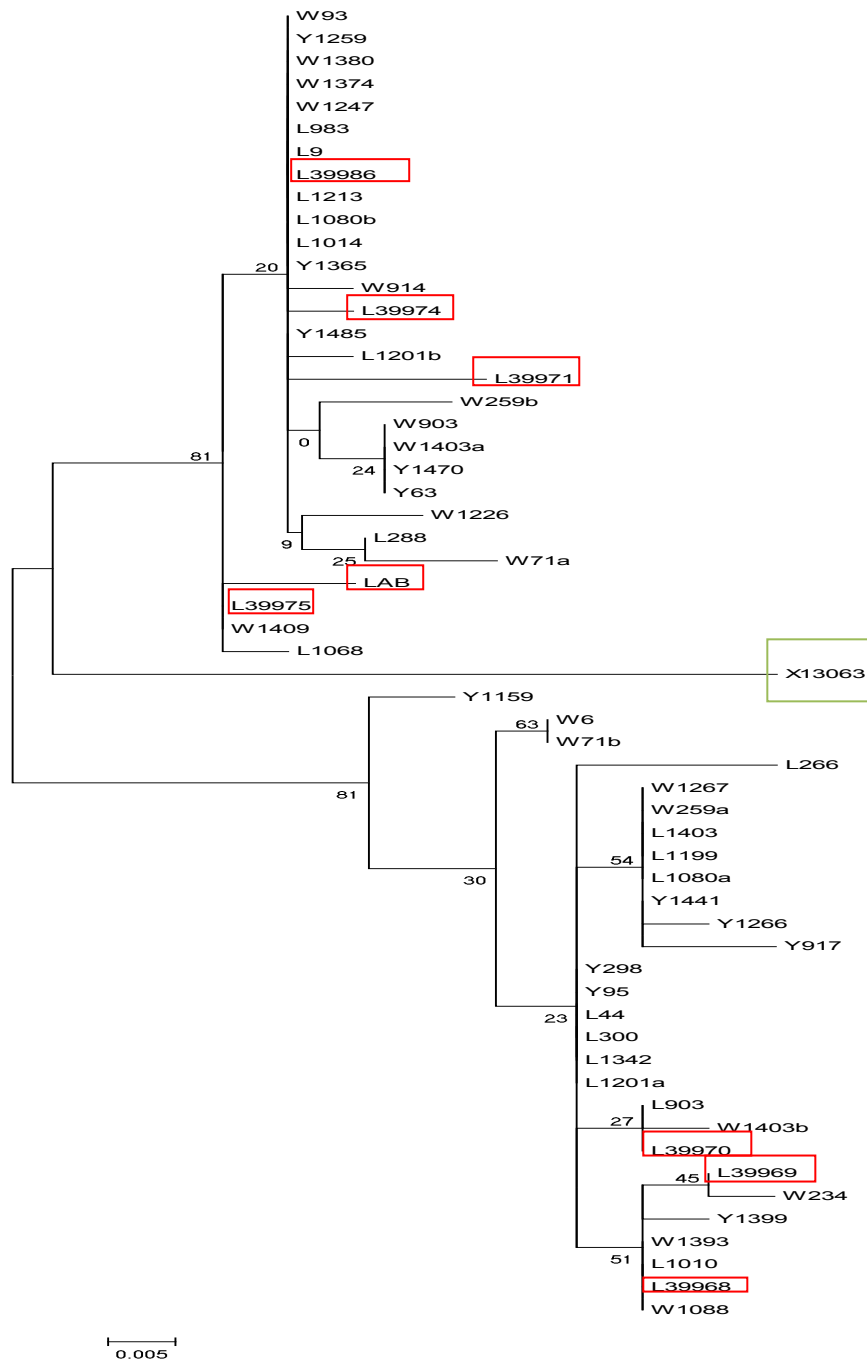


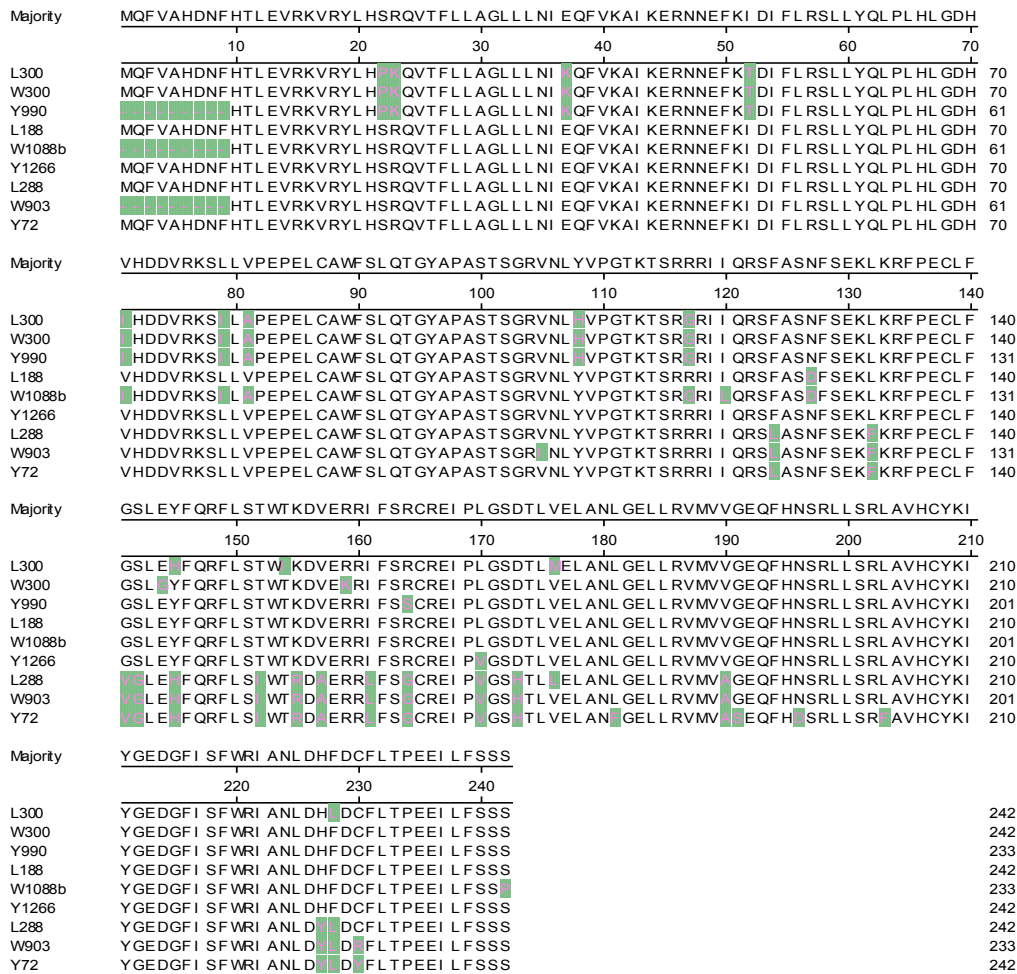
Figure 3.8 Maximum likelihood phylogenetic tree (abridged) of P3 amino acid sequences of *Turnip yellows virus* (TuYV) isolates from Lincolnshire (prefixed L), Warwickshire (prefixed W) and Yorkshire (prefixed Y) in England. Isolates in red boxes are GenBank accession numbers published by Hauser *et al.* (2000a) and the LAB isolate. X13063 in the green box is the GenBank accession number of isolate TuYV-FL published by Veidt *et al.* (1988). The scale bar signifies a genetic distance of 0.005 amino acid substitution per site.

3.3.3 Mixed genotypes

Of the 226 TuYV isolates sequenced in this study 10 were from plants infected by two different genotypes. Phylogenetic analyses of both P0 and P3 sequences showed that several plants were infected with isolates belonging to two distinct clades or genetic groups (i.e. mixed genotypes infections). In the P0 gene nucleotide phylogenetic tree, isolates L1408b, L1465a, W1403b and W1088a clustered in clade 1, whilst their counterparts from the same plant hosts, i.e. L1408a, L1465b, W1403a clustered in clade 3, and W1088b clustered in clade 2 (Figure 3.4). In the CP gene nucleotide phylogenetic tree, L1080a, W259a, L1210a and W1403b clustered in clade 1 whilst corresponding isolates from the same plant hosts (L1080b, W259b, L1210b, W1403a) clustered in clade 2 (Figures 3.6).

3.3.4 Alignments of P0 and P3 amino acid sequences

The deduced amino acid sequence alignment of the nucleotide sequence alignment of the P0 gene of TuYV isolates (appendix 3.3) is shown in Figure 3.9. There were 37 amino acid sites which differ from the consensus (Figure 3.9). Genotype 1 (clade 1) differs from genotype 3 (clade 3) at 22 amino acid sites. Genotype 2 (clade 2) shares the 5' region (1 to 123 amino acids sites) with genotype 3; and shares the 3' region (from 124 to 242 amino acid sites) with genotype 1.



Decoration 'Decoration #1': Shade (with dark green at 50% fill) residues that differ from the Consensus.

Figure 3.9 Alignment of P0 amino acid sequences of *Turnip yellows virus* (TuYV) isolates representing the three genetic groups (clades).

Genotype 1 isolates are L300, W300 and Y990; genotype 2 isolates are L188, W1088b and Y1266; genotype 3 isolates are L288, W909 and Y72. The shaded regions are the amino acid residues that differ from the consensus.

The deduced amino acid sequence alignment of P3 of TuYV isolates (appendix 3.4) is also shown in Figure 3.10. Clade 1 differs from clade 2 at nine amino acid sites (20, 34, 37, 49, 54, 95, 98, 153 and 164 positions). However, there were a total of 14 variable sites.

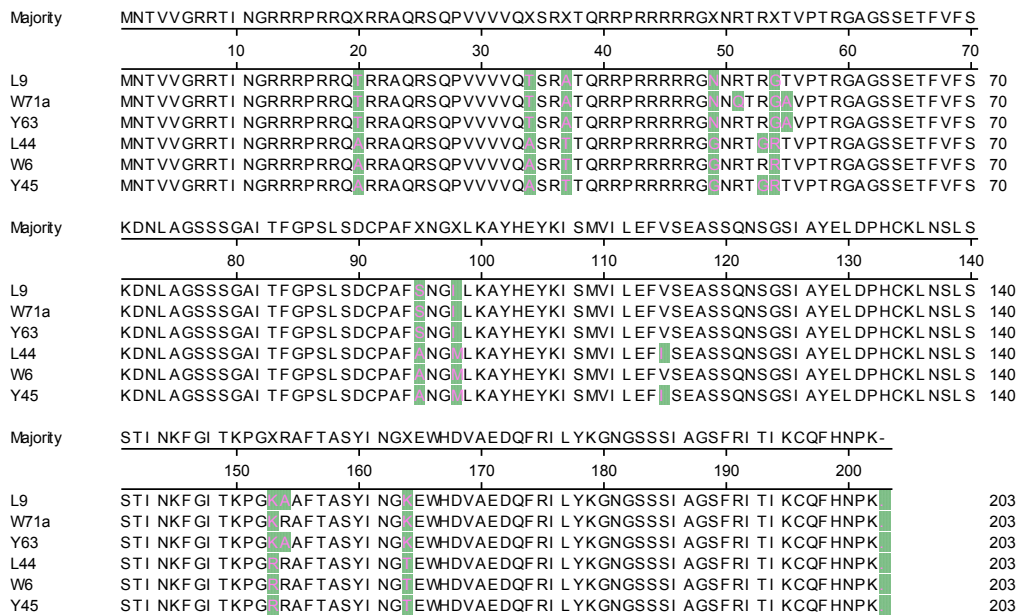


Figure 3.10 Alignment of P3 amino acid sequences of *Turnip yellows virus* (TuYV) isolates representing the two genetic groups (clades). Clade 1 isolates are L44, W6 and Y45; Clade 2 isolates are L9, W71a and Y63. The shaded regions are the amino acid residues that differ from the consensus.

3.3.5 Genetic diversity within P0 and P3 and different geographical regions

Analysis of genetic diversity within the P0 and the P3 showed that both genes were variable with high number of mutations, high number of polymorphic sites and very high haplotype diversity but low nucleotide diversity (Table 3.6a). However, the diversity in P0 gene ($S = 219$, $Eta = 266$, $\pi = 0.0331 \pm 0.002$, $h = 0.990 \pm 0.0012$) was higher than that of the P3 gene ($S = 135$, $Eta = 153$, $\pi = 0.0210 \pm 0.0015$; $h = 0.9795 \pm 0.0043$) (Table 3.6a).

Table 3.6a Genetic variability within ORF0 and ORF3 DNA sequences of *Turnip yellows virus* (TuYV) isolates

Dataset	Number of sequences	Number of polymorphic sites (S)	Total number of mutations (Eta)	Nucleotide diversity (π) ^a	Haplotype diversity (h) ^a
P0	226	219	266	0.0331 \pm 0.0020	0.9960 \pm 0.0012
P3	225	135	153	0.0210 \pm 0.0015	0.9795 \pm 0.0043

^a Mean \pm standard deviation

Genetic diversities in the three geographical regions were also estimated, based on P0 gene nucleotide sequence data (since P0 gene was found to be more variable than the coat protein gene). Results showed very high haplotype diversity, high number of mutations and high number of polymorphic sites but low nucleotide diversity in all the three regions. Lincolnshire had relatively highest haplotype diversity (0.9944), followed by Warwickshire (0.994), whilst Yorkshire had the lowest (0.9924). TuYV populations in Warwickshire had the highest number of mutations, highest polymorphic sites, and the highest nucleotide diversity, followed by Lincolnshire whilst Yorkshire had the lowest (Table 3.6b).

Table 3.6b Genetic diversity within the *Turnip yellows virus* (TuYV) populations in Lincolnshire, Yorkshire and Warwickshire based on 699 bp of the P0 gene of the viral genome.

County	N	H	S	Eta	Nucleotide diversity (π)	Haplotype diversity (h)
Lincolnshire	83	71	129	143	0.0330±0.0033	0.9944
Yorkshire	63	50	116	125	0.0269± 0.0035	0.9924
Warwickshire	80	69	135	149	0.0355± 0.0036	0.9940
Overall	226	174	229		0.0334	0.9960

Sample size (N), number of haplotypes (H), number of segregating sites (S), total number of mutations (Eta).

Of the 174 haplotypes identified for P0 gene, only 3 (1.7%) were shared among the three regions (Table 3.7). Seven haplotypes were shared between Lincolnshire and Warwickshire populations, whilst four each were shared between Lincolnshire and Yorkshire and between Warwickshire and Yorkshire (Table 3.7). 166 out of 174 haplotypes were restricted to specific geographical regions (i.e. they were not shared), an indication of high genetic differentiation between the TuYV populations at the three regions. The nucleotide sequences of the haplotypes shared between the three regions are shown in appendix 3.5.

Table 3.7 Number of haplotypes of *Turnip yellows virus* (TuYV) populations shared among the regions based on the P0 gene nucleotide sequences

Population 1	Population 2	Number of shared haplotypes	Haplotypes
Lincolnshire	Warwickshire	7 (4.0) ^a	Hap 2, Hap 15, Hap 27, Hap 31, Hap 42, Hap 48, Hap 109
Lincolnshire	Yorkshire	4 (2.3)	Hap 1, Hap 15, Hap 31, Hap 42
Warwickshire	Yorkshire	4 (2.3)	Hap 15, Hap 31, Hap 42, Hap 137
Lincolnshire, Warwickshire and Yorkshire		3 (1.7)	Hap 15, Hap 31, Hap42
Total number of haplotypes		174 (166) ^b	

^a Numbers in the parentheses are the percentage haplotypes

^b Number of unique haplotypes.

3.3.6 Recombination in the *Turnip yellows virus* (TuYV) isolates

The phylogenetic analyses revealed that the clustering of specific TuYV isolates differed between P0 and P3. The P0 gene phylogeny grouped the isolates into three main clades whilst analyses of the CP gene produced only two clades (Figures 3.3-3.6), indicating that the two phylogenetic trees were not congruent. These results are suggestive of recombination between the P0 and P3 in a number of the TuYV isolates.

To identify recombination events, P0 and P3 genes and concatenated sequences of all the TuYV isolates were analysed using seven different recombination detection methods available in the Recombination Detection Programme v3.44 (Martin *et al.*, 2010). The results suggested that L188, L1014, L1271, W1088b, Y917, Y1266 and

Y1485 were recombinants of haplotypes represented by the isolates Y72 and W1273 ($P \leq 1.271 \times 10^{-3}$), all detected within the P0 gene. One signal of recombination between P0 and P3 genes was detected; suggesting L187 as a recombinant of haplotype represented by the isolates L939 and Y917 ($P \leq 1.268 \times 10^{-2}$) (Table 3.8). The analyses also revealed several recombination breakpoints i.e. putative recombination “hot spots” within the P0 gene (8 sites), comprising position 159 in the sequence (corresponding to nucleotide 186 of the gene), positions 684 and 694 in the sequence (corresponding to nucleotides 711-721 of the P0 gene) and position 337 in the sequence (corresponding to nucleotide 364 within the gene). In the concatenated P0 and P3 gene sequence, only two breakpoint signals were detected i.e. position 533 in the 5' half of the P0 gene (corresponding to nucleotide 560) and position 1288 located near 3' end of the P3 gene (corresponding to nucleotide 589 of the gene).

Since recombination between genomes confounds attempts to estimate evolutionary rates (Gibbs *et al.*, 2010), these eight recombinants were excluded from subsequent evolutionary analysis.

Table 3.8 Recombination within and between P0 and P3 genes

Isolate	Analysed region	Recombination breakpoints		‘Parent-like’ isolates ^a		Methods ^b	<i>P</i> -values (range) ^c
		Beginning	Ending	Major	Minor		
L1014	P0	337	684	Y72	W1273	B,M,C,S, 3Seq	2.26 X 10 ⁻⁶ - 1.272 X 10 ⁻³
L1271	P0	348	684	Y72	W1273	B,M,C,S, 3Seq	2.26 X 10 ⁻⁶ - 1.272 X 10 ⁻³
W1088	P0	159	694	Y72	W1273	B,M,C,S, 3Seq	2.26 X 10 ⁻⁶ - 1.272 X 10 ⁻³
b Y917	P0	337	684	Y72	W1273	B,M,C,S, 3Seq	2.26 X 10 ⁻⁶ - 1.272 X 10 ⁻³
Y1266	P0	337	694	Y72	W1273	B,M,C,S, 3Seq	2.26 X 10 ⁻⁶ - 1.272 X 10 ⁻³
Y1485	P0	337	694	Y72	W1273	B,M,C,S, 3Seq	2.26 X 10 ⁻⁶ - 1.272 X 10 ⁻³
L187	(P0+P3) ^d	533	1288	L939	Y917	B, M, C, S, 3Seq	9.05 X 10 ⁻⁵ - 1.268 X 10 ⁻²

^a Major and minor parents are sequences that were used, along with the indicated recombinant, to identify recombination. Minor parent is apparently the contributor of the sequence within the indicated region whilst the major parent is the apparent contributor of the rest of the sequence. The identified “parental isolates” are not the actual parents but are haplotypes of the actual parents in the datasets.

^b Symbols representing the recombination detection methods out of a total of 7, which detected the recombinants, available in the RDP V.3.44 programme (Martin *et al.*, 2010): B = Bootscan, M = Maxchi, C = Chimaera, S = SiScan

^c Range of *P*-values for all the five recombination methods which detected the recombination signals.

^d (P0 + P3) is the concatenated nucleotide sequences between partial P0 gene (699 nt) and the complete P3 gene (609 nt).

Note: No recombination signal was detected within coat protein (P3 gene) nucleotide sequence dataset.

3.3.7 Nucleotide substitution rate in the *Turnip yellows virus* (TuYV) isolates

The mean evolutionary rate of the P0 gene was estimated to be 9.6 X 10⁻⁴ and 9.4 X 10⁻⁴ subs/site/year using constant size and exponential growth coalescent population models respectively. The mean substitution rate for the P3 gene was surprisingly higher than that of P0 gene, and was estimated to be 1.8 X 10⁻³ and 2.1 X 10⁻³

subs/site/year using constant size and exponential growth coalescent population models respectively (Table 3.9).

For P0 and P3, the time to the most recent common ancestor (TMRCA) was estimated so as to determine the time scale of the evolutionary history of the TuYV isolates. The TMRCA for P0 gene was 70 years with the 95% highest posterior density (HPD) ranging from 50 to 120 years. Interestingly, both constant and exponential population growth models produced the same mean divergence time of 70 years for P0 (Table 3.9). The TMRCA for the P3 was 12 years (ranges from 9 - 25 years) and 14 years (ranges from 12 - 25 years) for constant and exponential population growth models. It is also interesting to note that, both constant and exponential population growth models produced similar divergence times.

Table 3.9 Nucleotide substitution rate and TMRCA estimates for ORF0 of *Turnip yellows virus* (TuYV) isolates from oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire during 2007-10, 2008-9 and 2009-10 crop seasons

Gene	Divergence according to the indicated evolutionary model			
	Constant size (HKY+ γ_6 +I)		Exponential growth (HKY+ γ_6 +I)	
	Substitution rate ^a (HPD) ^b	TMRCA ^c (HPD)	Substitution rate (HPD)	TMRCA (HPD)
P0	9.6 X 10 ⁻⁴ (6.2 X 10 ⁻⁴ - 1.4 X 10 ⁻³)	70 (52 - 120)	9.4 X 10 ⁻⁴ (5.4 X 10 ⁻⁴ - 1.3 X 10 ⁻³)	70 (50 - 120)
P3	2.1 X 10 ⁻³ (1.1 X 10 ⁻³ - 3.4 X 10 ⁻³)	12 (9 - 25)	1.8 X 10 ⁻³ (1.1 X 10 ⁻³ - 2.6 X 10 ⁻³)	14 (12 - 25)

^a Mean nucleotide substitution rate (substitution/site/year). Values in parenthesis are the range of substitution rates (lower and upper 95% HPD values).

^b 95% HPD (highest posterior density) values.

^c TMRCA (Time of the most recent common ancestor), in years. Lower and upper 95% HPD values are indicated in the parenthesis.

3.3.8 Analyses of genetic distance and the natural selection within P0 and P3 genes of the *Turnip yellows virus* (TuYV) isolates

The overall mean genetic distances within and between the nucleotide sequence datasets for P0 and P3 genes were determined using Maximum Likelihood model (Tamura *et al.*, 2004) with gamma rate of variation among sites (shape parameter $r = 6.0$). The mean genetic distance within the TuYV isolates was higher for the P0 gene (0.034 ± 0.004) than for the P3 gene (0.02 ± 0.003) (Table 3.10).

The Maximum Likelihood method via the HyPhy package (Pond *et al.*, 2005), detected 77 codon positions in the P0 gene and 100 codon positions in the P3 gene which have undergone significant positive selection ($P < 0.05$) (Table 3.10). This provided strong evidence of heterogeneous selection pressures among codon sites in P0 and P3 genes. We also compared the overall selection intensity in the P0 and P3 genes. The results showed that the selection intensity was higher in the P0 gene than in the P3 gene (mean pairwise d_N/d_S were 0.479 and 0.299 for the P0 and P3 genes, respectively) (Table 3.10). However, overall, the values of the d_N/d_S ratio were low, i.e. $d_N/d_S < 1$, implying that both P0 and P3 genes were under negative selection.

Table 3.10 Mean pairwise genetic distance and the selective pressures within P0 and P3 genes of the *Turnip yellows virus* (TuYV) isolates

Gene	Mean genetic distance ^a	d _N	d _S	d _N /d _S (ω)	Total number of codons	Codon positions under positive selection ^b
P0 ^c	0.034 ± 0.004	0.5654	1.18	0.479	233	77
P3	0.02 ± 0.003	0.2688	0.8989	0.299	202	100

^a Mean ± standard error. Standard error was estimated by a bootstrap procedure of 1000 replicates. The overall genetic distance within and between P0 and P3 genes nucleotide sequences datasets were estimated using the Maximum likelihood model (Tamura *et al.*, 2004) with gamma rate of variation among sites (shape parameter $r = 6.0$).

^b Codons that have undergone positive selection ($P < 0.05$), rejecting hypothesis of neutral evolution. Maximum Likelihood analysis of natural selection codon-by-codon method was via HyPhy package (Sergei *et al.*, 2005) implemented in MEGA5 (Tamura *et al.*, 2011).

^c Only 699 out of 750 nucleotides of the P0 gene were analysed.

3.3.9 Neutrality tests

The results for the various neutrality tests are summarised in Table 3.11. Apart from Tajima's D test which was not significant ($P > 0.05$), the other tests (Fu and Li's D* and Fu and Li's F* tests) detected significant neutrality deviation ($P < 0.02$) for the TuYV populations.

Table 3.11 Neutrality test for ORF0 and ORF3 of *Turnip yellows virus* (TuYV)

Gene	Tajima's D	P-value	Fu and Li's D*	P-value	Fu and Li's F*	P-value
P0	-1.2236	> 0.05 ^b	-4.4580	< 0.02 ^a	-3.5371	< 0.02 ^a
P3	-1.5549	>0.05 ^b	-6.4395	<0.02 ^a	-4.8603	<0.02 ^a

^b $P > 0.05$ not significant

^a $P < 0.05$, significant at $P < 0.05$

3.3.10 Analyses of genetic structure of *Turnip yellows virus* (TuYV) sub-populations

As ORF0 region was the most variable compared to the ORF3, it was selected to further analyse both geographical (spatial) and temporal patterns of genetic differentiation among the TuYV populations using five test statistics, Snn, Hs, Ks, Z and chi-square (Hudson *et al.*, 1992; Hudson, 2000). All five test statistics showed significant differentiation between the TuYV populations in the three regions, Lincolnshire, Warwickshire and Yorkshire ($P < 0.01$) (Table 3.12).

Gene flow estimates gave low values of *Fst* and Nst statistics which indicated frequent movement of the TuYV populations between Lincolnshire, Warwickshire and Yorkshire ($Fst = 0.15952$; $Nst = 0.15939$) (Table 3.12). *Fst* values from Hudson *et al.* (1992) and Nst values from Lynch and Crease (1990) range from 0.0 for no differentiation to 1.0 for complete differentiation. When an absolute value of *Fst* is less than 0.33, it indicates frequent gene flow.

Table 3.12 Genetic differentiation and gene flow between *Turnip yellows virus* (TuYV) populations in Lincolnshire, Yorkshire and Warwickshire (Spatial genetic structure of TuYV populations) based on ORF0 nucleotide sequence dataset

Genetic differentiation			Gene flow	
Test statistics	Estimates	<i>P</i> -value ^a	Test statistics	Estimates
Snn ^b	0.48232	0.0000***	Nst ^d	0.15939
χ^2 (df = 552)	633.815	0.0089**	<i>Fst</i> ^e	0.15952
Hs ^c	0.99384	0.0040**		
Ks ^c	2.83464	0.0000***		
Z ^c	9.15447	0.0000***		

^a Probability obtained by permutation test with 1000 replicates.

*** Significant at $P < 0.001$, ** Significant at $P < 0.01$

χ^2 = Chi-square test (Workman and Niswander, 1970)

^b Snn = Nearest neighbour statistics (Hudson, 2000)

^c Hs, Ks, and Z = Sequence based statistics of Hudson *et al.* (1992) for detecting genetic differentiation of sub-populations.

^d Nst = Gene flow statistic (Lynch and Crease, 1990)

^c *Fst* = Gene flow statistic (Hudson *et al.*, 1992)

Pairwise comparisons of the genetic distances using the *Fst* and *Nst* statistics showed that the most frequent movement of TuYV populations occurred between Lincolnshire and Warwickshire (*Fst* = -0.00342; *Nst* = -0.00367), followed by the gene flow between Lincolnshire and Yorkshire populations (*Fst* = 0.00049; *Nst* = 0.00068). Movement of TuYV populations between Warwickshire and Yorkshire were the least frequent (*Fst* = 0.01310, *Nst* = 0.01327), indicating population substructuring (Table 3.13).

Table 3.13 Gene flow between *Turnip yellows virus* (TuYV) populations in Lincolnshire, Yorkshire and Warwickshire based on ORF0 nucleotide sequence dataset

Population 1	Population 2	<i>Fst</i>	<i>Nst</i>
Lincolnshire	Warwickshire	-0.00342	-0.00367
Lincolnshire	Yorkshire	0.00049	0.00068
Warwickshire	Yorkshire	0.01310	0.01327

Nst = Gene flow statistic (Lynch and Crease, 1990)

Fst = Gene flow statistic (Hudson *et al.*, 1992).

Similar to the spatial structure, all five test statistics (*Snn*, χ^2 , *Hs*, *Ks* and *Z*) indicated significant differentiation of TuYV populations over the three crop seasons, 2007-8, 2008-9 and 2009-10 ($P < 0.01$) (Table 3.14). Gene flow estimates indicated that there was frequent movement of TuYV populations between the three crop seasons (*Fst* = 0.15661, *Nst* = 0.15633) (Table 3.14).

Table 3.14 Genetic differentiation between *Turnip yellows virus* (TuYV) populations in the 2007-8, 2008-9 and 2009-10 crop seasons (Temporal genetic structure) based on ORF0 nucleotide sequence dataset.

Genetic differentiation			Gene flow	
Test statistics	Estimates	<i>P</i> -value ^a	Statistic	Estimates
Snn ^b	0.4863	0.0000 ***	Nst ^d	0.15633
χ^2 (df = 555)	642.748	0.0058 **	<i>Fst</i> ^e	0.15661
Hs ^c	0.99245	0.0000 ***		
Ks ^c	22.44878	0.0000 ***		
Z ^c	9.14666	0.0000 ***		

^a Probability obtained by permutation test with 1000 replicates.

*** Significant at $P < 0.001$, ** Significant at $P < 0.01$,

χ^2 = Chi-square test (Workman and Niswander, 1970)

^b Snn = Nearest neighbour statistics (Hudson, 2000)

^c Hs, Ks, and Z = Sequence based statistics of Hudson *et al.* (1992) for detecting genetic differentiation of sub-populations.

^d Nst = Gene flow statistic (Lynch and Crease, 1990)

^e *Fst* = Gene flow statistic (Hudson *et al.*, 1992)

Pairwise comparisons of genetic distances of TuYV populations between the crop seasons using the *Fst* and Nst statistics showed that the most frequent movement of population occurred between 2008-9 and 2009-10 crop seasons ($Fst = 0.0002$; Nst = -0.00017). This was followed by 2007-8 and 2009-10 populations ($Fst = 0.01209$; Nst = 0.01270) whilst 2007-8 and 2008-9 populations showed less frequent movement between them ($Fst = 0.03523$; Nst = 0.03607) (Table 3.15).

Table 3.15 Gene flow between *Turnip yellows virus* (TuYV) populations in the 2007-8, 2008-9 and 2009-10 crop seasons based on the ORF0 nucleotide sequence dataset.

Population 1	Population 2	<i>Fst</i>	<i>Nst</i>
2007-8	2008-9	0.03523	0.03607
2007-8	2009-10	0.01209	0.01270
2008-9	2009-10	0.0002	-0.00017

Fst = Gene flow statistic (Hudson *et al.*, 1992).

Nst = Gene flow statistic (Lynch and Crease, 1990)

3.3.11 Analysis of molecular variation (AMOVA)

AMOVA was performed to evaluate the contribution of various factors to the genetic differentiation of the TuYV populations. The total variation observed among the P0 gene nucleotide sequences was partitioned: (i) between all isolates from all the three regions (Lincolnshire, Warwickshire and Yorkshire) (ii) between isolates within the three regions and (iii) isolates within individual fields (Table 3.16). The contribution of the region to the total variation was not significant (-1.23% genetic variation, *Fst* = -0.0123, *P* = 0.999). The variation contributed by the TuYV isolates within regions was only 4.23% (*Fst* = 0.0417, *P* < 0.001), whilst the largest contribution to the variance (97.01%) was within TuYV isolates within individual fields (*Fst* = 0.03, *P* < 0.000).

AMOVA was also performed to determine the contribution of crop seasons, TuYV isolates (populations) within crop seasons and isolates within individual fields to the total variation observed in the TuYV populations. All the three factors contributed significantly to the total variance observed among the sequences (Table 3.16). TuYV isolates within fields contributed the highest to the total variance (96.6% of total variation, *Fst* = 0.0342, *P* < 0.001), followed by the isolates (populations) within

crop seasons (2.91% genetic variation, $F_{st} = 0.0293$, $P < 0.001$) whilst the variation in the isolates between the three crop seasons contributed the least (0.51% of total variation, $F_{st} = 0.0509$, $P = 0.0039$).

Table 3.16 AMOVA of *Turnip yellows virus* (TuYV) populations at different geographical regions and crop seasons (based on ORF0 nucleotide sequence dataset)

Source of variation	d. f. ^a	Variance component	% variation	Fixation indices (F -statistics)	P -value
a. Spatial analysis					
Between regions	2	-0.1346	-1.23	-0.0123	0.999 ns
Between isolates within regions	6	0.4624	4.23	0.0417	0.000 ***
Between isolates within fields	215	10.6141	97.01	0.03	0.000 ***
b. Temporal analysis					
Between crop seasons	2	0.05594	0.51	0.0051	0.004 **
Between isolates within crop season	6	0.32002	2.91	0.0293	0.000 ***
Between isolates within fields	215	10.6141	96.6	0.0342	0.000 ***

ns = not significant ($P > 0.05$); **Significant at $P < 0.01$; ***Significant at $P < 0.001$

^aDegree of freedom

3.4 Discussion

3.4.1 Genetic diversity in TuYV

Genetic variability of TuYV populations infecting oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire in England was analysed using the sequences encoding P0 and P3 of the viral genome. The results revealed that the TuYV isolates can be divided into two (for P3 gene) or three (for P0 gene) genetic groups (evolutionary divergent lineages) irrespective of the geographical origin or year of sampling. Previous studies showed variation in the TuYV isolates from oilseed rape and other brassica plant samples collected from England, France and Germany (de Miranda *et al.*, 1995; Schubert *et al.*, 1998; Hauser *et al.*, 2000a).

Clade 1 of the CP gene phylogenetic analysis consisted of the majority of isolates sequenced from oilseed rape and the previously published isolates from oilseed rape in England, i.e. L39968, L39969 and L39970 (de Miranda *et al.*, 1995) (see Figures 3.6 and 3.8). Clade 2 two consisted of 35 isolates sequenced from oilseed rape and previously published isolates from different brassica crops, i.e. L39971 (oilseed rape, France), L39974 (sprouts, England), L39975 (cauliflower, England), and L39986 (calabrese, England) (de Miranda *et al.*, 1995).

In the case of the P0 gene phylogenetic analysis (see Figures 3.3 and 3.7), clade 1 contained the majority of the isolates sequenced from oilseed rape (86.3%) and the published isolate BWYV-Col (AF168600, from oilseed rape, France). Clade 2 contained only seven of the isolates sequenced from oilseed rape (3.1%) and published isolate BWYV-Fev (AF168601, from field bean, France) (Hauser *et al.*, 2000a) and were all found to be recombinant strains, whilst genetic group three

contains 13.7% of the isolates sequenced from oilseed rape and two published isolates, viz TuYV-BN5 (AF168606, from oilseed rape, Germany) and TuYV-GB (AF168608, from oilseed rape, England) (Hauser *et al.*, 2000a). The English oilseed rape isolates sequenced did not cluster with TuYV-FL (X13063, from lettuce, France) (Veidt *et al.*, 1988), indicating a distance evolutionary relationship. This study has therefore clearly demonstrated diversity in TuYV isolates infecting oilseed rape in the three regions and P0 is more diverse than P3. This is in agreement with Hauser *et al.*, (2000a) who reported that P0 gene of poleroviruses is more diverse than the coat protein gene.

The nucleotide diversity (average number of nucleotide substitutions per site in each pair of sequence variants) was higher in the P0 gene than P3 gene of TuYV. This could be due to the greater number of mutations in the P0 gene than the P3 gene (Table 3.6a). It has been reported that mutation is the initial source of variation in populations (Drake *et al.*, 1998). Mutation and recombination are the two main types of errors that occur during replication of RNA viruses resulting in a high degree of variability (Domingo and Holland, 1997; Garcia-Arenal *et al.*, 2003) and these may account for the high sequence variants or haplotypes observed. This is due to the high error rates of RdRp (Garcia-Arenal *et al.*, 2003).

Despite the greater number of mutations and the subsequent high number of haplotypes recorded for the coat protein and P0 gene of the TuYV, the genetic diversity was low (0.03310 ± 0.0020 for the P0 gene and 0.0210 ± 0.0149 for the P3 gene), suggesting genetic homogeneity. This agrees with Garcia-Arenal *et al.* (2003) who proposed that in spite of high potential for genetic variation, populations of

plant viruses are not highly variable; and high mutation rates are not necessarily adaptive, as a fraction of the mutations are deleterious. It has been reported that analysed populations of plant viruses are genetically stable, and this is so regardless of the many haplotypes that may occur in the population (Garcia-Arenal *et al.*, 2003). Twenty-two out of 29 virus species listed by Garcia-Arenal *et al.* (2001) are reported to have genetic diversities of below 0.10. The high mutation rate in RNA viruses is due to the need for rapid replication of their chemically unstable RNA genome rather than being an evolutionary strategy (Drake and Holland, 1999). The relaxed polymerase fidelity in RNA viruses provides a source of sequence diversity that can allow virus quasispecies to form, enabling the virus to adapt successfully to changing environments (Barr and Fearn, 2010). On the contrary, Garcia-Arenal *et al.* (2001; 2003) have reported that the high mutation rates for RNA viruses may reflect an evolutionary strategy. The observation that both P0 and P3 genes of the isolates sequenced in this study, were under negative selection further lends support to the non-adaptive nature of mutations which occurred within them (Table 3.10). The negative selection occurring at the TuYV genome, which is necessary for maintaining the functional, encoded protein (as in P3 and P0 of TuYV) might have played a role in eliminating the deleterious variants. The P0 gene of TuYV plays an important role in RNA-silencing suppression (Pfeffer *et al.*, 2002) and mutation in this region is expected to seriously affect virus fitness, which in turn constrain genetic diversity and hence impact on $d_N:d_S$ ratio estimates (Holmes, 2003; Simmonds and Smith, 1999). Selection pressures associated with the maintenance of functional structures have also been documented for the CP of tobamoviruses (Garcia-Arenal *et al.*, 2001). The observed higher variability in P0 gene which was about twice that of CP gene could at least in part, be attributed to the higher negative

selection in the CP (lower d_N/d_S ratio) than the P0 gene (higher d_N/d_S). Genetic bottlenecks during vector transmission might also be an important factor for limiting the genetic variation in the TuYV population and result in founding populations that can lead to genetic drift (Li and Roossinck, 2004). Chare and Holmes (2006) have also shown that vector-borne RNA viruses are subject to greater selective constraints than those viruses transmitted by other routes, and hence, despite their high mutation rates, fitness trade-offs are commonplace in RNA virus evolution.

3.4.2 Recombination and evolution of *Turnip yellows virus*

Recombination can have a significant effect in driving evolution of virus population (Garcia-Arenal *et al.*, 2003) and generating genome diversity (Gibbs, 1995). The results of this study suggest that recombination may play a significant role in driving evolution of TuYV populations. The TuYV recombinant genotypes (genotype 2) were detected in oilseed rape crops in all three regions, and seemed to have resulted from genetic exchange between two distinct genotypes (genotypes 1 and 3, corresponding to clades 1 and 3, see Figure 3.4).

The evolution of *Luteoviridae* are characterised by relatively frequent intra- and interspecific recombination (Gibbs, 1995; Gibbs and Cooper, 1995; Stevens *et al.*, 2005) with recombination breakpoints common at gene boundaries but less within genes (Pagan and Holmes 2010). In this work, recombination breakpoints were detected mostly in the P0 gene (8 sites) and only one in P3 gene. This is a strong indication that P0 and P3 genes have different evolutionary histories. The significant phylogenetic incongruence in the P0 and P3 genes of TuYV observed in this study lends support to the idea that recombination may play a role in the evolution of the

virus. The detection of only one recombination break point within the coat protein gene could be due to the strong selection pressure acting on the gene. According to Pagan and Holmes (2010), recombination breakpoints that occur within some genes including such as the coat protein are strongly injurious such that they are rapidly purged by purifying selection.

The mean rate of nucleotide substitution among all isolates of TuYV ranged from 9.4×10^{-4} to 9.6×10^{-4} subs/site/year and 1.8×10^{-3} to 2.1×10^{-3} subs/site/year for P0 and P3 genes respectively. These were comparable to substitution rates previously estimated for TuYV by Pagan and Holmes (2010) which ranged from 1×10^{-4} to 1×10^{-3} subs/site/year and also comparable to some other plant RNA viruses (Gibbs *et al.*, 2010; Roossinck and Ali, 2007) and those observed in animal RNA viruses (Duffy *et al.*, 2008; Jenkins *et al.*, 2002). The coat protein is highly conserved in *Poleroviruses* including TuYV (Hauser *et al.*, 2000a) which suggests strong functional constraints (Gray and Gildow, 2003), and as such it was expected to have a lower rate of evolution than the P0 gene, as it was reported by Pagan and Holmes (2010) for TuYV and other species of the family *Luteoviridae*. But on the contrary, higher rates of evolution were observed for the coat protein gene than that of the P0 gene of the TuYV genome (Table 3.9); this could be a reflection of the heterogeneous selection pressures among codon sites in the coat protein gene where 100 out of 202 codons were under positive selection (see Table 3.10). These higher rates could be due to the fact that the isolates were sampled over very short time (2007-2009). It has been reported that sequences sampled over very short time periods tend to produce artificially inflated rates estimates, which reflects short-term mutation rates that include the circulation of transient deleterious mutation (i.e. polymorphisms),

rather than more meaningful long-term rates of nucleotide substitution that measure evolutionary dynamics following the action of purifying selection (Duffy *et al.*, 2008). Pagan and Holmes (2010) also observed very high substitution rate for the coat protein gene of *Cereal aphid-borne yellow virus* (CABYV) which was sampled over five years, and therefore concluded that the time scale of sampling has a major impact on the reliability of substitution rate estimates. It is therefore important to point out here that, since this study covered only three regions in England over only three years, the estimates of evolutionary rates, TMRCA and the number of distinct phylogenetic groups may be different if the study was extended to further regions of the UK, Europe and / or the rest of the world.

The higher TMRCA estimated for the P0 gene compared to that of P3 gene, suggested that the P3 gene is of more recent origin having diverged from its ancestor at around 9 to 25 years ago compared to the P0 gene whose time of divergence ranged between 50 years and 120 years ago. The TMRCA estimates for the P0 gene was comparable to a range of 26 years and 86 years previously estimated for P0 gene of TuYV and TMRCA estimates for other Poleroviruses such as BMYV and BChV (Pagan *et al.*, 2010). This is a clear indication that P0 and P3 genes are of different evolutionary history. The genus *Polerovirus* is believed to have evolved from a recombination event between a sobemovirus and an ancestor that provides the 3' properties (Gibbs, 1995; Mayo and Ziegler-Graff, 1996). Differences in the TMRCA between the P0 and P3 genes were also reflected in the topological incongruence in their phylogenetic trees. The TMRCA estimated for P0 gene and to some extent the coat protein gene using the constant and exponential population growth models, were similar, indicating no significant differences in the evolutionary models employed in

the coalescent analyses. This therefore suggests that any of the two demographic models (constant size and exponential growth) favourably fits the analysed nucleotide data.

3.4.3 Mixed genotype infection

There are several reports of high frequencies of mixed genotypes infections in many host-pathogen interactions (Hodgson *et al.*, 2001; Hodgson *et al.*, 2004; Read and Taylor, 2001; Schurch and Roy, 2004). On the contrary a limited number of mixed genotype infections were observed; 5, 3 and 2 in Lincolnshire, Warwickshire and Yorkshire respectively, out of 226 isolates sequenced (see Figures 3.3 and 3.5; Table 3.1). Mixed genotypes infections are important to virus evolution because they provide the precondition for recombination, which may contribute to the appearance of more severe virus strains (Ribeiro *et al.*, 2003). Hall and Little (2007) reported that despite their evolutionary importance, empirical studies of virus genetic variants in mixed infections are quite rare due, in part, to the lack of an effective method for quantifying the population size of closely related virus genotypes in a single host. Mutation and recombination events at the ORF0 and ORF3 regions of the TuYV genome may be associated with the observed mixed genotype infections in this study as has been reported for mixed genotypes infections of *Mycosphaerella graminicola* on wheat (Schurch and Roy, 2004).

3.4.4 *Turnip yellows virus* population structure

The significant neutrality deviation observed from the neutrality tests was an indication of population substructuring. All the neutrality tests gave negative values

(Table 3.10), suggesting that all the TuYV populations were in a state of active evolution.

The existence of significant population structure in the TuYV populations in oilseed rape at different regions and different crop seasons in England ($P < 0.01$), was an indication of limited gene flow between them. Most of the haplotypes (166 out of 174) showed a regionally distributed pattern with only few (8) among the three regions. This finding may suggest local / regional infections of TuYV in oilseed rape crops, which suggests that there is limited amount of spread between regions. However, estimates of gene flow parameters indicated very low values, e.g. $F_{st} < 0.033$ (Tables 3.11 and 3.13) indicating movement of TuYV populations between different regions and different crop seasons. This may explain the reason why the TuYV isolates did not cluster according to geographical region or year of sampling, and all the three genetic groups identified occurred in all the three geographical regions (Lincolnshire, Warwickshire and Yorkshire) (see Figures 3.4 and 3.6). This phenomenon of gene flow might be related to the characteristics of TuYV transmission and its wide host range. TuYV is transmitted by aphids, mainly *M. persicae* in the U.K. in a persistent manner, and can retain the virus for a long time once it is acquired (Gray, 1999; Gray and Gildow, 2003; Schliephake *et al.*, 2000; Stevens *et al.*, 2005). The aphids are therefore able to transmit the virus into a new host plant during their flight activity. Depending on time and altitude of flight, and the prevailing winds, long-flying aphids may originate from sources between 100 and 300 km away (Riley *et al.*, 1995). It has been reported that as the aphids migrate from their source in order to avoid overcrowding or deteriorating habitat (Watt and Dixon, 1981), or to locate primary or secondary host (Moran and Whitham, 1990),

the dispersive morphs i.e. the winged aphids act as a bridge between one habitat or seasonal cycle to the next (Castle *et al.*, 2008). This may account for the observed movement of TuYV populations (gene flow) between regions and between crop seasons as observed in this study. The differences in gene flow among TuYV populations in the three regions could at least in part be attributable to the spatial distribution during migration of the *M. persicae* vector, which according to Taylor (1975) is dependent on the wind.

The relatively higher haplotype frequency observed in Lincolnshire compared to Warwickshire and Yorkshire could be due to infections of the oilseed rape crops in the region with TuYV isolates transmitted from several host plants by the *M. persicae* vector. Lincolnshire is a heart of vegetable production in England, and the several brassica crops in the region could serve as alternate host of TuYV. Variation in the TuYV isolates from different host plants has been documented (de Miranda *et al.*, 1995; Schubert *et al.*, 1998; Hauser *et al.*, 2000a).

The AMOVA analysis showed that the most important component in the observed variability in TuYV populations is due to the variation within populations in the individual fields. This accounted for 97% and 96.6% of the total variance observed in the spatial and temporal analyses respectively. This may explain why populations at different regions were highly differentiated, with a limited gene flow between them, resulting in a fewer number of haplotypes shared between the regions. Of the 174 haplotypes identified for P0 gene sequences, 166 haplotypes were restricted to specific geographical regions (i.e. they were not shared) (Table 3.6). This may also be true for TuYV populations at different crop seasons. For plant RNA viruses,

predominantly negative selection, population bottlenecks during movement and transmission, and population differentiation during plant growth and development have been shown to be responsible for maintaining population diversity (Li and Roosnick, 2004).

CHAPTER 4:

ISOLATING AND DIFFERENTIATING GENOTYPES OF *TURNIP YELLOW*S VIRUS (TuYV) INFECTING OILSEED RAPE IN ENGLAND USING GROUP SPECIFIC PRIMERS AND POLYMERASE CHAIN REACTION

4.1 Introduction

Turnip yellows virus (TuYV; genus *Polerovirus*, family *Luteoviridae*) (D'Arcy and Domier, 2005) is the most important virus infecting oilseed rape in the UK (Walsh, 1986; Stevens *et al.*, 2008). Molecular studies into the genetic variation and structure of TuYV infecting oilseed rape in England based on the P0 gene of the virus, led to the identification of three distinct clades /genetic groups (chapter three, section 3.3.2). Clade 1 was the most prevalent among them (86.3% of isolates sequenced), closely related to the published isolate BWYV-Col (AF168600) found in oilseed rape in France (92.9 - 99.1% nucleotide identities). This was followed by clade 3 (13.7%), closely related to published isolates TuYV-GB (AF168608) obtained from oilseed rape in England and TuYV-BN5 (AF168606) obtained from oilseed rape in Germany (92.1 - 99.4% nucleotide identities). Clade 2 isolates, which were all of recombinants origin, formed just 3.1% of field isolates analysed, very close to published isolate BWYV-Fev (AF168601) found in field bean from France (Hauser *et al.*, 2000a) (93.9 - 97.1% nucleotide identities).

It is however not known, which of the three genetic groups is more virulent or destructive to oilseed rape. There is the need for rapid and reliable method for

routine detection and discrimination of the different genotypes of TuYV circulating in the oilseed rape crops in England. In addition to its application for diagnostic purposes, genotype- specific detection would be very useful for epidemiological studies of TuYV in oilseed rape, and for studies evaluating resistance of oilseed rape to TuYV infection.

There have been numerous studies on the use of PCR-based methods for detecting and/or discriminating member species of the genus *Polemovirus* infecting oilseed rape or beet (Jones *et al.*, 1991; de Miranda *et al.*, 1995; Schubert *et al.*, 1998; Hauser *et al.*, 2000b). However, there was no information of the discrimination of TuYV isolates infecting oilseed rape in the UK or any other country. Methods based on PCR are often used for the detection of members of the family *Luteoviridae* because they are claimed to be more sensitive and accurate than serological methods (Balaji *et al.*, 2003; D'Arcy *et al.*, 1989), which frequently fail to detect infection, due to the low concentration of the members of *Luteoviridae* in plants and can be non-specific due to the common cross-reactivity of antisera (Chomic *et al.*, 2010a)

The primary objective of the work described in this chapter was to develop an RT-PCR assay with genotype-specific primers to discriminate the three genetic groups of TuYV isolates identified in the oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire (see chapter three). The genotype specific-primers were designed from the P0 gene nucleotide sequences from which the phylogenetic analysis revealed the three genetic groups.

4.2 Materials and Methods

4.2.1 Field survey for *Turnip yellows virus* (TuYV) isolates

Using a line transect sampling method (Buckland *et al.*, 2001), the leaves of 48 plants were sampled from three crop fields in each of two oilseed rape-growing regions of England, i.e. Lincolnshire (Eastern) and Warwickshire (Midlands) in the winter period of the 2010-11 crop season. The Lincolnshire fields were near Long Sutton and the Warwickshire fields were near Gaydon. The locations of the fields were determined using a Global Positioning System (Garmin E-Trex GPS Receiver, Garmin Corporation Olathe, KS, USA) as follows: 52.10°N 1.27°W, 52.11°N 1.27°W, and 52.10°N 1.26°W for fields 1, 2, and 3 sampled in Warwickshire; whereas the longitudes/latitudes of the three fields in Lincolnshire were 52.48°N 0.14°E, 52.47°N 0.14°E, and 52.47°N 0.13°E respectively.

The sampling procedure involved estimating the length and breadth of each field in order to divide the field into six equally-spaced transects with eight equally-spaced-samples collected per transect. The fields were exceptionally large so only a proportion of the fields were sampled.

4.2.2 Detection of *Turnip yellows virus* in the field samples using ELISA

The presence of TuYV in the leaf samples were tested by standard triple antibody sandwiched enzyme-linked immunosorbent assay (TAS-ELISA) as described in chapter two (section 2.2.2), with some modifications as follows. The primary antibody used was rabbit IgG (AS-0049), secondary antiserum was the mouse monoclonal antibody (AS-0049/1), and the tertiary alkaline phosphatase conjugated antiserum was rabbit anti-mouse (RAM-AP), all from DSMZ (Braunschweig,

Germany). Portions of each leaf sample tested were stored at both -5°C and -80°C for further studies.

The cumulative numbers of *M. persicae* caught between August (crop sowing) and November from 2007 to 2010 in the Rothamsted insect survey suction traps closest to the sampling sites (Kirton in Lincolnshire, and Wellesbourne in Warwickshire) were determined from the weekly suction trap aphid catches.

4.2.3 RNA extraction

Total plant RNA was prepared by using RNeasy[®] Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of the purified RNA was determined using a NanoDrop[®] ND-1000 spectrophotometer (Thermo Scientific), and then stored at -80°C until needed.

4.2.4 Primer design

A slight modification to the method used by Gutierrez-Aguirre *et al.* (2009) was used to design three sets of primers specific to the three genotypes identified from chapter 3. TuYV P0 gene sequences from isolates representing the three different genotypes (i.e. genotypes 1, 2 and 3) and some published sequences from NCBI database were analysed. Multiple sequence alignment was performed using the ClustalW software (Thompson *et al.*, 1994) implemented in MEGA 5 software (Tamura *et al.*, 2011). Forward and reverse primers were designed in sequence areas which differed most between the different genotypes, within the P0 gene with the help of PrimerSelect algorithm of DNASTAR Lasergene 8 software (Burland, 2000). These regions were conserved among isolates of the same genotype within the P0 gene (Figure 4.1). The possible occurrence of homo- and heterodimer formation was

assessed for each primer pair by estimating the thermodynamic parameters using DNASTAR Lasergene 8 software (Burland, 2000). Information about the primers is presented in Table 4.1 and Figure 4.1.

Table 4.1 P0 gene primers used in the RT-PCR assay to discriminate three distinct genotypes of *Turnip yellows virus* (TuYV).

Primer	Sequence (5' - 3')	Orientation	Position in the sequence	Genotype specificity
AB23	AAAGTCAGATACCTCCACCCAAAG	Forward	46 - 69	Genotype 1
AB17 ^a	AAAGTCAGGTACCTCCAYTCRCG	Forward	46 - 68	Genotypes 2 and 3
AB24 ^b	CTTTAGTCCATGTTGATAGAA	Reverse	446 - 466	Genotypes 1 and 2
AB18	TCTCTAGTCCAGATAGACAGAAAT	Reverse	444 - 467	Genotype 3

The expected amplicon size for the primer pairs AB23-AB24 and AB17-AB24 is 420 bp and that of AB17-AB18 is 421 bp.

^a AB17 is the forward primer for both genotypes 2 and 3.

^b AB24 is the reverse primer for both genotypes 1 and 2.

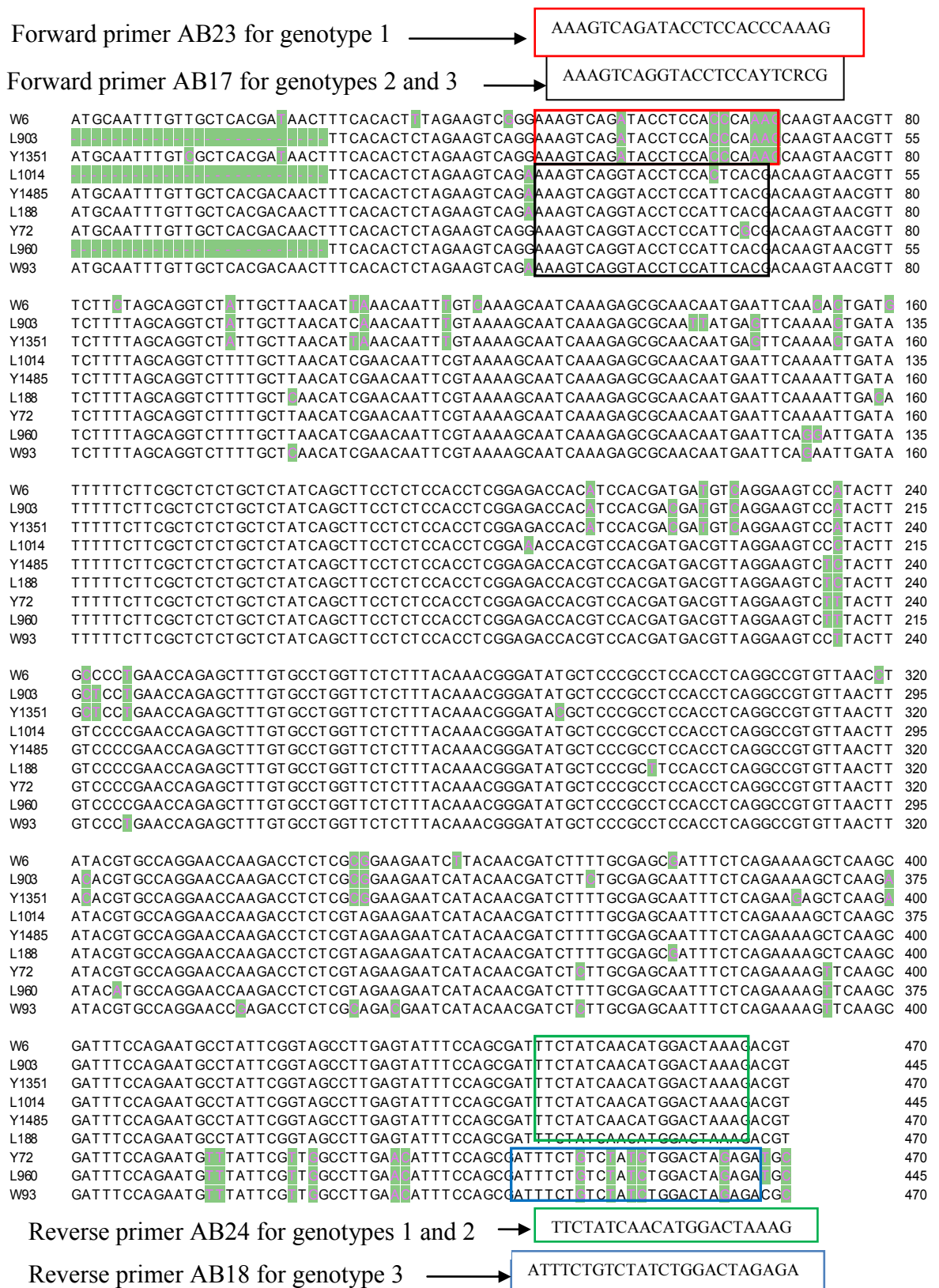


Figure 4.1 Design of the RT-PCR assay of the P0 gene of *Turnip yellow virus* (TuYV).

The alignment shows part of the P0 gene nucleotide sequences of selected field isolates of TuYV representing the three different genotypes. Primer sequences for

each amplicon are highlighted in the boxes. The forward primer for genotype 1 is depicted in the red box; black box for both genotypes 2 and 3. The reverse primer for both genotypes 1 and 2 is depicted in the green box, and the reverse primer for genotype 3 is depicted in the blue box.

4.2.5 Optimisation of RT-PCR conditions for the genotype-specific primers

cDNA was synthesized from purified RNA samples of known genotypes (isolate Y1351 for genotype 1; Y1485 for genotype 2; LAB for genotype 3) by a slight modification of the method described in chapter three (section 3.2.3), using reverse primers AB18 for genotype 2 and AB24 for genotypes 1 and 2.

5 μ L the cDNA was added to 45 μ L PCR reaction mixture containing 5 μ L 10x PCR buffer (Invitrogen), 1.5 mM magnesium chloride, 0.2 mM dNTP, 2 units of Taq-DNA Polymerase (Invitrogen), 10 pmol of each of specific primers AB23-AB24, AB17-AB24 and AB17-AB18 for genotype 1, 2 and 3 respectively. The PCR reaction mixture was incubated in a pre-warmed thermal cycler under the following conditions: one cycle for 5min at 95°C; 30 cycles at 95°C for 30s (denaturation), 65°C for 1 min (annealing) and 72°C for 1 min (extension) and one cycle at 72°C for 10 min. The RT-PCR products (15 μ L) were separated by electrophoresis in 1.5% agarose gel stained with GelRed (Biotium Inc.) in 1.0xTBE buffer and visualised on a UV-transilluminator. A 1 Kb plus DNA ladder (Invitrogen) was included to determine the size of amplified products.

4.2.6 Detecting and differentiating *Turnip yellows virus* (TuYV) isolates with different genotypes from field samples.

Using the optimised RT-PCR conditions determined in section 4.2.5 above, primer pairs AB23-AB24, AB17-AB24 and AB17-AB18 were used for the identification of

TuYV genotypes 1, 2 and 3 respectively from the field samples collected. Twenty four field isolates were initially selected for this analysis following the TAS-ELISA carried out in section 4.2.2. Samples with very low virus concentrations as determined by the ELISA were not selected for the RT-PCR assay. RNA from a healthy oilseed rape plant (cv Mikado) was used as negative control. RNA from leaf samples infected with TuYV of known genotypes (used in the primer optimisation, section 4.2.5) was used as positive controls for the three different specific genotypes. All three primer pairs were used to amplify each cDNA sample.

4.2.7 Transmission of the *Turnip yellows virus* (TuYV) isolates with different genotypes to oilseed rape plants.

Non-viruliferous *Myzus persicae* Mp1S clone were starved for one and half hours and allowed to feed overnight on the leaf samples infected with TuYV of known genotypes. Leaf pieces with the aphids feeding on were then transferred onto oilseed rape plants (cv Mikado) in an insectary under 16 h photoperiod at $20 \pm 2^{\circ}\text{C}$. Three weeks after challenging the Mikado oilseed rape plants, an ELISA was performed to test for TuYV infection. Isolates of the TuYV genotypes were maintained in oilseed rape plants through serial transfer onto healthy plants using *M. persicae*, until needed for further studies.

4.2.8 Verification of the genotypes of *Turnip yellows virus* (TuYV) isolates from field samples.

To ensure that the TuYV genotypes isolated from field oilseed plants were pure (i.e. single genotype) RT-PCR assays using the genotype specific primers (see Table 4.1) was performed using the method described in section 4.2.6. For verification of the

identity of these isolates maintained in Mikado plants, the RT-PCR products were sequenced as described in chapter 3 (section 3.2.6) using the genotype specific primers.

4.2.9 Sequence comparisons of P0 gene of the *Turnip yellows virus* (TuYV) isolates maintained in oilseed rape cv. Mikado

In order to compare the nucleotide and amino acid sequences of the P0 gene of the TuYV isolates obtained from field plants, the isolates belonging to the two genotypes were amplified using the generic primers (AB1-AB3) which produced a product for the whole 750 nt P0 gene. The methods used in chapter 3 (sections 3.2.3 to 3.2.6) were employed, but the RT-PCR products were directly sequenced without cloning.

DNASTAR Lasergene software (Burland, 2000) was used to visualise the chromatogram, evaluate the quality of each nucleotide in the sequence, detect and evaluate nucleotide changes and construct a consensus for each amplicon. Both the non-coding and primer sequences were discarded from the alignments. Final editing of the nucleotide sequences was done using BioEdit v7.0.5 (Hall, 2005). Additional sequences published by Veidt *et al.* (1988) and Hauser *et al.* (2000a) were retrieved from GenBank, checked and added to the data set. Multiple alignments of the sequences were done using the ClustalW programme (Thompson *et al.*, 1994) implemented in MEGA version 5.2 (Tamura *et al.*, 2011). Alignments were also adjusted manually to ensure correct reading frames. After editing, 726 nt out of 750 nt of the P0 gene sequence alignment was analysed for sequence comparisons and phylogenetic studies.

4.2.10 Sequence comparisons and phylogenetic analyses

Nucleotide and the deduced amino acid sequence identities were determined using BioEdit v7.0.5 (Hall, 2005). A Maximum likelihood tree was constructed using the Hasegawa-Kishino-Yano (HKY) model with a discrete Gamma distribution, with 5 rate categories, using MEGA 5 software (Tamura *et al.*, 2011). The HKY model was identified using MEGA 5 as the one that best fitted the nucleotide sequence data. Statistical validity of the clusters obtained was verified after 1000 trials of bootstrap analysis.

4.3 Results

4.3.1. Incidence of *Turnip yellows virus* (TuYV) in oilseed rape

No TuYV was detected in any of the three oilseed rape fields in Warwickshire, whereas the virus was detected in all three fields in Lincolnshire (Table 4.2). The highest TuYV incidence was recorded for field 1 (27.1%), followed by field 3 (20.8%) whilst field 2 had the lowest incidence (16.7%).

Table 4.2 The incidence of *Turnip yellows virus* (TuYV) in oilseed rape crops in Warwickshire and Lincolnshire during the winter of the 2010-11 crop season.

Location		Percent TuYV incidence (%)
County	Field	
Warwickshire	1	0
	2	0
	3	0
Lincolnshire	1	27.1 (13)*
	2	16.7 (8)
	3	20.8 (10)

*Value in the parenthesis is the actual number of infected plants out of a total of 48 plants sampled per field.

4.3.2 Cumulative numbers of *Myzus persicae* caught in the Rothamsted insect survey suction traps

Table 4.3 shows the cumulative August-November *M. persicae* numbers caught in the Rothamsted insect survey suction traps in Lincolnshire (Kirton) and Warwickshire (Wellesbourne) between 2007 and 2010. In each year, Lincolnshire had higher cumulative number of *M. persicae* than Warwickshire. The highest *M. persicae* flight activities occurred in 2009, where Lincolnshire had cumulative

number of 476 and Warwickshire 45. This was followed by 2007 (253 in Lincolnshire and 43 in Warwickshire), and 2010 (122 in Lincolnshire and 38 in Warwickshire) whilst lowest cumulative *M. persicae* numbers occurred in 2008 (17 in Lincolnshire and 6 in Warwickshire).

Table 4.3 Cumulative August to November *Myzus persicae* numbers caught in the Rothamsted Insect Survey suction traps in Lincolnshire (Kirton) and Warwickshire (Wellesbourne) from 2007 to 2010.

Year	August to November cumulative <i>Myzus persicae</i> numbers	
	Lincolnshire	Warwickshire
2007	253	43
2008	17	6
2009	476	45
2010	122	38

Source: Aphis News, Rothamsted Insect Survey, Rothamsted Research, UK

4.3.3 RT-PCR assay design

The aim of this study was to isolate TuYV isolates of the three different genetic groups (genotypes) identified in chapter 3. In order to do this it was necessary to design a set of RT-PCR assays capable of specific detection of all three TuYV genetic groups identified in oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire in chapter 3. At annealing temperature of 65°C and magnesium chloride concentration of 1.5 mM, the primers AB23-AB24 and AB17-AB24 produced strong bands of 420 bp each with genetic groups 1 and 2 respectively whilst primers AB17-AB18 produced a strong band of 421 bp with genetic group 3 (Figure 4.2).

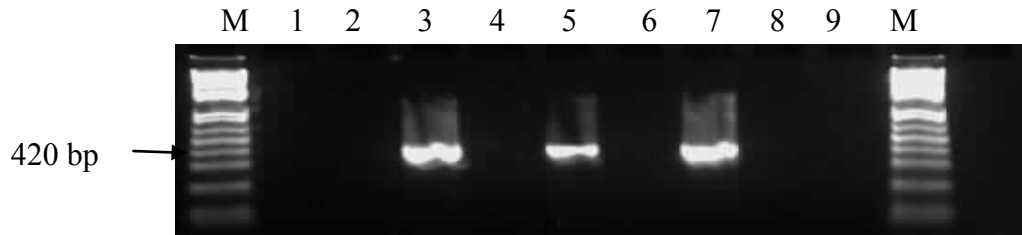


Figure 4.2 RT-PCR amplification products (420 bp and 421 bp) using *Turnip yellow virus* (TuYV) genotype specific primers AB23-AB24, AB17-AB24 and AB17-AB18.

Lanes M correspond to 1kb plus DNA ladder; lanes 1, 4, 7 correspond to sample LAB isolate (genotype 3); lanes 2, 5, 8 correspond to sample Y1485 isolate (genotype 2); and lanes 3, 6, 9 correspond to sample Y1351 isolate (genotype 1). Lanes 1 – 3 correspond to AB23-AB24 assay; lanes 4 – 6 correspond to AB17-AB24 assay and lanes 7 – 9 correspond to AB17-AB18 assay.

4.3.4 Genotype discrimination of different *Turnip yellow virus* (TuYV) isolates

As shown in Table 4.4, the primers detected the corresponding specific genotypes efficiently. The AB23-AB24 assay which was designed to detect only genotype 1, detected isolates Y1351 (positive control), and TuYV in samples L1808, L1843, L1851, L1875, L1876, L1890, L1937 and L1944. The AB17-AB18 primers detected LAB isolate (positive control) and TuYV in sample L1906. The AB17-AB24 primers designed to detect genotype 2 isolate, detected only Y1485 (positive control) but none of the field isolates. Samples L1802, L1803, L1805, L1809, L1815, L1824, L1830, L1834, L1850, L1903, and L1910 (forming 45.8% of the test samples) were detected by both AB23-AB24 and AB17-AB18 primer pairs, indicating they were mixtures of genotypes 1 and 3. Samples L1811, L1904, L1907 and L1920 were not detected by any of the three primer pairs.

Table 4.4 Detection of *Turnip yellows virus* (TuYV) isolates with different genotypes in oilseed rape using AB23-AB24, AB17-AB24 and AB17-AB18 assays.

Plant number	RT-PCR assay			Genotype identified
	AB23-AB24 (genotype 1)	AB17-AB24 (genotype 2)	AB17-AB18 (genotype 3)	
L1802	+ ^a	- ^b	+	Mixed (1 and 3)
L1803	+	-	+	Mixed (1 and 3)
L1805	+	-	+	Mixed (1 and 3)
L1808	+	-	-	Genotype 1
L1809	+	-	+	Mixed (1 and 3)
L1811	-	-	-	Undetermined ^c
L1815	+	-	+	Mixed (1 and 3)
L1824	+	-	+	Mixed (1 and 3)
L1830	+	-	+	Mixed (1 and 3)
L1834	+	-	+	Mixed (1 and 3)
L1843	+	-	-	Genotype 1
L1850	+	-	+	Mixed (1 and 3)
L1851	+	-	-	Genotype 1
L1875	+	-	-	Genotype 1
L1876	+	-	-	Genotype 1
L1890	+	-	-	Genotype 1
L1903	+	-	+	Mixed (1 and 3)
L1904	-	-	-	undetermined
L1906	-	-	+	Genotype 3
L1907	-	-	-	undetermined
L1910	+	-	+	Mixed (1 and 3)
L1920	-	-	-	undetermined
L1937	+	-	-	Genotype 1
L1944	+	-	-	Genotype 1
LAB (control)	-	-	+	Genotype 3
Y1351 (control)	+	-	-	Genotype 1
Y1485 (control)	-	+	-	Genotype 2
Negative control ^d	-	-	-	None

^a + Positive, ^b - negative, ^c RT-PCR was negative with all the three primer pairs.

^d Healthy oilseed rape cv Mikado was used as negative control.

4.3.5 *Turnip yellows virus* (TuYV) isolates propagation in oilseed rape plants

Five isolates of genotype 1 (L1808, L1843, L1851, L1876 and L1937) and the one isolate of genotype 3 (L1906) were selected for propagation using *M. persicae* in oilseed rape (cv. Mikado) plants in an insectary room under a 16-h day at 20 ± 2°C. Three weeks after transfer of *M. persicae* to recipient oilseed rape plants, ELISA showed that all of the oilseed rape plants were infected with TuYV, indicating that

the selected TuYV isolates had been successfully transmitted to the oilseed rape plants (Figure 4.3). Isolate L1851 recorded the highest ELISA absorbance of 1.61, whilst isolate L1876 had the lowest absorbance 0.42.

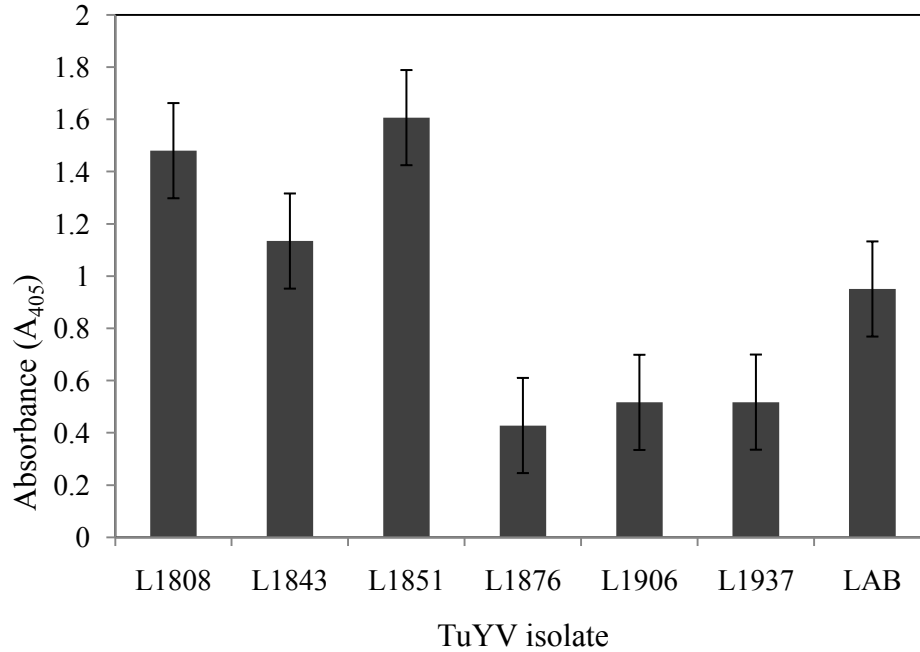


Figure 4.3 ELISA absorbance values of oilseed rape plants (Mikado) infected with six isolates of *Turnip yellows virus* (TuYV) and LAB control. The absorbance (A_{405}) for uninfected control was 0.18. The error bar represents the standard error of the mean.

4.3.6 Genetic purity of *Turnip yellows virus* (TuYV) obtained from field plants in Lincolnshire.

In order to ensure that the field isolates propagated in Mikado plants were single genotypes, RT-PCR was performed using the genotype-specific primers and total plant RNA extracted from the Mikado plants infected with the isolates. The AB17-AB18 primer pairs produced 421 bp RT-PCR products with only isolates Y1259 (positive control), LAB and L1906 but not with the other isolates and the negative control (Figures 4.4a). The AB23-AB24 primer pairs produced 420 bp RT-PCR products with isolates Y1351 (positive control), L1808, L1843, L1851, L1876 and

L1937 but none with isolates Y1259, LAB and L1906 or the negative control (Figure 4.4a). The AB17-AB24 primer pairs only produced 420 bp RT-PCR product with isolate Y1485 (positive control) and none with the other isolates and the negative control (Figure 4.4b). The identity of each isolate was confirmed by sequencing.

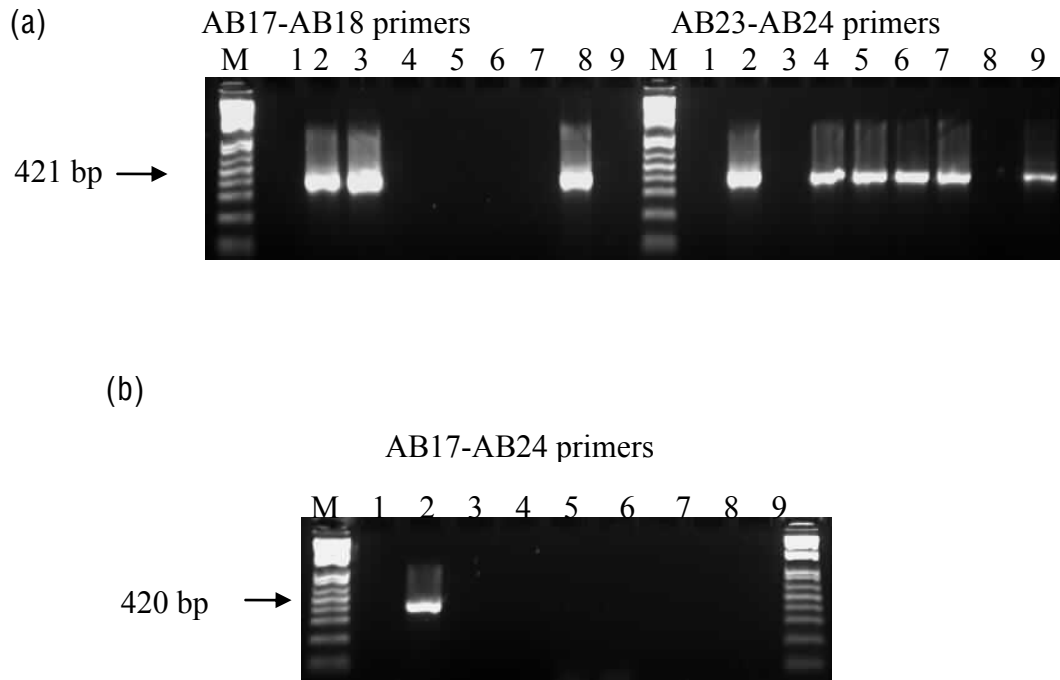


Figure 4.4 RT-PCR amplification products using *Turnip yellows virus* (TuYV) genotype-specific P0 gene primers: (a) AB17-AB18 (421 bp amplicon) and AB23-AB24 (420 bp amplicon) and (b) AB17-AB24 primers (420 bp amplicon). For each assay, lane M, 1 kb+ ladder; 1, sterilised distilled water (negative control); 2, positive control; 3, LAB; 4, L1808; 5, L1843; 6, L1851; 7, L1876; 8, L1937; 9, L1906. The target gene was the 750 bp P0 gene of the TuYV. Positive control for AB17-AB18 primer pair was isolate Y1259 (a genetic group 3 isolate); isolate Y1351 for AB23-AB24 primer pair, and isolate Y1485 for AB17-AB24 primer. Apart from the control (Y1485), no products were expected with AB17-AB24 primer pair.

4.3.7 Phylogenetic analysis of *Turnip yellows virus* (TuYV) isolates

Phylogenetic analysis revealed that the isolates were clustered into two main clades (genetic groups) (Figure 4.5). Clade 1 consisted of the isolates of genotype 1 (L1808, L1843, L1851, L1876, L1937) and clustered with the previously published isolate AF168600 (the isolate BWYV-Col from France) (Hauser *et al.*, 2000a) sharing

nucleotide and amino acid identities ranging from 96.6 - 99.5% and 94.6 - 99.5% respectively with each other (Table 4.5). The genotype 3 isolates (L1906 and LAB) clustered with the previously published sequence of AF168608 (the isolate TuYV-GB from England) (Hauser *et al.*, 2000a) in clade 2 and shared nucleotide and amino acid sequence identities ranging between 97.9% - 98.6% and 97.1% - 97.9% respectively with each other (Table 4.5).

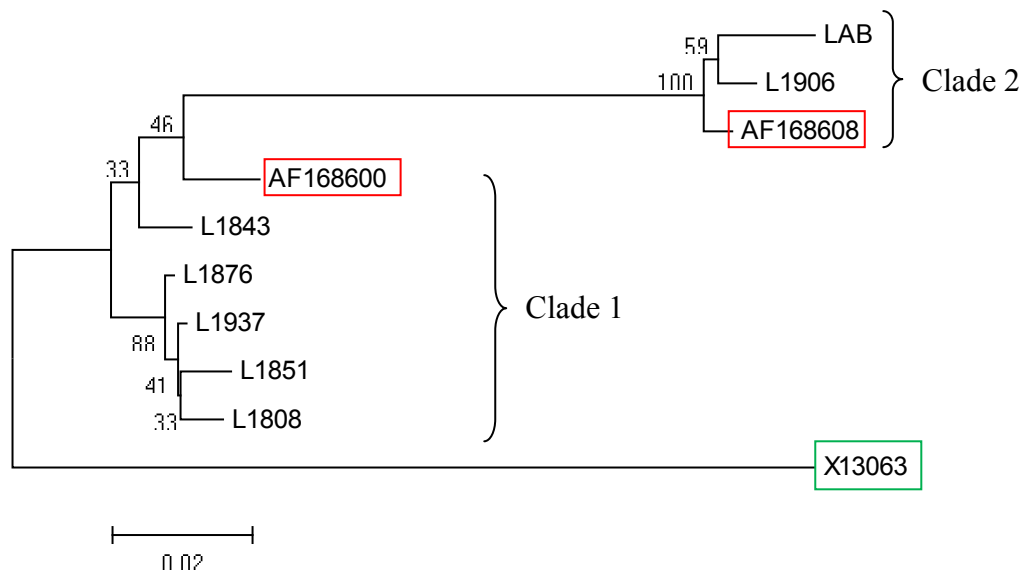


Figure 4.5 Maximum likelihood tree of nucleotide sequences of 726 bp of the P0 gene of *Turnip yellows virus* (TuYV) isolates showing two genetic groups. The scale bar signifies a genetic distance of 0.02 nucleotide substitutions per site. AF168600 and AF168608 depicted in red boxes are the GenBank accession numbers for isolates BWYV-Col and TuYV-GB published by Hauser *et al.* (2000a). X13063 in the green box is the GenBank accession number for isolate TuYV-FL published by Veidt *et al.* (1988) and it served as an out-group.

Table 4.5 Nucleotide and amino acid sequence identities of *Turnip yellows virus* (TuYV) isolates based on 726 bp fragment of the P0 gene.

		<i>Amino acid sequence identity</i>									
Isolate		1	2	3	4	5	6	7	8	9	10
1	L1808	-	95.4	99.1	99.5	99.1	95.8	88.0	88.0	88.0	84.7
2	L1843	97.2	-	95.4	95.8	96.2	94.6	89.6	88.4	89.2	83.8
3	L1851	98.6	97.5	-	99.5	99.1	95.8	88.8	88.0	88.0	83.8
4	L1876	99.0	97.9	99.0	-	99.5	96.2	88.4	88.4	88.4	84.2
5	L1937	99.1	98.0	99.1	99.5	-	96.6	88.8	88.0	88.0	84.7
6	AF168600	96.9	97.2	96.6	97.3	97.5	-	90.0	88.8	90.0	84.2
7	L1906	92.5	93.3	92.4	92.8	92.6	93.3	-	97.1	97.5	78.5
8	LAB	92.2	92.6	91.8	92.5	92.1	92.6	98.2	-	97.9	77.2
9	AF168608	92.8	93.2	92.1	93.1	92.6	93.9	98.6	97.9	-	78.5
10	X13063	88.0	88.0	87.4	88.0	88.1	83.7	84.7	83.7	84.9	-

Nucleotide sequence identity

The values in the lower diagonal of the table are the percent nucleotide sequence identities whilst the values at the upper diagonal are the corresponding deduced amino acid sequence identities. AF168600 and AF168608 are GenBank accession numbers of TuYV for isolates BWYV-Col and TuYV-GB respectively (Hauser *et al.*, 2000a). X13063 is the GenBank accession number for isolate TuYV-FL published by Veidt *et al.* (1988)

4.3.8 Amino acid sequence alignment

An alignment of the predicted amino acid sequences of the TuYV isolates propagated in oilseed rape plants was produced using DNASTAR Lasergene software (Burland, 2000). The alignment clearly showed the differences in amino acid sequences between clade 1 (L1808, L1843, L1851, L1876, L1937) and clade 2 (L1906, LAB) isolates. The sequences differ at several amino acid residues (35 amino acid residues) distributed throughout the P0 gene fragment. Of the 35 variable

amino acid sites, clade 1 (genotype 1) isolates differ from clade 2 (genotype 3) isolates at 20 amino acid sites (Figure 4.6).

L1808	MQFVAHDNFHTLEVRKVRYLHPKQVTFLLAGLLLNIKQFVKAIKERNNEFKTDVFLRSLLYQLPLHLGDH	70
L1843	MQFVAHDNFHTLEVRKVRYLHPKQVTFLLAGLLLNIKQFVKAIKERNNEFKTDVFLRSLLYQLPLHLGDH	70
L1851	MQFVAHDNFHTLEVRKVRYLHPKQVTFLLAGLLLNIKQFVKAIKERNNEFKTDVFLRSLLYQLPLHLGDH	70
L1876	MQFVAHDNFHTLEVRKVRYLHPKQVTFLLAGLLLNIKQFVKAIKERNNEFKTDVFLRSLLYQLPLHLGDH	70
L1937	MQFVAHDNFHTLEVRKVRYLHPKQVTFLLAGLLLNIKQFVKAIKERNNEFKTDVFLRSLLYQLPLHLGDH	70
LAB	MQFVAHDNFHTLEVRKVRYLHSSRQVTFLLAGLLLNIEQFVKAIKERNNEFKTDVFLRSLLYQLPLHLGDH	70
L1906	MQFVAHDNFHTLEVRKVRYLHSSRQVTFLLAGLLLNIEQFVKAIKERNNEFKTDVFLRSLLYQLPLHLGDH	70
L1808	IHDDVRKSI LAPEPELCAWFSLQTGYAPASTSGRVNLHVPYGTKTSRGRILQRSFASDFSEKLRKFPPECLF	140
L1843	IHDDVRKSI LAPEPELCAWFSLQTGYAPASTSGRVNLHVPYGTKTSRGRILQRSFASDFSEKLRKFPPECLF	140
L1851	IHDDVRKSI LAPEPELCAWFSLQTGYAPASTSGRVNLHVPYGTKTSRGRILQRSFASDFSEKLRKFPPECLF	140
L1876	IHDDVRKSI LAPEPELCAWFSLQTGYAPASTSGRVNLHVPYGTKTSRGRILQRSFASDFSEKLRKFPPECLF	140
L1937	IHDDVRKSI LAPEPELCAWFSLQTGYAPASTSGRVNLHVPYGTKTSRGRILQRSFASDFSEKLRKFPPECLF	140
LAB	VHDDVRKSLVPEPELCAWFSLQTGYAPASTSGRVNLHVPYGTKTSRGRIVQRSASNFSEKLRKFPPECLF	140
L1906	VHDDVRKSLVPEPELCAWFSLQTGYAPASTSGRVNLHVPYGTKTSRGRIVQRSASNFSEKLRKFPPECLF	140
L1808	GSL EYFQRFLSTWTKDVERRIFSRCREIPLGSDTLVELANLGELLRVMVVGGEQFHNSRLLSRLAVHCYKI	210
L1843	GSL EYFQRFLSTWTKDVERRIFSRCREIPLGSDTLVELANLGELLRVMVVGGEQFHNSRLLSRLAVHCYKI	210
L1851	GSL EYFQRFLSTWTKDVERRIFSRCREIPLGSDTLVELANLGELLRVMVVGGEQFHNSRLLSRLAVHCYKI	210
L1876	GSL EYFQRFLSTWTKDVERRIFSRCREIPLGSDTLVELANLGELLRVMVVGGEQFHNSRLLSRLAVHCYKI	210
L1937	GSL EYFQRFLSTWTKDVERRIFSRCREIPLGSDTLVELANLGELLRVMVVGGEQFHNSRLLSRLAVHCYKI	210
LAB	VGF EYFQRFLSIFWTRDAERRIFSRCREIPVGSHTLVELANLGELLRVMVVGGEQFHNSRLLSRLAVHCYKI	210
L1906	VGF EYFQRFLSIFWTRDAERRIFSRCREIPVGSHTLVELANLGELLRVMVVGGEQFHNSRLLSRLAVHCYKI	210
L1808	YGEDGFI SFWRIANLDHFDCFLTPEEILFSSS	242
L1843	YGEDGFI SFWRIANLDHFDCFLTPEEILFSSS	242
L1851	YGEDGFI SFWRIANLDHFDCFLTPEEILFSSS	242
L1876	YGEDGFI SFWRIANLDHFDCFLTPEEILFSSS	242
L1937	YGEDGFI SFWRIANLDHFDCFLTPEEILFSSS	242
LAB	YGEDGFI SFWRIANLDYLDHFDCFLTPEEILFSSS	242
L1906	YGEDGFI SFWRIANLDYLDHFDCFLTPEEILFSSS	242

Figure 4.6 Amino acid sequences of 726 nt P0 gene of *Turnip yellows virus* (TuYV) genotypes 1 and 3.

The shaded regions are the amino acid residues that differ from the consensus.

4.4 Discussion

4.4.1 Incidence of *Turnip yellows virus* in oilseed rape

ELISA of leaf samples collected from 2010-11 oilseed rape crops in Lincolnshire and Warwickshire have shown relatively low TuYV incidences ranging from 0 (recorded in Warwickshire) to 27.1% (in Lincolnshire). This however, is comparable to the TuYV incidences in oilseed rape crops in Warwickshire and Lincolnshire in the 2008-9 crop season which ranged between 0% and 24% but lower than the incidences of up to 100% recorded in the autumn of 2009-10 in oilseed rape crops in Lincolnshire and Warwickshire (refer to chapter 2). Varying incidences of TuYV infection in oilseed rape crops have also been reported in the UK, ranging from 0 to 100% (Hardwick *et al.*, 1994; Hill *et al.*, 1989; Jay *et al.*, 1999; Walsh *et al.*, 1989). The higher TuYV incidences recorded in the 2009-10 crop season compared to that of the 2008-9 crop season was attributed to the higher flight activity of the *M. persicae* vectors in the 2009-10 crop season than in the 2008-9 season (chapter 2). Autumn incidences of TuYV in the oilseed rape crops in Lincolnshire, Warwickshire and Yorkshire were closely associated with the cumulative numbers of *M. persicae* caught in the suction traps in these regions between August and November (chapter 2, Figure 2.4). It is therefore likely that the low incidence recorded in this study could be attributable to low flight activity of *M. persicae* vectors in the autumn of 2010 (Table 4.3).

4.4.2 Discrimination of *Turnip yellows virus* (TuYV) genotypes

RT-PCR assays involving three sets of primers that amplified a 420 bp or 421 bp of P0 region of TuYV were developed and tested for discrimination of different

genotypes of TuYV. The results clearly showed that the primer pairs were capable of detecting and discriminating TuYV genotypes found in oilseed rape crops in England in 2007-2009 (see Table 4.3). Genotype 1 isolates were found to be the most common isolates (8 out of 9 isolates excluding mixed genotypes; 19 out of 20 for all isolates) infecting oilseed rape in England whilst genotype 3 was far less common (1 out of 9, excluding the mixed genotypes; 12 out of 20 for all isolates). Genotype 2, which was a recombinant of genotypes 1 and 3, was not detected. This finding therefore supports the previous results on the phylogenetic analysis of TuYV infecting oilseed rape in England where genotype 1 was found to be the most prevalent TuYV isolate, followed by genotype 3, with genotype 2 far less (chapter three, Figure 3.2). The failure of the RT-PCR assays to detect genotype 2 was a further indication that it is a rare genotype of TuYV infecting oilseed rape crops in the regions surveyed.

PCR-based methods have been developed for detection and discrimination of members of the family *Luteoviridae* (Mayo and D'Arcy, 1999; Chomic *et al.*, 2010a, 2010b) but this is the first report of the use of RT-PCR to discriminate genotypes of TuYV infecting any crop. The RT-PCR assay developed had the additional advantage of detecting mixed genotype infections. Eleven out of 20 isolates analysed (55%) were of mixed genotypes, consisting of genotypes 1 and 3. These results agree with the previous studies (see chapter three, section 3.3.2) where mixed genotype infections were observed. Mixed genotype infections have been previously reported to be common in plant-pathogen systems (Hodgson *et al.*, 2004; Schurch and Roy, 2004). A surprisingly high proportion of mixed genotypes (55%) were detected using the genotype specific primers compared to about 4.6% observed in my earlier

work (chapter 3). There are several possible reasons for this discrepancy. The use of genotype-specific primers which amplified between 420 and 421 bp of the P0 gene could be more efficient in detecting the presence of mixed genotypes than the degenerate primers used in the previous work (i.e. chapter 3) which targeted the entire 750 bp of the P0 gene. Advances in techniques to distinguish pathogen genotypes have revealed high frequencies of mixed-genotype infections in many host-pathogen interactions (Hodgson *et al.*, 2004; Hodgson *et al.*, 2001; Read and Taylor, 2001). Co-infection and super-infection have been postulated as two main pathways of multiple infection (Miralles *et al.*, 2001; Saldana *et al.*, 2003). In co-infection, two or more genotypes invade the host simultaneously or within a short time interval. In super-infection, different genotypes infect the host at different times through the *M. persicae* vector. Genotype x environment interactions has been reported to promote genotypic diversity in plant viruses (van Molken and Stuefer, 2011). It could be possible that the temperatures in the autumn of 2010 might have favoured the co-existence of the two different isolates of different genotypes instead of single genotype. Classical kin-selection models predict that single-genotype infections can exploit host resources prudently to maximise fitness, but that selection favours rapid exploitation when co-infecting genotypes share limited resources (Hodgson *et al.*, 2004). According to Roossinck (2005), different viral genotypes can be in obligate symbiotic relationships with each other and can co-evolve, meaning that when one changes the other changes to adapt to the change of the first.

It is worth noting that the published isolate BWYV-Col (AF168600) obtained from oilseed rape in France (Hauser *et al.*, 2000a) belongs to genotype 1. In the earlier work (chapter three), published isolate TuYV-BN5 (AF168606), obtained from

oilseed rape in Germany (Hauser *et al.*, 2000a), was found to belong to genotype 3, whilst published isolate BWYV-Fev (AF168601), obtained from field bean in France (Hauser *et al.*, 2000a), belonged to genotype 2. This clearly demonstrates that the different genotypes exist in other countries and hence the RT-PCR assays developed have the potential to discriminate not only TuYV genotypes in oilseed rape in England, but also in other countries such as France and Germany and possibly in other host plants including field bean.

4.4.3 Propagation of *Turnip yellows virus* (TuYV) isolates belonging to different genotypes

Five field isolates of genotype 1 (L1808, L1843, L1851, L1876, L1937) and one of genotype 3 (L1906) have been successfully propagated in oilseed rape plants in the insectary. The results of the RT-PCR assay (Figure 4.5), the phylogenetic analysis of nucleotide sequences of 726 bp fragment of P0 gene (Figure 4.6) and the corresponding amino acid sequence alignment (Figure 4.7) all clearly confirmed the genotype identities of the isolates propagated. Amino acid identities between genotypes 1 and 3 ranged between 88% and 89.6% (see Table 4.5), indicating they belong to two distinct genetic groups, and possibly in different species based on 10% or more amino acid sequence difference criterion used for species discrimination of members of the family *Luteoviridae* proposed by D'Arcy and Domier (2005).

The seven TuYV isolates maintained in the oilseed rape plants may have different levels of within host accultation as suggested by the ELISA absorbance values (Figure 4.4). Although it has been usually assumed that virulence is a consequence of within-host replication of the parasite, viral strains may be highly virulent without

experiencing large accumulation as a consequence of immunopathological host responses (Lafforge *et al.*, 2011). Nevertheless, they are important pre-requisites for identifying resistance to TuYV in oilseed rape in England. Further research is needed to identify the genetic structure of TuYV populations in other regions of the U.K. and to propagate genotype 2 TuYV isolates. This is necessary in developing oilseed rape cultivar(s) with broad-spectrum resistance against TuYV. Knowledge of evolution of plant virus is important for the development of efficient and stable control strategies, as often, there is evolution of resistance breaking genotypes which renders the control measures ineffective (Garcia-Arenal *et al.*, 2001).

CHAPTER 5:

INTERACTIONS BETWEEN *BRASSICACEAE* AND *TURNIP YELLOWS VIRUS*

5.1 INTERACTIONS BETWEEN A *BRASSICA NAPUS* DIVERSITY FIXED FOUNDATION SET (DFFS) AND *TURNIP YELLOWS VIRUS* (TuYV)

5.1.1 Introduction

Turnip yellows virus (TuYV) has been reported to cause yield losses of up to 45% in oilseed rape crops in the UK (Impey, 2010; Stevens, 2010) and it is considered to be one of the reasons why oilseed rape does not achieve its full yield potential in the UK (Stevens *et al.*, 2008). Management of TuYV in oilseed rape has not been effective due to the wide host range of the virus and the resistance of *M. persicae* vectors to the insecticides approved for use in the UK (Stevens, 2010; Stevens *et al.*, 2008). Recently, there has been a high percentage of *M. persicae* carrying modified acetyl cholinesterase (MACE; resistance to pirimicarb) and knock down resistance (kdr; resistance to pyrethroids) in the UK (Collier, 2009; Insecticide Resistance Action Group-UK, 2008). Survey work carried out between 2007 and 2010 to assess the incidence of TuYV in oilseed rape crops in England (Lincolnshire, Warwickshire and Yorkshire), as part of this thesis, revealed that the seed treatments such as Chinook and Modesto which are supposed to offer better protection than the foliar spray against TuYV-carrying aphids are also not effective. High TuYV incidences of up to 100% in the oilseed rape crops planted with Chinook- or Modesto-treated seed were recorded (chapter 2). It has been reported that when up to

72% of *M. persicae* carry the TuYV it is difficult to prevent widespread primary infection of oilseed rape with the virus, regardless of seed treatment and / or foliar sprays used (Stevens *et al.*, 2008). This is clear evidence that controlling TuYV with either foliar sprays or seed treatments against the *M. persicae* vector is not effective. Better control options are therefore required.

The best option to prevent yield losses caused by virus infection in oilseed rape is the breeding of cultivars resistant to TuYV (Dreyer *et al.*, 2001). Breeding for resistance against TuYV may involve introgressing resistance genes into the current commercial oilseed cultivars in the UK. Resistance sources could potentially be identified by screening *B. napus* gene pool for resistance to TuYV infection. One gene pool source is the *B. napus* diversity fixed foundation set (DFFS) developed by the Defra funded Oilseed rape Genetic Improvement Network, OREGIN, UK (<http://www.oregin.info/>). DFFS was defined as “an informative set of genetically fixed lines representing a structured sampling of diversity across a genepool” (Teakle, 2009). Since the plant lines in the *B. napus* DFFS are mostly genetically homozygous (fixed) immortal lines (Teakle, 2009), any resistance trait identified will be true to type. Data obtained are cumulative, allowing long-term comparative analysis. In addition, fixed lines enable experimental trials to be established with replicate plants (King, 2011). As such, they may be interpreted to provide insights into contribution and interaction of genetic, environmental and developmental components of variation (King, 2011).

The aim of this study was to screen part of the OREGIN *B. napus* DFFS for resistance to TuYV.

5.1.2 Materials and methods

5.1.2.1 Plant materials

Variation in susceptibility of *B. napus* to TuYV infection was studied in 27 accessions of the OREGIN *B. napus* DFFS described in Table 5.1. The accessions were chosen to encompass parents of mapping populations, double haploid (DH) populations, different morphotypes and different geographical origins.

Table 5.1 Background information on *Brassica napus* DFFS accessions evaluated for susceptibility to *Turnip yellows virus* (TuYV).

Originating organisation's accession name	Crop type	Genetic status	Country of origin
Apex	Winter OSR	unspecified	Denmark
Bienvenu DH4	Winter OSR	DH	France
Capricorn DH1	Winter OSR	DH	Great Britain
Darmor- <i>bzh</i>	Winter OSR	Inbred	France
Major DH	Winter OSR	DH	France
Ningyou 7	Winter OSR	DH	China
Rafal DH1	Winter OSR	DH	France
Tapidor DH	Winter OSR	DH	France
Jet Neuf	Winter OSR	unspecified	France
Victor	Winter OSR	unspecified	Sweden
Stellar DH	Spring OSR	DH	Canada
Westar DH10	Spring OSR	DH	Great Britain
Yudal	Spring OSR	DH	Korea
Hanna	Spring OSR	unspecified	Sweden
Vige DH1	DH Swede	DH	Norway
Judzae	Swede landrace	unspecified	Korea
Bronowski DH1	Spring forage rape	DH1	Poland
Q100	synthetic	DH	---
Brauner Schnittkohl	Siberian kale	unspecified	Germany
Canard	Winter forage rape	unspecified	Great Britain
Couve Nabica	cauve nabica	unspecified	Portugal
Moana, Moana rape	Fodder rape	unspecified	New Zealand
Sarepta	Winter OSR	unspecified	France
Monty-028DH	Spring OSR	DH	Australia
Kavla Podzemna	Swede	unspecified	Yugoslavia
Rumena Maslena			
Sensation NZ	Swede	unspecified	New Zealand
Dwarf Essex	Forage rape	unspecified	Great Britain

5.1.2.2 Virus isolate

The LAB isolate of TuYV used in chapters 3 and 4 was used for this experiment. This isolate has been maintained in oilseed rape (cv. Mikado) in an insectary under 16 h photoperiod at $20 \pm 2^{\circ}\text{C}$ by serial transmission using *M. persicae*.

5.1.2.3 Plant cultivation

Forty-eight seeds of the *B. napus* DFFS accessions listed in Table 5.1 were sown directly in 16 FP7 pots in Levington's M2 peat compost (i.e. 3 seeds per pot) and grown in an insect-proof air-conditioned glasshouse at 18°C for the duration of the experiment. After germination, the seedlings were thinned out leaving one per pot.

5.1.2.4 Infection Procedure

Eight plants of each accession were challenged with TuYV using viruliferous *M. persicae* and the other eight mock-inoculated with non-viruliferous aphids when they were at 3 - 4 true leaf stage (3 weeks post sowing). Leaf pieces of oilseed rape with about 10 viruliferous aphids or non-viruliferous aphids (*M. persicae* Mp1s clone) were placed on each plant for an inoculation access period of 7 days. The Mp1s clone of *M. persicae* was used because it is susceptible to insecticides. The mock inoculation with non-viruliferous *M. persicae* was done to assess the direct feeding effect of the aphids on the TuYV-infected plants. Plants from the mock-inoculated pots were used as controls.

The plants were then sprayed with pirimicarb (Aphox: Syngenta) at 0.5 g/L followed by cypermethrin (Cleancrop Pyrimet: United AgriProducts) at 1 ml/L and then chlorpyrifos (Equity: Dow Agrosiences) at 1 ml/L to ensure that all the aphids were

killed. Both the TuYV-inoculated and mock-inoculated plants were in the same house but were covered separately immediately after inoculation with insect-proof lutrasil cages to prevent the viruliferous aphids from infesting the glasshouse and the mock-inoculated plants.

5.1.2.5 Experimental design and layout

There were eight plants of each *B. napus* accession for both TuYV-infected and uninfected (mock-inoculated). Alpha design (Patterson and Williams, 1976) was used to arrange the plants (432 experimental units) in a north-south direction on a bench in the glasshouse, with infected plants placed in the same orientation as uninfected plants. Each replicate was sub-blocked into 3 with 9 plants each (1 plant of each accession in each of 3 sub-blocks).

The plants were watered when necessary. A sulphur fungicide (Thiovit Jet: Novartis/Sandoz) at 2.0 g/L was sprayed to control powdery mildew infection in the glasshouse.

5.1.2.6 Data taken and analysis

The following data were recorded 6 weeks after challenging the plants with TuYV

Vegetative yield data

Fresh weight per plant

This was determined by weighing the plant harvested at soil level in a pan balance. After recording the total fresh weight, one leaf each was taken for ELISA serology. The remaining plant was re-weighed for total dry weight calculations.

Dry weight per plant

This was determined by drying the plants in an oven at 85°C for 4 days, allowing to cool at room temperature and then weighing using pan balance. Total dry weight per plant was then calculated using ratio of total fresh weight to the fresh weight minus one leaf.

Test for Turnip yellows virus infection (ELISA)

The relative amount of TuYV in each accession was determined using TAS-ELISA as described in chapter 2, with a few modifications. The measurement was done 6 weeks after challenging the plants with TuYV using *M. persicae*. The primary antibody used was rabbit IgG (AS-0049, DSMZ, Braunschweig, Germany), secondary antiserum was the rat monoclonal antibody MAFF 24 (Stevens *et al.*, 1995), and the tertiary alkaline phosphatase conjugated antiserum was goat anti-rat (A8438, Sigma-Aldrich Ltd., Poole, UK). Absorbance values (A_{405nm}) were measured with a Biochrom Anthos 2010 microplate reader (Biochrom Ltd., Cambridge, U.K.) as described in chapter two.

Statistical analysis

Using GenStat Release version 12.1 (Payne *et al.*, 2009), analysis of variance was carried out and the means separated using least significant differences (LSD). Correlation coefficients were calculated to determine the relationship between ELISA absorbance values (level of TuYV) and fresh weight and dry weight losses.

5.1.3 Results

5.1.3.1 The levels of *Turnip yellows virus* (TuYV) detected in plants

Results from the study of variation in the resistance reactions of 27 accessions of *B. napus* DFFS to TuYV infections are presented in Table 5.2. All the *B. napus* accessions were infected by TuYV. There were significant differences in the quantity of TuYV detected (ELISA absorbance values) in the *B. napus* accessions ($F_{26, 176} = 5.84$; $P < 0.01$). Rafal DH1 had the highest absorbance value of 1.985 ± 0.318 , indicating the most susceptible accession whilst Yudal had the lowest absorbance value of 0.485 ± 0.09 .

5.1.3.2 Dry weight of plants

Effects of TuYV on the mean dry weight of the *B. napus* accessions are shown in Table 5.2. Infected plants had lower mean dry weights than the mock-inoculated plants ($F_{1, 163} = 160.15$; $P \leq 0.001$). The ANOVA also showed a significant effect of accession on dry weights recorded ($F_{26, 163} = 17.87$; $P \leq 0.01$), indicating that the accessions differ in their average dry weights. The ANOVA further revealed a significant interaction between accession and infection status ($F_{26, 163} = 2.11$; $P \leq 0.01$), indicating that the effect of TuYV infection on dry weight varies between accessions. Significant reductions in mean dry weight due to TuYV infection occurred in 16 out of the 27 accessions evaluated (Table 5.2). Monty-028DH recorded the highest percentage dry weight loss (45.34%) whilst Dwarf Essex was not affected (0.54%).

Table 5.2 *Turnip yellows virus* (TuYV) infection on *Brassica napus* accessions, the quantity of virus detected and the effect on dry weight.

Accession	Absorbance ^a (A _{405nm})	Mean dry weight (g)		Reduction in dry weight (%)
		Uninfected	TuYV-infected	
Apex DH	1.654 ± 0.310 ^b	2.58	2.02* ^c	21.71
Bienvenu DH4	1.956 ± 0.322	2.45	1.94*	20.82
Brauner Schnittkohl	1.307 ± 0.198	3.58	2.54*	29.05
Bronowski DH1	1.533 ± 0.211	2.07	1.39*	32.85
Canard	1.632 ± 0.207	2.82	2.47	12.41
Capricorn DH1	1.599 ± 0.219	2.65	1.74*	34.34
Couve Nabica	1.145 ± 0.299	2.67	2.44	8.61
Darmor- <i>bzh</i>	1.264 ± 0.619	1.82	1.73	4.95
Dwarf Essex	1.414 ± 0.299	1.84	1.85	-0.54
Hanna	1.139 ± 0.308	2.54	2.08*	18.11
Jet Neuf	1.464 ± 0.226	2.72	2.36	13.24
Judzae	0.940 ± 0.295	2.24	1.51*	32.59
Kavla Podzemna	1.853 ± 0.232	2.44	2.01	17.62
Major DH	1.975 ± 0.224	2.74	1.92*	29.93
Moana	1.627 ± 0.240	2.11	1.33*	36.97
Monty-028DH	1.368 ± 0.278	1.56	0.85*	45.34
Ningyou 7	1.344 ± 0.244	2.75	2.51	8.73
Q100	1.779 ± 0.374	2.50	1.77*	29.2
Rafal DH1	1.985 ± 0.318	2.57	1.68*	34.63
Sarepta	0.812 ± 0.133	3.85	2.77*	28.05
Sensation NZ	1.706 ± 0.242	1.70	1.49	12.35
Stellar DH	1.719 ± 0.261	2.43	1.93*	20.58
Tapidor DH	1.530 ± 0.252	2.02	1.94	3.96
Victor	1.447 ± 0.238	3.19	2.51*	21.32
Vige DH1	1.668 ± 0.238	1.60	1.11	30.63
Westar DH10	1.663 ± 0.227	3.89	2.53*	34.96
Yudal	0.485 ± 0.090	2.58	2.31	10.47
Mean		2.52	1.95* ^d	

*Difference between dry weights of uninfected and TuYV-infected plants was significant ($P < 0.05$).

^a LSD for comparing mean absorbance values between accessions was 0.411 at d.f. = 163, $P < 0.05$.

^b Means ± standard error.

^c LSD for comparing uninfected and infected dry weights within accession was 0.456 at d.f. of 163, $P < 0.05$.

^d LSD for comparing uninfected and infected dry weights for the mean of all accessions was 0.088 at d.f. of 163, $P < 0.05$.

LSD for comparing average dry weights between accessions was 0.3454 at d.f. of 177, $P < 0.05$.

5.1.3.3. Fresh weight

The ANOVA for the effect of TuYV on mean fresh weight of the *B. napus* accessions indicated significant differences between accessions ($F_{26,163} = 12.66$; $P < 0.001$) and within infection status ($F_{1, 163} = 143.51$; $P < 0.001$). The ANOVA also showed a significant interaction between accession and infection status ($F_{26, 163} = 2.08$; $P = 0.003$), an indication that the effect of TuYV infection on fresh weight varies between the accessions. The TuYV infection resulted in a significant loss ($P < 0.05$) in fresh weight of 13 out of the 27 accessions (Table 5.3). Monty-028DH had the highest fresh weight loss (38.28%) whilst Dwarf Essex was unaffected (0.01%).

Table 5.3 Effect of *Turnip yellows virus* (TuYV) infection on fresh weight of *Brassica napus* accessions

Accession	Mean fresh weight (g)		Reduction in fresh weight (%)
	Mock-inoculated	TuYV-infected	
Apex DH	22.53	18.13* ^a	18.51
Bienvenu DH4	22.49	18.63*	17.16
Brauner Schnittkohl	30.04	21.50*	28.43
Bronowski DH1	17.28	13.68	20.83
Canard	23.18	20.59	11.17
Capricorn DH1	24.83	16.88*	32.02
Couve Nabica	23.24	21.37	8.05
Darmor- <i>bzh</i>	17.41	15.81	9.19
Dwarf Essex	17.17	17.28	-0.01
Hanna	21.84	16.69*	23.58
Jet Neuf	23.57	20.79	11.79
Judzae	22.06	17.30	21.58
Kavla Podzemna	20.71	18.03	12.94
Major DH	23.62	17.27*	26.88
Moana	20.30	14.17*	30.20
Monty-028DH	16.09	9.938	38.28
Ningyou 7	24.57	23.74	3.37
Q100	20.30	15.43*	23.99
Rafal DH1	23.41	16.64*	28.92
Sarepta	30.76	22.29*	27.54
Sensation NZ	17.22	16.16	6.16
Stellar DH	17.84	14.47	18.89
Tapidor DH	19.40	18.07	6.86
Victor	25.99	21.70*	16.51
Vige DH1	16.59	12.50*	24.65
Westar DH10	32.45	21.33*	34.27
Yudal	22.57	20.29	10.10
Mean	22.15	17.80* ^b	

* Difference between fresh weights of uninfected and TuYV-infected plants was significant at 5% probability level.

^a LSD for comparing uninfected and infected fresh weights within accession was 3.726 at d.f. of 163.

^b LSD for comparing uninfected and infected fresh weights for the mean of all accessions was 0.717 at d.f. of 163.

LSD for comparing mean fresh weights between cultivars was 2.794 at d.f. of 177.

5.1.3.4 Relationship between infection and fresh weights and dry weights

There was a positive but non-significant correlation between the ELISA absorbance values and dry weight losses ($r = 0.103$; d.f. = 25; $P > 0.05$) (Figure 5.1). There was also a positive and non-significant correlation between the mean absorbance values and mean fresh weight loss ($r = 0.037$; d.f. 25; $P > 0.05$) (Figure 5.2).

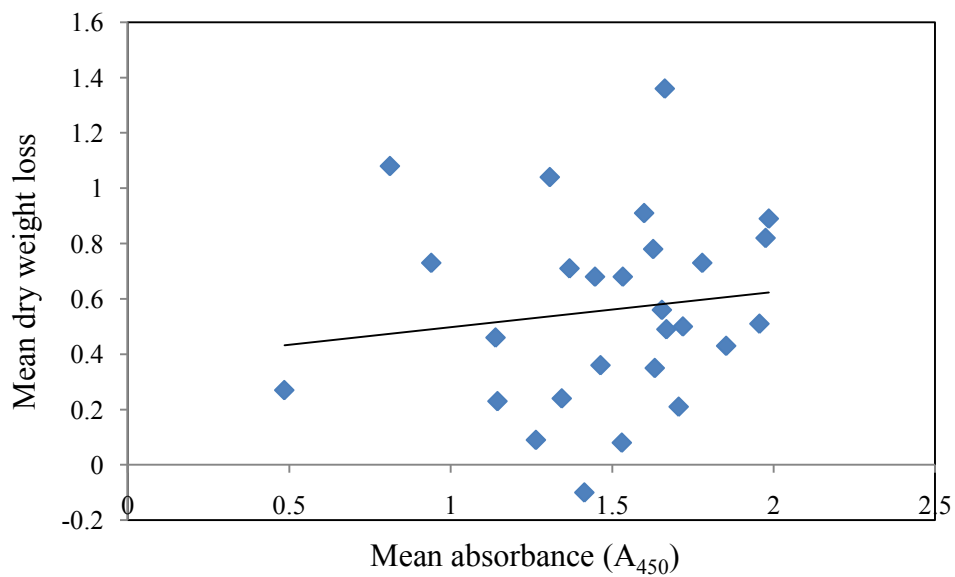


Figure 5.1 Correlation between mean ELISA absorbance values and mean dry weights of *B. napus* accessions. ($r = 0.103$; d.f. = 25; $P > 0.05$).

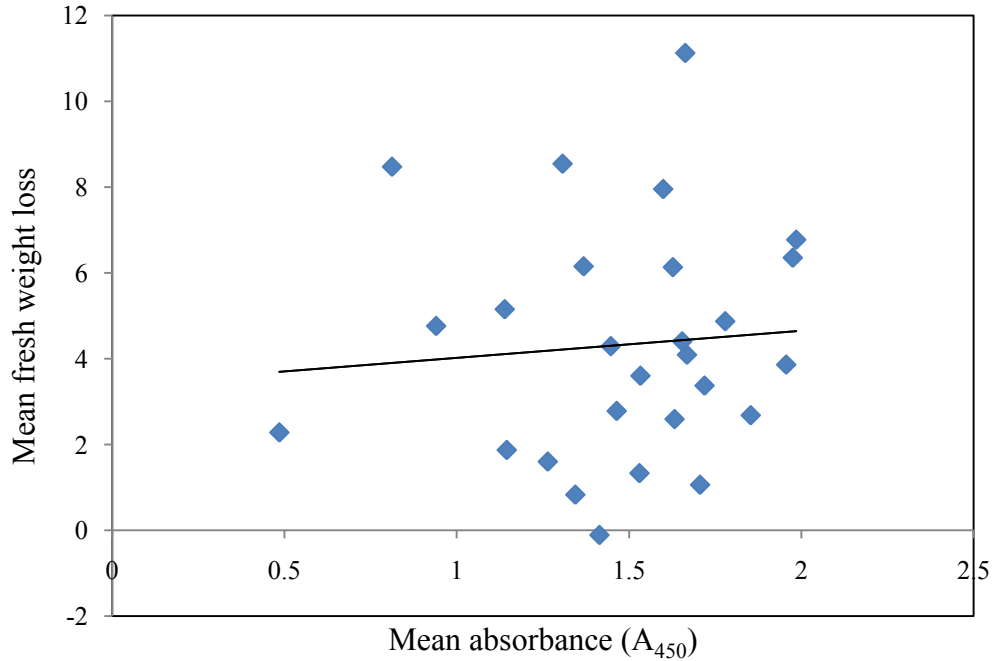


Figure 5.2 Correlation between mean absorbance values and mean fresh weights of *B. napus* accessions. ($r = 0.037$; d.f. = 25; $P > 0.05$).

5.1.4 Discussion

The reaction of the 27 accessions of *B. napus* to TuYV infection has revealed a range of variation in the TuYV accumulation of the accessions to the virus. This finding is comparable to that of Pagan *et al.* (2010) where there was genetic variation in the accumulation of virus in 20 *A. thaliana* genotypes infected with *Cauliflower mosaic virus* (CaMV) and *Cucumber mosaic virus* (CMV). They attributed this variation in virus accumulation to the interactions between the host (i.e. *A. thaliana*) and the virus (i.e. CaMV and CMV) genotypes. None of the accessions were found to be resistant to the TuYV infection in terms of the ELISA absorbance (virus titre values) measured. The accession Yudal however had the lowest virus accumulation among the accessions, indicating that it was less susceptible to the TuYV infection than the

rest. The results of this experiment were similar to those of Graichen and Peterka (1999) who found that all the 650 genotypes of both summer and winter oilseed rape tested were susceptible to TuYV. From a large number of resynthesised oilseed rape lines tested (Gland, 1980) only one line, R54 that had resistance to TuYV (Graichen, 1994). The TuYV resistance in R54 represented a quantitative reduction in virus titre (Juergens *et al.*, 2010), as found in other luteovirus – plant interactions such as BYDV and barley (Niks *et al.*, 2004).

TuYV infection resulted in a reduction in both dry weights and fresh weights of most of the accessions. This is an indication that TuYV infection caused reduction in growth of the *B. napus* accessions. TuYV has also been reported to cause a significant reduction in dry weights of leaves, stalks and racemes in winter oilseed rape at some sample dates (Jay *et al.*, 1999). According to Hull (2002) plants infected with virus will become stunted on a dry weight basis at least, by reducing the availability of the products of carbon fixation. Rafal DH1 which had the highest level of TuYV accumulation suffered significant dry weight and fresh weight losses whereas both fresh weight and dry weight losses recorded for Yudal, which had the lowest level of infection, was not significant. This may suggest that Yudal exhibited partial resistance / tolerance to the TuYV infection, whilst Rafal DH1 might be the most susceptible accession. A further evaluation of the partial resistance / tolerance in Yudal, including determination of the effect of the TuYV on growth and fitness yield (seed yield) of TuYV is needed to investigate this further.

Lack of significant association between ELISA absorbance and dry and fresh weight losses suggests that the levels of TuYV accumulated in the accessions did not have a

consistent effect on the growth of all accessions. This observation could be due to the genetic variation in susceptibility and / or tolerance of the accessions to the TuYV infection. This result may suggest that either virus accumulation or fresh weight and dry weight losses, at the vegetative stage is / are not appropriate predictive criteria to determine how TuYV affects the overall fitness of the *B. napus* accessions. A further evaluation of the effect of TuYV on the yield (seed yield) of oilseed may therefore be necessary.

5.2 THE EFFECTS OF *TURNIP YELLOWES VIRUS* (TuYV) ON THE GROWTH AND YIELD OF SOME OILSEED RAPE CULTIVARS

5.2.1 Introduction

In the previous experiment 27 accessions of *B. napus* DFFS were screened for resistance to TuYV infection (section 5.1). The accessions varied in their susceptibility to TuYV infection based on ELISA absorbance values. Yudal was found to accumulate low levels of TuYV whilst Rafal DH1 accumulated the highest levels. There was no relationship between the levels of virus (ELISA absorbance values) detected in each accession and the vegetative yields (i.e. fresh and dry weights). It is not known whether the virus will affect the fitness (seed yield) of these accessions.

Currently, there is limited information on TuYV resistance in *B. napus* in the UK or elsewhere. The only published data on TuYV resistance was based on the resynthesised oilseed rape line, R54 (Graichen, 1994). The resistance was derived from a Chinese cabbage (A genome) (Gland, 1980). In Australia, *B. napus* cultivars-Tranby, Trigold and Stubby had resistance to BWYV by aphid transmission, yet 1, 2 and 3 plants respectively out of 68 plants each were found to be infected with the virus (Coutts and Jones, 2000) indicating they are not completely resistant to BWYV.

TuYV resistance identified in R54 was found to be heritable (Graichen and Peterka, 1999) and a major quantitative trait locus involved in the resistance has been

identified (Dreyer *et al.*, 2001; Juergens *et al.*, 2010). If the reduced TuYV accumulation and unaffected vegetative growth identified in Yudal results in unaffected seed yield, then Yudal will be either a good cultivar for oilseed rape growers in region where TuYV is prevalent and / or a good source of resistance for breeding new TuYV-resistant oilseed rape cultivars.

The main aim of the research described in this section was to determine the influence of TuYV infection on the seed yield of Yudal in order to assess its resistance / tolerance to the virus.

The study was also aimed to:

1. Compare the impact of TuYV infections on the growth and yield of oilseed rape cultivars which have different virus accumulation levels (ELISA absorbance values).
2. Determine the relationship between the TuYV accumulation levels (ELISA absorbance values), vegetative and seed yields in order to find out whether there is any relationship between the two former traits and the latter.

5.2.2 Materials and methods

5.2.2.1 Plant material

Four *B. napus* DFFS accessions from the previous experiment (section 5.1; Westar DH10, Tapidor DH, Rafal DH1 and Yudal) and a current commercial cultivar (Castille) were used in this experiment. Rafal DH1 and Yudal represent extremes of variation in accumulation of TuYV detected in section 5.1. Rafal DH1 had the

highest level of TuYV accumulation and significant high dry weight loss, whilst Yudal had the lowest level of TuYV accumulation and low dry weight loss. Both Tapidor DH and Westar DH10 had intermediate levels of TuYV accumulation, and low and high dry weight losses respectively (Table 5.2). Westar DH10 and Yudal are spring oilseed rape cultivars whilst Rafal DH1 and Tapidor DH are winter oilseed rape cultivars.

5.2.2.2 Virus isolate

The LAB isolate of TuYV used in section 5.1.2.2 was used in this experiment.

5.2.2.3 Plant cultivation

Seeds of the five oilseed rape accessions were sown in FP9 pots in M2 peat compost (Scotts Levington, UK) and grown in an insect-proof air-conditioned glasshouse at 18°C for three weeks.

5.2.2.4 Infection procedure

The oilseed rape plants were infected as in section 5.1.2.4.

5.2.2.5 Experimental design and layout

Each of the five oilseed rape lines, both TuYV-infected and uninfected (mock-inoculated with non-viruliferous aphids) had 8 replicates. Two sets of the above treatments were included, one set for vegetative yield assessment and the other for seed yield assessment, making a total of 160 plants. The treatments were laid out in a Split plot design on a bench in the glasshouse, with infected and uninfected plants facing in a north-south direction on the bench.

5.2.2.6 Vernalisation of plants

Three weeks after infection with TuYV, the winter oilseed rape lines (Castile, Tapidor DH, and Rafal DH1) were vernalised in a cold room at 5°C under natural light for 12 weeks to induce flowering. Yudal (a spring oilseed rape which behaves as early flowering winter type in temperate climates) was given 4 weeks vernalisation but Westar another spring oilseed rape readily flowered without vernalisation. After vernalisation, the plants were transferred into an insect-proof air-conditioned glasshouse at 18°C until the end of the experiment.

5.2.2.7 Cultural practice

Plants were watered as necessary. The plants were sprayed with the fungicide (Thiovit at 2.0g/L) to control powdery mildew infection.

5.2.2.8 Data taken and analysis

Vegetative parameters

In addition to the fresh weight and dry weight which was determined six weeks after challenging the plants with TuYV as described in section 5.1.2.6, plant height at senescence was also measured.

ELISA

The relative amount of TuYV in each accession was determined using TAS-ELISA as described in section 5.1.2.6. The primary antibody used was rabbit IgG (AS-0049, DSMZ, Braunschweig, Germany), the secondary antiserum was the rat monoclonal antibody MAFF 24 (Stevens *et al.*, 1995), and the tertiary alkaline phosphatase

conjugated antiserum was goat anti-rat (A8438, Sigma-Aldrich Ltd., Poole, UK). Absorbance values ($A_{405\text{nm}}$) were measured with a Biochrom Anthos 2010 microplate reader (Biochrom Ltd., Cambridge, U.K.).

Seed yield

The following yield parameters - number of branches per plant, number of pods per plant, number of seeds per pod, total seed yield - were recorded using the method described by Jay *et al.* (1999) with some modifications. The number of primary branches was counted on each of the 8 plants of each cultivar. Counts were also made of the number of pods produced by each of the 8 plants; the number of seeds per pod was counted for 8 pods from each plant. These pods were then threshed by hand to obtain seed numbers. The remaining pods were threshed and winnowed to obtain the seed yield for each plant by weighing on an electronic balance.

Data analysis

Using GenStat Release version 12.1 (Payne *et al.*, 2009), an analysis of variance (ANOVA) was performed. Count data on number of branches per plant, number of pods per plant and number of seeds per pod were square root-transformed in order to homogenise variances between treatments, using Genstat Release version 12.1 (Payne *et al.*, 2009). Correlation coefficients were calculated for all variables (characters) across all genotypes in order to determine the relationship between the characters.

5.2.3 Results

5.2.3.1 *Turnip yellows virus* infection and mean plant height

TuYV infected all the oilseed rape cultivars tested, with mean absorbance values from TAS-ELISA ranging from 1.505 to 2.464 (Table 5.4). Tapidor DH had the highest absorbance value of 2.464 whilst Yudal had the lowest (1.505). The ANOVA showed significant differences between the cultivars in terms of the degree of virus accumulation ($F_{28, 56} = 6.48$; $P < 0.001$). The level of TuYV accumulation in Tapidor DH (2.464) was not significantly different from that in Castille (2.07) and Rafal DH1 (2.006) but was significantly different ($P < 0.01$) from those in Westar DH10 (1.599) and Yudal (1.505), which did not differ significantly from each other.

The effects of TuYV infection on the mean plant height of oilseed rape cultivars are presented in Table 5.4. An ANOVA revealed that inoculated plants had on average significantly shorter plant height than mock-inoculated plants ($F_{1, 63} = 20.75$; $P < 0.001$). There was also a significant effect of cultivar on plant height ($F_{4, 63} = 16.11$; $P < 0.001$), indicating that cultivars differ on their mean plant heights whether infected or not. However, the ANOVA did not show a significant interaction between cultivar and infection status ($F_{4, 63} = 2.09$; $P = 0.092$), indicating that the effect of TuYV infection on plant height did not vary among the accessions (Table 5.4). However, TuYV infection resulted in the highest reduction in mean plant height in Westar DH10 (12.91%), followed by Castille (12.16%), Tapidor DH (7.43%) and Yudal (5.39) whilst Rafal DH1 had the lowest (0.61).

Table 5.4 ELISA absorbance (virus accumulation) and mean plants heights of oilseed rape cultivars challenged with *Turnip yellows virus* (TuYV) for six weeks

Cultivar	Absorbance	Mean plant height (cm)		
	(A ₄₀₅) ^a	Mock-inoculated	TuYV-infected	% Reduction
Castille	2.074	115.1	101.1*	12.16
Rafal DH1	2.006	114.5	113.8	0.61
Tapidor DH	2.464	105.0	97.2	7.43
Westar DH10	1.599	134.4	117.1	12.91
Yudal	1.505	116.8	110.5	5.39
Mean		117.2	108 ^b	

^a LSD for comparing mean absorbance values between cultivars was 0.4414 at d.f.= 28, $P < 0.05$

^b LSD for comparing overall mean heights of mock-inoculated and TuYV-infected plants was 4.67 at 63 d.f.

*Difference between mean plant heights of mock-inoculated and TuYV-infected plants was not significant ($P > 0.05$).

LSD for comparing mean plant heights between cultivars was 6.38 at 63 d.f.

5.2.3.2 Fresh weight and dry weight

The ANOVA of the effect of TuYV infection on fresh weights of oilseed rape cultivars showed significant differences between the cultivars ($F_{4, 63} = 13.65$; $P < 0.001$), indicating that the cultivars differ on their mean fresh weights independent of whether they were infected. The ANOVA also showed that infection had a significant effect on fresh weight ($F_{1, 63} = 5.81$; $P = 0.019$) but non-significant cultivar - infection interaction effect ($F_{4, 63} = 1.57$; $P = 0.205$) indicating that infection and cultivar acted independently on each other. However, Westar DH10 had the highest reduction of fresh weight (15.62%), followed by Rafal DH1

(13.18%), Tapidor DH (8.1%), Yudal (3.26%), whilst Castille had the lowest (3.08%) (Table 5.5).

ANOVA indicated significant differences in mean dry weights between cultivars and infection (cultivars: $F_{4, 63} = 12.99$; $P < 0.001$ and infection status: $F_{1, 63} = 16.47$; $P < 0.01$), but their interaction effect was not significant ($F_{4, 63} = 0.97$; $P = 0.455$) (Table 5.5). This suggests that on the average the infected plants had lower dry weight than the controls and also the cultivars differed on their average dry weights irrespective of infection. However, the variation in dry weights between uninfected and TuYV-infected plants for each cultivar was not sufficiently large to detect significance effect in their interaction. Rafal DH1 had the highest reduction in dry weight (20.79%), followed by Westar DH10 (18.86%), Tapidor DH (18.16%) and Castille (9.11%), whilst that of Yudal was reduced by 4.15% due to TuYV infection.

Table 5.5 Mean fresh weight and mean dry weight of oilseed rape cultivars challenged with *Turnip yellows virus* (TuYV) and mock challenged.

Cultivar	Mean fresh weight (g)			Mean dry weight (g)		
	Uninfected	Infected	% Reduction	Uninfected	Infected	% Reduction
Castille	29.55	28.64 [*]	3.08	6.04	5.49 [*]	9.11
Rafal DH1	30.73	26.68	13.18	6.06	4.80	20.79
Tapidor DH	26.18	24.06	8.10	4.68	3.83	18.16
Westar DH10	39.05	32.95	15.62	6.84	5.55	18.86
Yudal	33.17	32.09	3.26	6.50	6.23	4.15
Mean	31.52	29.10 ^a		6.02	5.18 ^b	

* Difference between TuYV-infected and uninfected (mock-inoculated) plants within cultivar was not significant ($P > 0.05$).

^a Overall mean fresh weight difference between mock-inoculated and TuYV-infected plants was significant (LSD = 2.005; d.f. = 63; $P < 0.05$).

^b Overall mean dry weights difference between mock-inoculated and TuYV-infected plants was significant (LSD = 0.415; d.f. = 63; $P < 0.05$).

LSD for comparing mean fresh weights between cultivars was 3.170 at 63 d.f.

LSD for comparing mean dry weights between cultivars was 0.656 at 63 d.f.

5.2.3.3 Numbers of branches and pods per plant

ANOVA indicated significant differences within cultivars ($F_{4, 63} = 32.33$; $P < 0.001$) and infection status ($F_{1, 63} = 6.04$; $P = 0.017$) for the number of branches, but their interaction effect was not significant ($F_{4, 63} = 1.59$; $P = 0.187$). This suggests that the variation between the uninfected relative to infected plants across cultivars was not sufficiently large to detect any significant interaction effect. However, Castille had the highest reduction (28.43%), followed by Westar DH10 (26.04%), and Tapidor DH (7.84%) and Rafal DH1 (4.27%) whilst mean number of branches in Yudal remained unchanged irrespective of the virus infection (Table 5.6).

ANOVA showed significant differences in the mean number of pods per plant within both cultivars and infection: cultivars ($F_{4, 63} = 29.54$; $P < 0.001$) and infection status ($F_{1, 63} = 18.70$; $P < 0.001$) but there was no significant interaction effect ($F_{4, 63} = 1.14$; $P = 0.344$), indicating that the variation in pod numbers between uninfected and infected plants across cultivar was not large enough. However, Castille suffered highest reduction in pod numbers (41.58%), followed by Tapidor DH (22.65%), Westar DH10 (20.33%) and Rafal DH1 (17.26%) (Table 5.6). Yudal had the lowest reduction in pod number (2.99%) due to TuYV infection (Table 5.6).

Table 5.6 Mean number of branches and mean number of pods of oilseed rape plants challenged with *Turnip yellows virus* (TuYV) and mock challenged.

Cultivar	Mean number of branches			Mean number of pods		
	Uninfected	Infected	% Reduction ^a	Uninfected	Infected	% Reduction
Castille	3.00 (9.25)	2.57 (6.62) ^{a*}	28.43	6.24 (40.4)	4.78 (23.6) ^{a*}	41.58
Rafal DH1	2.37 (5.62)	2.32 (5.38)	4.27	8.77 (77.6)	8.00 (64.2)	17.26
Tapidor DH	2.49 (6.38)	2.41 (5.88)	7.84	8.64 (75.5)	7.59 (58.4)	22.65
Westar DH10	1.83 (3.38)	1.57 (2.50)	26.04	7.73 (60.0)	6.89 (47.8)	20.33
Yudal	2.01 (4.12)	2.03 (4.12)	0.00	8.34 (70.2)	8.18 (68.1)	2.99
Mean	2.34 (5.75)	2.18 (4.90) ^b		7.94 (64.8)	7.09 (52.4) ^c	

* Difference between mock-inoculated and TuYV-infected plants was not significant ($P > 0.05$). Values in the parenthesis are the actual mean; values outside brackets are the square root-transformed means.

^a Per cent reductions were calculated based on the actual means.

^b Difference in the overall mean number of branches between mock-inoculated and infected plants was significant (LSD = 0.1304; d.f. = 63; $P < 0.05$).

^c Difference in the overall mean number of pods between mock-inoculated and infected plants was significant (LSD = 0.3941; d.f. = 63; $P < 0.05$).

LSD for comparing mean number of branches between cultivars was 0.2062 at 63 d.f.

LSD for comparing mean number of pods between cultivars was 0.6231 at 63 d.f.

5.2.3.4 Number of seeds per pod and seed yield

The ANOVA showed that the mock-inoculated plants had on average significantly higher numbers of seeds per pod than the infected plants ($F_{1, 63} = 34.04$; $P = 0.001$). Cultivars also had a significant effect on the seed yield ($F_{4, 63} = 5.64$; $P < 0.05$), indicating that the cultivars differ in their mean seed yield, whether infected, or not. The ANOVA showed a non-significant cultivar – infection interaction effect ($F_{4, 63} = 2.24$; $P = 0.074$), indicating that the effect of TuYV infection on the number of seeds per pod did not vary between the accessions. The mean number of seeds per pod were reduced by 21.96% in Castille, 11.47% in Rafal DH1 and 9.93% in Tapidor DH due to TuYV infection, whilst those of Westar DH10 and Yudal were higher (6.13% and 1.02% respectively) in TuYV infected plants (Table 5.7).

The effect of TuYV infection on seed production of the five oilseed rape cultivars are also summarised in Table 5.7. The ANOVA showed significant differences within both main factors (cultivar: $F_{4, 63} = 20.76$; $P < 0.001$ and infection status: $F_{1, 63} = 14.18$; $P = 0.001$) but no significant interaction effect ($F_{4, 63} = 1.38$; $P = 0.253$). However, Castille suffered significantly highest yield loss of 44.72%, followed by Tapidor DH (30.44%), Rafal DH1 (22.61%) and Yudal (9.64%) whilst Westar DH10 recorded the lowest (3.15%) due to TuYV infection.

Table 5.7 Mean number of seeds per pod and mean seed yield (g) of oilseed rape cultivars challenged with *Turnip yellows virus* (TuYV) and mock-challenged.

Cultivar	Mean number of seeds per pod			Mean seed yield (g)		
	Uninfected	Infected	% Change ^a	Uninfected	Infected	% Reduction
Castille	3.80 (14.62)	3.34 (11.41) ^{*b}	21.96	1.79	0.99 [*]	44.72
Rafal DH1	4.90 (24.07)	4.61 (21.31)	11.47	3.88	3.00	22.61
Tapidor DH	4.41 (19.44)	4.18 (17.51)	9.93	2.66	1.85	30.44
Westar DH10	3.77 (14.36)	3.90 (15.24)	6.13	2.23	2.16	3.15
Yudal	4.32 (18.69)	4.34 (18.88)	1.02	2.07	1.87	9.64
Mean	4.24	4.07 ^c		2.53	1.97 ^d	

* Difference between mock-inoculated and TuYV-infected plants within a cultivar was significant ($P > 0.05$).

^a Per cent change (relative to uninfected plants) was calculated based on the actual means.

^b Values in the parenthesis are the actual means; those outside are the square root transformed means.

^c Difference in the overall mean seeds per pod between mock-inoculated and infected plant was significant (LSD = 0.1413; d.f. = 63; $P < 0.05$).

^d Difference in the overall mean seed yield between mock-inoculated and infected plants was significant (LSD = 0.4628; d.f. = 63; $P < 0.05$).

LSD for comparing mean number of seeds per pod between cultivars was 0.2234 at 63 d.f.

LSD for comparing mean seed yield per plant between cultivars was 0.4535 at 63 d.f.

5.2.3.4 Relationships between growth and yield traits

The results of correlation coefficients between the traits studied are shown in Table 5.8. Significant positive correlations were observed between mean seed yield and mean plant height ($r = 0.340$; d.f. = 38; $P < 0.05$), pod number ($r = 0.566$; d.f. = 38; $P < 0.01$) and seeds per pod ($r = 0.566$, d.f. = 38; $P < 0.001$). There were however no significant correlations between seed yield and ELISA absorbance ($r = 0.166$; d.f. = 38; $P > 0.05$). A significant and positive relationship was found between pod number and seeds per pod ($r = 0.408$; $P < 0.01$). There was a highly significant and positive correlation between fresh weight loss and dry weight loss ($r = 0.942$; $P < 0.001$). There was a positive, but non-significant correlation between mean fresh weight and number of branches ($r = 0.087$; d.f. = 38; $P > 0.05$). There were also a negative but

non-significant relationships between ELISA absorbance and fresh weight ($r = -0.038$; d.f. = 38; $P > 0.05$), dry weight ($r = -0.035$; d.f. = 38; $P > 0.05$).

Table 5.8 Correlations between the vegetative and economic yield parameters of oilseed rape cultivars.

1	Absorbance	-							
2	Fresh weight	-0.038	-						
3	Dry weight	-0.035	0.942***	-					
4	Plant height	-0.094	0.123	-0.004	-				
5	Branches	-0.002	0.087	0.088	0.349*	-			
6	Pod number	0.182	-0.145	-0.190	0.247	0.353*	-		
7	Seeds/pod	0.278*	0.218	0.201	-0.077	0.212	0.408**	-	
8	Seed yield	0.166	0.097	0.026	0.340*	0.229	0.566**	0.386*	-
	Characters	1	2	3	4	5	6	7	8

*Significant at $P < 0.05$, **Significant at $P < 0.01$, ***Significant at $P < 0.001$
Number of observations, $n = 40$,

5.2.4 Discussion

5.2.4.1 *Turnip yellows virus* (TuYV) resistance testing

The study revealed that all the five oilseed rape accessions tested were infected and varied in the levels of TuYV accumulated in them. Yudal once again recorded the lowest mean absorbance value (virus titre). This result agrees with the previous result work (section 5.1), where Yudal was found to be the least TuYV susceptible among the 27 *B. napus* accessions screened. This suggests that Yudal could possess some reproducible resistance. However, whilst the mean absorbance value for Yudal

in the first test was as low as 0.489 that of the recent test was 1.505, indicating that the virus titre / concentration in Yudal can vary between experiments. This finding is comparable to that of Juergens *et al.* (2010) who observed that the virus titres of DH oilseed rape lines derived from the resistant line R54 were very low during the 2004-5 and 2005-6 crop seasons but were very high during 2006-7 crop season. They therefore concluded that TuYV resistance of 'R54' was not a complete type of resistance but rather represented a quantitative reduction of the virus titre. TuYV resistance / tolerance in Yudal is not complete but rather a quantitative reduction in virus concentration relative to other accessions. This type of resistance can be influenced by environmental factors (Juergens *et al.*, 2010) and is controlled by a single major gene together with additional contributing genes in R54 (Dreyer *et al.*, 2001; Graichen, 1998).

5.2.4.2 The effect of *Turnip yellows virus* (TuYV) on the growth of plants

The significant reduction in plant height of some of the oilseed rape accessions due to TuYV infection clearly suggests that TuYV infection can cause stunted growth in oilseed rape depending upon the cultivar. Walsh *et al.* (1989), Jay *et al.* (1999) and Stevens (2010) have also reported of significant reductions in plant height of oilseed rape crops infected with TuYV in England. Other examples of viruses reducing plant heights have been reported in other plant-virus pathosystems including BYDV in winter wheat (Yount *et al.*, 1985), *Banana streak virus* in banana (Daniells *et al.*, 2001) and TuMV in *Brassica juncea* (Guo *et al.*, 2004). Three biochemical mechanisms by which virus infection could cause stunted growth in plants have been described by Hull (2002): changes in the activity of growth hormones, a reduction in the availability of the products of carbon fixation and a reduction in the uptake of

nutrients. A significant reduction in chlorophyll and hence photosynthetic efficiency has been observed in sweet potato infected with sweet potato virus (Hahn, 1979) and in stem mustard infected with TuMV (Guo *et al.*, 2004). Similar reasons may account for the observed reduction in fresh and dry weights of the infected oilseed rape plants. Reduction in vegetative yields (fresh and dry weights or biomass) have been reported in several virus-plant pathosystems, including the effect of PLRV on potato (Watson and Wilson, 1956), paracrinkle virus on King Edward potato (Kassanis and Schwabe, 1961), TuYV on oilseed rape (Jay *et al.*, 1999), and TuMV on *B. juncea* (Guo *et al.*, 2004).

5.2.4.3 Numbers of branches, pods, seeds per pod and seed yield

Depending upon the cultivar, TuYV infection resulted in a reduction in the number of branches from 0% and 28.43%. This agrees with an observation made by Jay *et al.* (1999) where TuYV-infected oilseed rape plants produced fewer branches than control plants. A positive correlation between the number of branches and seed yield of oilseed rape been observed by Ozer *et al.* (1999) and Tuncturk and Ciftci (2007), suggesting that reductions in seed yield could partly be due to the reduction in the number of branches caused by TuYV infection.

It has been reported that TuYV-infection has no significant effect on the numbers of pods produced on the main raceme or on the branches (Jay *et al.*, 1999). It was also observed in this work that the mean numbers of pods per TuYV-infected plant were not significantly different from those of mock-inoculated plants of the same cultivar. It is however noteworthy that infection resulted in fewer numbers of pods per plant of up to 48.45% compared to the uninfected plants. This is consistent with the report

of Stevens (2010) which stated that TuYV-infected oilseed plants produce fewer pods than the uninfected plants.

The percentage seed yield losses observed in this study, which ranged between 3.15% in cv Westar and 44.72% in cv Castille, are comparable to the previous reports of yield losses in oilseed rape in the UK infected by TuYV, ranging from 0 to 45% (Walsh *et al.*, 1989; Hardwick *et al.*, 1994; Jay *et al.*, 1999; Impey, 2010; Stevens and Clark, 2009). It is also comparable to the yield loss of up to 50% reported in oilseed rape in Australia (Jones *et al.*, 2007), and yield losses of between 12 and 34% recorded in oilseed rape crops in Germany (Graichen and Schliephake, 1999). My current work has therefore confirmed the report that TuYV infection can be one of the reasons why oilseed rape cannot achieve its yield potential (Stevens *et al.*, 2008).

Even though TuYV infection caused reduction in seed yield in the oilseed rape accessions, indicating that the virus can exert selection on oilseed rape, the effect of the virus on fitness or yield varied between the accessions. However, the relative reduction in seed yield between the accessions due to TuYV infection cannot be explained by the virus accumulation (ELISA absorbance values) in all the accessions, indicating that the cultivars may vary for trait that mediates the effect of virus on seed yield (fitness), i.e. susceptibility / tolerance trait. This is supported by the non-significant correlation between virus accumulation (ELISA absorbance) and seed yield. Walsh *et al.* (1989) observed no correlation between percentage BWYV infection and seed yield.

There were no significant correlations between the vegetative yields (fresh weight, dry weight) and seed yield. This agrees with Degenhart and Kondra (1984), who observed no consistent trend in the relationship between seed yield and growth characters. Plant height was however significantly and positively correlated with seed yield and number of pods per plant, but was positive although not significantly correlated with number of seeds per pod. This suggests that TuYV infection mediated the reduction in seed yield by causing stunting in growth which contributed to a reduction in the number of pods per plant.

5.3 THE INTERACTION BETWEEN *ARABIDOPSIS THALIANA* ACCESIONS AND *TURNIP YELLOWS VIRUS* (TuYV)

5.3.1 Introduction

Apart from the work described in sections 5.1 and 5.2 little is known about the resistance of oilseed rape cultivars grown in the U.K. to TuYV. In Germany, a resynthesised oilseed rape line, R54, was found to be resistant to TuYV. However, progenies from a cross between R54 and commercial cultivars were shown to possess an incomplete type of resistance. My current work (sections 5.1 and 5.2) only identified one cultivar, Yudal with partial resistance, whilst another one, Westar DH10 was found to be tolerant to the virus. There is therefore the need to search for more stable sources of resistance to TuYV infection. *A thaliana* is a useful tool for investigating TuYV-*Brassicaceae* interactions. The entire genome of this model plant has been sequenced and numerous mutants have been well characterised. This model system has the potential to successfully aid the genetic dissection of oilseed rape-TuYV interactions (Stevens *et al.*, 2008). This will also help to broaden the understanding of the complex relationships between TuYV and plants resistance as not all genes that confer resistance (biochemical or physiological) will necessarily increase yield (host fitness) as has been observed by Kover and Schaal (2002). For instance, the resistance studies carried out in the previous section of this chapter (i.e. section 5.2) showed no significant correlation between the levels of TuYV accumulation (i.e. the absorbance values) and the seed yield in the oilseed rape cultivars.

A. thaliana has also been used to broaden an understanding involving pathogen- host plant interactions. Infection of *A. thaliana* with turnip vein clearing virus (TVCV), a positive-sense RNA tobamovirus, has since been used as a model system for studying virus-plant interactions (Sheng *et al.*, 1998). The interaction between *A. thaliana* and *Pseudomonas syringae* has been an important model in the study of genetic basis of plant–pathogen interactions (Kover *et al.*, 2005). *A. thaliana* has been identified as a host for TuYV thus providing a valuable model system to study virus-host interactions (Stevens *et al.*, 2005).

The main aim of this work was to screen *A. thaliana* accessions against TuYV infection, in order to seek resistance. It was also aimed at determining the effect of TuYV on the growth and seed production of *A. thaliana*.

5.3.2 Materials and methods

5.3.2.1 Plant materials

The interactions of plants to TuYV infections was studied on 20 *A. thaliana* accessions, described in Table 5.9. The accessions were made up of ecotypes of wide geographical origins and also included extremes of *A. thaliana* genetic diversity based on data from microsatellite (Innan *et al.*, 1997) and AFLP markers (King *et al.*, 1993). Col-0 and Ler-0 were included because they are accessions commonly used in studies of disease resistance in *A. thaliana*.

Table 5.9 List of the *Arabidopsis thaliana* accessions used, their stock numbers and their geographical origin.

Ecotype	Stock *	Collection site
Bur-0	CS6643	Ireland
Can-0	CS6660	Canary Islands
Col -0	CS6673	USA
Ct-1	CS6674	Italy
Edi -0	CS6688	Scotland
Hi-0	CS6736	Netherlands
Kn-0	CS6792	Lithuania
Ler-0	CS20	Germany
Mt-0	CS1380	Libya
No-0	CS6805	Germany
Oy-0	CS6824	Norway
Po-0	CS6839	Germany
Rsch-4	CS6850	Russia
Sf-2	CS6857	Spain
Wil-2	CS6889	Russia
Ws-0	CS91	Russia
Ws-3	N1682	Wassilewskija (Belarus)
Ws-eds1	-	-
Wu-0	CS6897	Germany
Zu-0	CS6902	Germany

*Stock number of the *A. thaliana* accession from Arabidopsis Information Management System.

Ecotype Ws-3 was obtained from Nottingham Arabidopsis Stock Centre; Ws-eds1 (a mutant of Ws ecotype) from Falk *et al.* (1999); others from Kover and Schaal (2002), all provided for this study by Professor Eric Holub of the University of Warwick, UK.

5.3.2.2 Virus isolate

The LAB isolate of TuYV used in section 5.2.2.2 was used in this experiment.

5.3.2.3 Plant cultivation

A total of 1440 seeds (72 seeds of each accession) were sown in FP7 pots in M2 peat compost (Scotts Levington, UK) and then randomly distributed among 16 trays with 20 pots per tray. All pots were stratified at 4°C for 5 days in the dark before being placed to germinate and grow in a growth room under 10-h photoperiod at 20 ± 2°C.

Seven days after germination, the plants were thinned out leaving one plant per pot. Tray positions in the growth chamber were rotated every week to minimise the effect of micro environmental variation.

5.3.2.4 Inoculation procedure

Plants were challenged with TuYV as described in section 5.1.2.4

5.3.2.5 Experimental design and layout

Each of the 20 accessions both TuYV-infected and uninfected (mock-inoculated) had 8 replications. Two sets of the above treatments were raised: one set for ELISA serology to test for resistance / susceptibility and the other for vegetative and seed yield measurements. The treatments were laid out in a Split plot design on a bench in the growth room, separating infected and non-infected plants.

5.3.2.6 Cultural practice

Plants were watered when necessary.

5.3.2.7 Data taken

Test for resistance

Eight weeks after challenging the plants with TuYV, the relative amount of TuYV (i.e. virus accumulation) in each accession was determined using ELISA as described in section 5.2.2.8 with some modifications. The primary antibody used was rabbit IgG (AS-0049) and the secondary antiserum was the mouse monoclonal antibody (AS-0049/1), all from DSMZ (Braunschweig, Germany). Tertiary alkaline

phosphatase conjugated antiserum was goat anti-mouse (A3562, Sigma-Aldrich Ltd., Poole, U.K.).

Absorbance values ($A_{405\text{nm}}$) were measured with a Biochrom Anthos 2010 microplate reader (Biochrom Ltd., Cambridge, U.K.) as described in chapter 2.

After plants had been tested by ELISA, the day length was increased to 16 hours to induce flowering.

Vegetative yield

The effect of TuYV infection on growth related fitness of the *A. thaliana* accessions was assessed by measuring plant height and rosette diameter of infected and mock-inoculated plants. Rosette diameter was measured for each infected and mock-inoculated plant before senescence. Time to senescence varied among the accessions, from as early as 2 to a maximum of 4 months after inoculation.

The plant height at senescence and the number of branches (primary and secondary branches together) were also determined for each infected and mock-inoculated plant.

Seed yield

Because *A. thaliana* is an annual plant, the effect of TuYV infection on fitness can be estimated by total seed production, as described by Kover and Schaal (2002) in determining the effect of *P. syringae* on the fitness of *A. thaliana*. The number of fruits produced by a plant has previously been shown to correlate closely with total seed production (Mauricio and Rausher, 1997). To estimate seed production, four

fruits (siliques) from each plant were collected and the number of seeds in each counted. Thus seed production which represents plant fitness was estimated by multiplying the number of fruits produced per plant by the average number of seeds per fruit bases on four fruits, for that accession. The number of fruits per each plant was counted for both infected and mock-inoculated plants.

5.3.2.8 Data analysis

Using GenStat Release version 12.1 (Payne *et al.*, 2009), analysis of variance was carried out and the significance of difference between means determined using least significant differences (LSD). Data on the number of branches per plant, the number of fruits per plant and the number of seeds per pod were transformed using square root transformation before ANOVA was performed. Correlation coefficients were calculated for all variables (traits) across all accessions in order to determine the relationships between them.

5.3.3 Results

5.3.3.1 Infection of *Arabidopsis thaliana* accessions with *Turnip yellows virus* (TuYV)

Twenty *A. thaliana* accessions were tested for accumulation of TuYV (i.e. ELISA absorbance) (Table 5.10). All assayed accessions were susceptible to TuYV. The ANOVA showed highly significant differences in virus accumulation among the accessions ($F_{19, 114} = 3.71$; $P < 0.001$). Sf-0 had the highest mean virus accumulation of 1.013 ± 0.028 whilst Ler-0 had the lowest value of 0.679 ± 0.035 .

Table 5.10 shows the effect of TuYV infection on the plant heights of *A. thaliana* accessions. An ANOVA showed significant differences within both main factors (accession: $F_{19, 229} = 33.50$; $P < 0.001$ and infection status: $F_{1, 229} = 480.38$; d.f. = 1; $P < 0.001$) as well as a significant interaction effect ($F_{19, 229} = 5.59$; $P < 0.001$) indicating that the two main factors were not acting independently of each other. The mean plant heights of the mock-inoculated plants were significantly higher ($P < 0.05$) than that of the TuYV-infected plants in 18 of the *A. thaliana* accessions, indicating that TuYV significantly caused stunted growth in many accessions. Edi-0 had the highest reduction in height (74.49%) whilst Mt-0 had the least reduction (1.66%) due to TuYV infection.

Table 5.10 *Turnip yellows virus* (TuYV) infection on *Arabidopsis thaliana* accessions, the quantity of virus detected and the effect on plant height

Accession	Absorbance (A_{405nm})	Mean plant height (cm)		% Reduction
		Uninfected	Infected	
Bur-0	0.902 ± 0.039	27.60	12.33* ^a	55.33
Can-0	0.844 ± 0.050	30.39	15.12*	50.25
Col -0	0.903 ± 0.026	33.38	24.38*	26.96
Ct-1	0.863 ± 0.034	45.78	29.28*	36.04
Edi -0	0.856 ± 0.031	20.50	5.23*	74.49
Hi-0	0.869 ± 0.050	37.38	29.10*	22.15
Kn-0	0.931 ± 0.037	43.63	29.68*	31.97
Ler-0	0.679 ± 0.035	32.25	16.98*	47.35
Mt-0	0.759 ± 0.071	36.54	32.28	11.66
No-0	0.818 ± 0.069	48.49	34.63*	28.58
Oy-0	0.930 ± 0.046	46.38	20.13*	56.60
Po-0	0.846 ± 0.040	54.89	37.19*	32.25
Rsch-4	0.905 ± 0.054	37.70	32.44	13.95
Sf-2	1.013 ± 0.028	50.55	38.30*	24.23
Wil-2	0.869 ± 0.017	40.63	24.94*	38.62
Ws-0	0.918 ± 0.039	31.66	13.46*	57.49
Ws-3	0.798 ± 0.035	51.38	33.76*	34.29
Ws-eds1	0.776 ± 0.066	54.68	29.56*	45.94
Wu-0	0.773 ± 0.049	44.14	33.94*	23.10
Zu-0	0.802 ± 0.044	41.46	11.13*	73.15
Mean		40.47	25.19* ^b	

*Difference between uninfected and infected plants was significant at $P < 0.05$.

^a LSD for comparing uninfected and infected plant height within accession was 6.141 at d.f of 237.

^b LSD for comparing average uninfected and infected plant height was 1.373 at d.f. of 237.

LSD for comparing mean plant heights between accessions was 4.342 at d.f. of 237.

5.3.3.2 Rosette diameter and number of branches

The effects of TuYV infection on the mean rosette diameter of the *A. thaliana* accessions are presented in Table 5.11. The ANOVA showed significant differences within both main factors (accession: $F_{19, 229} = 10.37$; $P < 0.001$ and infection status: $F_{1, 229} = 104.33$; $P < 0.001$) as well as significant interaction effect ($F_{19, 229} = 1.90$; $P = 0.014$). Rosette sizes of the mock-inoculated plants were significantly higher than

the infected plants in 12 of the accessions (Ct-0, Edi-0, Hi-0, Kn-0, Ler-1, Oy-0, Po-0, Rsch-0, Wil-0, Ws-0, Ws-eds1, and Wu-0) ($P < 0.05$).

The effect of TuYV infection on mean number of branches of the *A. thaliana* accessions are shown in Table 5.11. The mock-inoculated plants recorded higher number of branches than the TuYV-infected plants in all the accessions. The ANOVA showed significant differences within both main factors (accession: $F_{19, 229} = 8.66$; $P < 0.001$ and infection status: $F_{1, 229} = 93.91$; $P < 0.001$) but no significant interaction effect ($F_{16, 229} = 1.14$; $P = 0.322$). Oy-0 recorded the highest reduction in the number of branches due to TuYV infection (41.92%) whilst Zu-0 had the least (11.34) (Table 5.11)

Table 5.11 Effect of *Turnip yellows virus* (TuYV) infection on mean rosette diameter (cm) and mean number of branches of *Arabidopsis thaliana* accessions.

Accession	Rosette diameter (cm)			Number of branches		
	Uninfected	Infected	% Reduction	Uninfected	Infected	% Reduction ^a
Bur-0	8.75	8.99 ^b	2.74	2.05 (3.75)	1.77 (2.33) ^c	37.87
Can-0	7.65	6.91	9.67	2.81 (7.43)	2.52 (6.01)	19.11
Col -0	9.35	8.74	6.52	2.69 (6.750)	2.32 (5.00)	25.92
Ct-1	10.20	8.24*	19.22	2.39 (5.250)	2.09 (4.00)	23.81
Edi -0	10.05	7.39*	26.47	2.77 (7.25)	2.30 (4.81)	33.66
Hi-0	11.44	9.19*	19.67	2.88 (7.88)	2.47 (5.63)	28.55
Kn-0	10.35	8.42*	18.65	2.62 (6.38)	2.47 (5.63)	11.76
Ler-0	7.06	4.78*	32.29	2.71 (6.97)	2.42 (5.55)	20.37
Mt-0	9.46	8.73	7.72	2.62 (6.41)	2.50 (5.88)	8.26
No-0	9.21	8.31	9.77	2.67 (6.63)	2.27 (4.75)	28.36
Oy-0	11.20	8.39*	25.09	2.60 (6.25)	2.01 (3.63)	41.92
Po-0	11.65	9.40*	19.31	2.66 (7.00)	2.44 (5.50)	21.42
Rsch-4	10.31	8.04*	22.02	2.83 (7.50)	2.64 (6.50)	13.33
Sf-2	10.55	10.18	3.5	2.64 (6.50)	2.19 (4.37)	32.77
Wil-2	9.77	7.85*	19.65	2.47 (5.63)	2.21 (4.37)	22.38
Ws-0	9.94	8.39*	15.59	2.64 (6.50)	2.49 (5.74)	11.69
Ws-3	8.71	8.64	0.69	2.45 (5.63)	2.21(4.50)	20.07
Ws-eds1	10.63	8.69*	18.25	2.59 (6.25)	2.25(4.63)	25.92
Wu-0	9.23	7.28*	21.13	2.83 (7.56)	2.72 (6.88)	8.99
Zu-0	10.18	9.31	8.55	2.47 (5.64)	2.34 (5.00)	11.34
Mean	9.78	8.29 ^d		2.62 (6.46)	2.33 (5.04) ^e	

*Difference between uninfected and infected plants was significant ($P < 0.005$).

^a Percentage reduction in the number of branches was calculated based on the actual means.

^b LSD for comparing mean rosette diameter of uninfected and infected plants within accession was 1.28 at d.f. of 273.

^c Difference between number of branch of uninfected and infected plants was not significant ($P > 0.05$).

^d LSD for comparing average rosette diameter of uninfected and infected plants was 0.29 at d.f. of 229.

^e LSD for comparing average number of branches of uninfected and infected plants was 0.058 at d.f. of 229.

LSD for comparing mean rosette diameters between accessions was 0.9101 at d.f. of 273.

LSD for comparing mean numbers of branches between accessions was 0.9205 at d.f. of 229.

5.3.3.5 Number of pods per plant

The infected plants produced on the average significantly fewer number of pods than the mock-inoculated plants ($F_{1, 273} = 195.59$; $P < 0.001$) (Table 5.12). There was also significant effect of accession on pod production ($F_{19, 273} = 16.41$; $P < 0.001$), indicating that accessions differ on the average the number of pod produced irrespective of whether they are infected. In addition, the ANOVA showed a significant interaction between accession and infection ($F_{19, 273} = 6.58$; $P < 0.001$), indicating that the effect of TuYV infection on pod production varies among the accessions (Table 5.12). All the eight plants each of accessions Bur-0, Can-0 and Ler-0 challenged with TuYV died before pod formation, suggesting that they were very susceptible to the virus infection.

Table 5.12 Effect of *Turnip yellows virus* (TuYV) infection on mean number of pods of *Arabidopsis thaliana* accessions

Accession	Mean number of pods		% Change ^a
	Uninfected	Infected	
Bur-0	8.99 (81.2)	0.71 (0.0) ^b	100
Can-0	12.91 (192.6)	0.71 (0.0)*	100
Col -0	12.98(169.8)	9.12 (88.1)*	48.11
Ct-1	11.84 (140.1)	9.90 (101.4)	27.62
Edi -0	13.33(182.2)	1.87 (12.5)*	93.25
Hi-0	14.67 (233.4)	11.64(137.9)*	40.92
Kn-0	15.36 (238.5)	10.97 (121.0)*	49.27
Ler-0	6.99 (90.6)	0.71 (0.0)*	100
Mt-0	12.38 (174.0)	11.07 (124.2)	28.62
No-0	14.89 (232.5)	19.52 (115.1)*	50.49
Oy-0	14.37 (209.4)	9.26 (86.2)*	58.83
Po-0	13.58 (186.6)	11.58 (135.5)	27.38
Rscl-4	18.67 (356.6)	11.70 (148.2)*	58.44
Sf-2	12.84 (165.9)	10.88 (126.9)	23.51
Wil-2	12.58 (167.2)	13.01 (168.9)	1.02
Ws-0	14.19 (208.9)	2.15 (18.8)*	91.00
Ws-3	13.79 (199.5)	10.92 (120.8)	39.45
Ws-eds1	16.90 (292.1)	11.15 (134.8)*	53.85
Wu-0	13.20 (199.4)	11.76(141.0)	29.29
Zu-0	9.89 (128.1)	10.50 (111.1)	13.27
Mean	13.22	8.51* ^c	

*Difference between uninfected and infected plants was significant at $P < 0.05$.

^a Percentage change in the number of pods was calculated based on the actual means

^b LSD for comparing uninfected and infected number of pods within accession was 2.99 at d.f of 273.

^c LSD for comparing average number of pods between uninfected and infected plants was 0.663 at d.f. of 273.

LSD for comparing mean number of pods between accessions was 2.097 at d.f. of 273.

5.3.3.6 Number of seeds per pod and total seed production

Effects of TuYV on mean number of seeds per pod and total seed production are summarised in Table 5.13. The ANOVA revealed that the infected plants produced on average significantly fewer number of seeds per pod (2.95) than the mock-

inoculated plants (4.38) ($F_{1, 273} = 155.88$; $P < 0.001$). There was also a significant effect of accession on the mean number of seeds produced per pod ($F_{19, 273} = 17.31$; $P < 0.001$), indicating that accessions differ on their average number of seeds per pod independent of whether they were infected. Furthermore, the ANOVA showed a significant interaction between accession and infection ($F_{19, 273} = 9.51$ $P < 0.001$), indicating that the effect of TuYV infection on the number of seeds per pod varies among accessions (Table 5.13). Infection caused significant reduction in number of seeds per pod in 12 of the 20 accessions, ranging from 1.1% in Po-0 to 100% in Bur-0, Can-0 and Ler-0 (where the plants died before senescence due to TuYV infection).

The ANOVA of the effect of TuYV infection on seed production revealed that the infected plants produced on average significantly fewer seeds than the uninfected plants ($F_{1, 273} = 126.11$; $P < 0.001$). Accession also had significant effect on seed production ($F_{19, 273} = 5.06$; $P < 0.001$). The ANOVA further showed a significant interaction between accession and infection status ($F_{19, 273} = 2.41$; $P = 0.001$), indicating that the effect of TuYV on seed production varies among the accessions (Table 5.13). This reduction ranged between 13.65% in Ct-1 and 100% in Bur-0, Can-0 and Ler-0. Infection resulted in a significant reduction in seed production in 10 out of the 20 accessions.

Table 5.13 Effects of *Turnip yellows virus* (TuYV) infection on the number of seeds per pod and seed production.

Accession	Number of seeds per pod			Seed production		
	Uninfected	Infected	% Change ^a	Uninfected	Infected	% Change
Bur-0	4.52 (20.54)	0.00* ^b	100	1647	0.00 ^c	100
Can-0	3.23 (11.93)	0.00*	100	2575	0.00*	100
Col -0	4.46 (20.10)	3.76 (14.38)*	28.46	3577	1290	63.94
Ct-1	3.94 (15.58)	4.32 (18.84)	17.30	2191	1892	13.65
Edi -0	5.03 (25.70)	0.63 (3.13)*	87.82	4721	312*	93.39
Hi-0	5.10 (26.35)	4.13 (17.54)*	33.43	6106	2549*	58.25
Kn-0	4.59 (21.85)	4.32 (19.13)	12.45	5210	2398*	53.97
Ler-0	2.24 (10.58)	0.00 (0.00)*	100	2158	0.00*	100
Mt-0	3.89 (17.78)	3.64 (13.34)*	24.97	3667	1669	54.49
No-0	4.49 (20.51)	3.43 (12.21)*	40.47	5328	1678*	68.51
Oy-0	5.19 (27.38)	2.89 (8.45)*	69.14	5775	761*	86.82
Po-0	4.61 (22.50)	4.6 (22.75)	1.10	4037	3087	23.53
Rsch-4	4.79 (23.35)	4.74 (22.74)	2.61	8171	3376*	58.68
Sf-2	4.66 (21.79)	4.46 (20.03)	8.08	3590	2625	26.88
Wil-2	4.45 (20.15)	4.12 (17.08)	15.24	3477	2904	16.48
Ws-0	4.81 (23.91)	0.41 (1.31)	94.52	5502	197*	96.42
Ws-3	3.83 (15.58)	3.12 (9.83)*	36.91	3360	1198	64.35
Ws-eds1	5.15 (27.50)	3.55 (12.95)*	52.90	8508	1729*	79.68
Wu-0	5.19 (34.98)	3.89 (15.16)*	56.66	7698	2129*	72.34
Zu-0	3.45 (16.25)	3.01 (9.13)*	43.82	2858	1011	64.63
Mean	4.38	2.95* ^c		4508	1540* ^d	

*Difference between uninfected and infected plants was significant at $P < 0.05$.

^a Percentage change in the number of seeds per pod was calculated based on the back-transformed data.

^b LSD for comparing uninfected and infected seeds/pod within an accession was 1.006 at d.f. of 273.

^c LSD for comparing average uninfected and infected seeds/pod was 0.225 at d.f. of 273.

^c LSD for comparing uninfected and infected seed production within accession was 2326.5 at d.f. of 273.

^d LSD for comparing average uninfected and infected seed production was 520.2 at d.f. of 273.

LSD for comparing mean number of seeds per pod between accessions was 0.7113 at d.f. of 273.

LSD for comparing mean seed production between accessions was 1645.1 at d.f. of 273.

5.3.3.7 Correlations among traits

Table 5.14 shows the correlations coefficients among the growth and yield traits studied. ELISA absorbance values were not significant but positively correlated with plant height, rosette size, seeds per pod and seed production but positively and non-significantly correlated with number of branches and number of pods per plant ($P > 0.05$). There was a significant and positive correlation between plant height and rosette size ($r = 0.326$, d.f = 38; $P < 0.05$), number of pods per plant ($r = 0.363$; d.f = 38; $P < 0.05$), and seed production ($r = 0.340$; d.f = 38; $P < 0.05$). There was a significant positive correlation between seed production and number of pods per plant ($r = 0.795$; d.f = 38; $P < 0.001$) and number of seeds per pod ($r = 0.808$; d.f = 38; $P < 0.001$). There was also a significant and positive correlation between number of pods and seeds per pod ($r = 0.347$; d.f = 38; $P < 0.05$).

Table 5.14 Correlation coefficients

1	Absorbance	-						
2	Plant height	-0.070	-					
3	Rosette size	-0.158	0.326*	-				
4	Branches	0.221	0.115	0.267	-			
5	Pod number	0.120	0.363*	0.192	0.168	-		
6	Seeds/pod	-0.123	0.254	0.201	0.085	0.347*	-	
7	Seed production	-0.096	0.340*	0.202	0.112	0.795***	0.808***	-
	Characters	1	2	3	4	5	6	7

* Significant at $P < 0.05$; *** Significant at $P < 0.001$. Sample size (n) = 40

5.3.4 Discussion

5.3.4.1 Resistance testing

The study revealed narrow range of continuous variation in the susceptibility of TuYV infection among the *A. thaliana* accessions. With the absorbance values ranging between 0.679 ± 0.035 (occurred in Ler-0) and 1.013 ± 0.028 (recorded for Sf-0), it is clear that all the accessions were highly susceptible to TuYV infection, indicating that *A. thaliana* is a good host of TuYV. Stevens *et al.* (2005) have also identified *A. thaliana* as a host of TuYV. The observed quantitative variation in virus accumulation among the accessions could be due to different interaction effects between different host genotypes and that of TuYV. These accessions, except Ws-eds1, have also been found to exhibit continuous variation in the susceptibility to *P. syringae* infection (Kover and Schaal, 2002). Eighteen accessions of *A. thaliana* were also found to differ in their tolerance to *Cucumber mosaic virus* (CMV) (Pagan *et al.*, 2008).

5.3.4.2 Effect of TuYV on growth of *A. thaliana*

Virus infections have been found to reduce growth related fitness in *A. thaliana* in terms of its rosette size (Kover *et al.*, 2005) and plant height (Sheng *et al.*, 1998). In this study, infection of 20 *A. thaliana* accessions with TuYV caused significant reduction in their plant heights, rosettes sizes and number of branches, at varying levels. That is the reduction in plant growth caused by infection was not uniform but varied among the accessions. These could be as a result of the accessions varying in tolerance, i.e., some plant has higher fitness (i.e. higher plant height, rosette size) despite higher degrees of infection (absorbance values). For instance Sf-0 had the highest virus titre level or virus accumulation whilst Ler-0 had the lowest but Ler-0

suffered higher reduction in plant height than Sf-0 (Table 5.10), indicating that Sf-0 is more tolerant to TuYV infection than Ler-0. The varying degree at which TuYV affected the growth related traits could be attributed to how the virus infection resulted in the reduction in their growth hormones, photosynthetic abilities and nutrients uptake (Hull, 2002).

5.3.4.3 Seed production

TuYV infection resulted in reduction in seed production by reducing all the yield components i.e. number of branches, number of pods per plants and number of seed per pod in all the accessions. This further indicates that *A. thaliana* is good host of TuYV, and hence suitable for further detailed resistance studies. Seed production was tightly correlated with the number of pods per plant and the number of seeds per pod but was not significantly correlated with absorbance values or virus accumulation. This indicates that even though TuYV infection reduced the plants fitness (seed production), the degree to which they were infected (i.e. level of virus accumulation) was not correlated with the amount of fitness lost (i.e reduction in seed production including number of pods produced and the number of seeds per pod). This suggests that the effect of TuYV on seed production is mediated by other factors than the one that determines virus accumulation in the plants. For instance, Sf-0 had higher degree of infection (absorbance value of 1.013 ± 0.028) than Ws-0 (0.918 ± 0.039), Edi-0 (0.856 ± 0.031) and Ler-0 (0.679 ± 0.035) yet Sf-0 suffered lower fitness loss (26.88% reduction in seed production) than Ws-0 (96.42%), Edi-0 (93.39%) and Ler-0 (100%). Similar observations were also made by Kover *et al.* (2002) when 19 accessions of *A. thaliana* were screened for resistance and tolerance to *P. syringae* infection. It was therefore concluded that resistance traits in *A.*

thaliana are not good predictors of fitness, and that tolerance traits play an important role in mediating plant fitness under infection (Kover and Schaal, 2002). In this work, Sf-0 together with Ct-1, Wil-2 and Po-0 also with fitness losses of 13.65%, 16.42 and 23.53% respectively may be tolerant to TuYV infection. This tolerance may be involved in modification of life history traits in response to TuYV infection by allocating more resources into producing more branches per plant, more pods per plant and more seeds per pod leading into more seed production. It has been reported that tolerance to pathogen infection involves alteration of host's life history (Agnew *et al.*, 2000) including reproductive efforts (Christie *et al.*, 1996; Sorci *et al.*, 1997) by modified resource allocation to increase the production of reproductive structures and progeny (Pagan *et al.*, 2008). Pathogens usually affect fitness-related traits, and hence have important economical effects on crops by reducing growth and yield (Wolfe, 2000).

This work has therefore revealed that TuYV accumulations in plants are not good predictors of fitness in *A. thaliana*.

CHAPTER 6:

A QUANTITATIVE TRAIT LOCUS (QTL) ANALYSIS OF PARTIAL RESISTANCE OF OILSEED RAPE (*BRASSICA NAPUS*) TO *TURNIP YELLOW*S VIRUS (TuYV) AND ASSESSMENT OF THE SPECTRUM OF RESISTANCE

6.1 A QUANTITATIVE TRAIT LOCUS ANALYSIS OF PARTIAL RESISTANCE OF OILSEED RAPE CV YUDAL AGAINST *TURNIP YELLOW*S VIRUS (TuYV)

6.1.1 Introduction

Plant viral diseases cause serious economic losses in many major crops by reducing yield and quality and often determine whether and when a crop is planted in a cropping system (Kang *et al.*, 2005). The most effective and sustainable approach to the prevention of virus disease is through the deployment of genetic resistance targeted against the virus directly or, in theory, against their vectors (Maule *et al.*, 2007). The first step in the study of genetics of viral resistance is to determine whether the resistant response is inherited and if so, the number of genes involved and their mode of inheritance (Kang *et al.*, 2005). Qualitative resistance is often controlled by major genes, which are often inherited dominantly, less frequently recessively (Do Vale *et al.*, 2001). Quantitative resistance (also referred as partial, polygenic and field resistance) is often controlled by multiple genes / quantitative trait loci (Do Vale *et al.*, 2001; Kou and Wang, 2010).

Quantitative trait locus (QTL) mapping is a highly effective approach for studying genetically complex forms of plant disease resistance (Young, 1996). With QTL mapping, the roles of specific resistance loci can be described, race-specificity of partial resistance genes can be assessed and interactions between resistance genes, plant development and the environment can be analysed (Young, 1996). QTL mapping involves testing molecular markers throughout a genome for the likelihood they are associated with a QTL. Individuals in a mapping population (doubled-haploid, F2, backcross, recombinant inbred lines) are analysed in terms of DNA marker genotypes and the phenotype of interest (Young, 1996).

Experiments carried out in chapter five (sections 5.1 and 5.2) of this thesis have demonstrated that oilseed rape cv. Yudal has partial resistance to TuYV infection. TuYV-resistance in oilseed rape has been found to be heritable (Dreyer *et al.*, 2001; Juergens *et al.*, 2010). In Germany, TuYV resistance genes identified in the progeny of a resynthesised oilseed line, R54, were introgressed into modern oilseed rape breeding material (Graichen and Peterka, 1995; Graichen and Peterka, 1999). It is therefore desirable to map QTL for quantitative resistance in Yudal, with the eventual aim of characterising genes for TuYV resistance. Knowledge of the number, location, effects, and identities of such genetic loci (QTL) can assist the selection of improved agricultural crops (Broman and Sen, 2009) including TuYV-resistant oilseed rape cultivar. This would form the foundation for a subsequent marker assisted selection programme.

The aim of this study was to identify the QTL involved in the partial resistance of Yudal to TuYV infection, and to define a set of linked markers.

6. 1.2 Materials and methods

6.1.2.1 Plant material

A doubled-haploid (DH) population DYDH (Darmor x Yudal) of 118 individuals derived from microspores of a single F1 plant originating from the cross of Darmor-*bzh* with Yudal (Foisset *et al.*, 1997) was used for trait analysis. Yudal is less susceptible (partially resistant) to TuYV whilst Darmor-*bzh* is highly susceptible to TuYV (see chapter 5, section 5.1). Darmor-*bzh* is a dwarf isogenic line (B₃F₃) derived through the introgression of the dwarf *Bzh* gene into Darmor background (Foisset *et al.*, 1995). Darmor is a French winter oilseed rape cultivar, whilst Yudal is a spring Korean oilseed rape cultivar that behaves as an early-flowering winter type in temperate climates.

6.1.2.2 Resistance test

Virus isolate

The TuYV isolate (LAB) described in chapter three was used as the inoculum.

Plant cultivation

Four seeds of each DH line were sown directly into FP7 pots in M2 peat compost (Scott Levingtons, UK) and grown in an insect-proof air-conditioned glasshouse at 18 °C ± 2 °C for three weeks. After germination, the plants were thinned out, leaving one plant per pot.

Due to limited space in the glasshouse, the DYDH population was divided into two sub-populations (SP1 and SP2). SP1 comprised all 118 genotypes and was sown in September 2010. SP2 comprised 115 lines (three of the original lines were not available) and were sown in December 2010.

Inoculation of plants

The plants were challenged with TuYV as described in chapter 5 (section 5.1.2.4)

Experimental design and layout

The plants were arranged in a randomised complete block design with four replications per DH line on a bench in the glasshouse at $18 \pm 2^{\circ}\text{C}$ and maintained for six weeks post inoculation. SP2 was grown in a glasshouse at $20 \pm 2^{\circ}\text{C}$ for further six weeks post inoculation.

TAS-ELISA

The virus concentration in each plant was determined by standard triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) as described in chapter two, section 2.2.2, with a few modifications. The primary antibody used was rabbit IgG (AS-0049), secondary antiserum was the mouse monoclonal antibody (AS-0049/1), and the tertiary alkaline phosphatase conjugated antiserum was rabbit anti-mouse (RAM-AP), all from DSMZ (Braunschweig, Germany).

6.1.2.3 Linkage map and genotype data

Genotype data for the DYDH population and a linkage map (labelled DY0703b) were received from Regine Delourme, INRA, France, and Graham Teakle,

University of Warwick, UK. The map has a total length of 1928 cM and is composed of 266 markers [predominantly amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) marker types] assembled into 20 linkage groups (LG). The longest group was assigned C3 (DY17-N13, 185 cM) and the shortest group C5 (N15, 27 cM). The greatest inter-marker distance was 39 cM and the smallest was 0.8 cM. The average inter-marker distance was 9.54 cM.

6.1.2.4 Statistical analysis

To assess the reproducibility of the resistance tests (SP1 and SP2), Pearson's correlation coefficients and coefficient of variation were calculated using GenStat release version 12 (Payne *et al.*, 2009). The ELISA absorbance data from experiments SP1 and SP2 were analysed using Residual Maximum Likelihood (REML) analysis (Patterson and Thompson, 1971; Van Dongen *et al.*, 1999), implemented in GenStat. Due to uneven sample size as a result of some plants failing to establish in the glasshouse, REML analysis was appropriate. The frequency distribution of the titre values in the DYDH population were checked using histograms drawn using EXCEL (Microsoft Corporation, 2007).

6.1.2.5 Detection of QTL (Genetic linkage analysis)

The genetic map data, locus genotype data and the predicted mean ELISA absorbance values from REML analysis were used as input data for QTL analysis. The QTL analysis was performed using R/qtl (Broman *et al.*, 2003) implemented in the R statistical package (<http://www.r-project.org>), via interval mapping using the EM algorithm (Broman *et al.*, 2003). A permutation test (1000 permutations) was performed to determine LOD significance thresholds to determine the significance

($P = 0.20, 0.05, 0.01, \text{ or } 0.001$) of QTLs identified. A 1 and 2 LOD confidence interval was calculated to define the QTL identified, followed by calculation of an approximate 95% Bayes credible-interval. A bootstrap-based confidence interval was also calculated using 1000 iterations. These confidence intervals enabled the determination of the most likely interval that contained the QTL. Linked markers were then nominated to define the QTL interval.

QTL analyses were also performed using MapQTL version 6 (Van Ooijen, 2009), as described by Zwart *et al.* (2008). Briefly, putative QTLs were initially identified using interval mapping. A genome-wide LOD significance threshold ($P < 0.05$) was calculated using 1000 permutations. The markers closest to the major QTL peak were then selected as co-factors in a multiple-QTL model implemented in the MQM mapping procedure of MapQTL. Markers were removed as cofactors if their LOD values dropped below the significance thresholds.

The software MapChart version 2.2 (Voorrips, 2002), was used to visualise QTL intervals and their locations on the DY0703b linkage map.

6.1.3 Results

6.1.3.1 Resistance tests

A total of eight replicates per DH line were tested for resistance and infection determined by TAS-ELISA using replicates SP1 and SP2. Even though the frequency distribution showed comparable maximum values (1.743 for SP1 and 1.806 for SP2) (Table 6.1), the distribution for SP1 was strongly skewed towards the

left side, indicating that most plants had low virus titres as determined by TAS-ELISA (Figure 6.1). About 39% of the DH lines in SP1 had titre values ranging from 0.000 to 0.399 whilst none of the SP2 population was within this range. The distribution of SP2 population was almost Gaussian but slightly skewed towards right, indicating that most DH plants had higher titre levels (ELISA absorbance values), ranging between 0.400 and 1.799 (Figure 6.1). The mean virus accumulation in SP2 (0.943) was higher than that of SP1 (0.621) (Table 6.1). Both SP1 and SP2 sub-populations showed continuous genotypic distributions, indicating quantitative accumulation of TuYV, and hence a polygenic control of TuYV susceptibility in oilseed rape. A 1:1 distribution of susceptible to resistant plants, as would have been expected for monogenic segregation in DH population, was not observed.

ANOVA performed separately for each of the two subpopulations (SP1 and SP2) revealed significant differences between the DH lines (SP1: $F_{119, 172} = 3.12$; $P < 0.001$; SP2: $F_{115, 221} = 2.59$; $P < 0.001$). This indicates that the DH lines differ in their susceptibility to TuYV infection.

Table 6.1 Distribution of Absorbance values from TAS-ELISA on Darmor-*bzh* x Yudal DH populations (SP1 and SP2) challenged with *Turnip yellows virus* (TuYV)

Statistic / parent	Absorbance (A_{405})	
	Subpopulation 1 (SP1)	Subpopulation 2 (SP2)
Darmor- <i>bzh</i>	1.329	1.059
Yudal	0.110	0.481
Observed population maximum	1.743	1.806
Estimated population mean	0.621	0.9434
Observed population minimum	0.089	0.450
Standard deviation	0.397	0.326

The means of the parents (Darmor-*bzh* and Yudal) were included for comparison with those of the population.

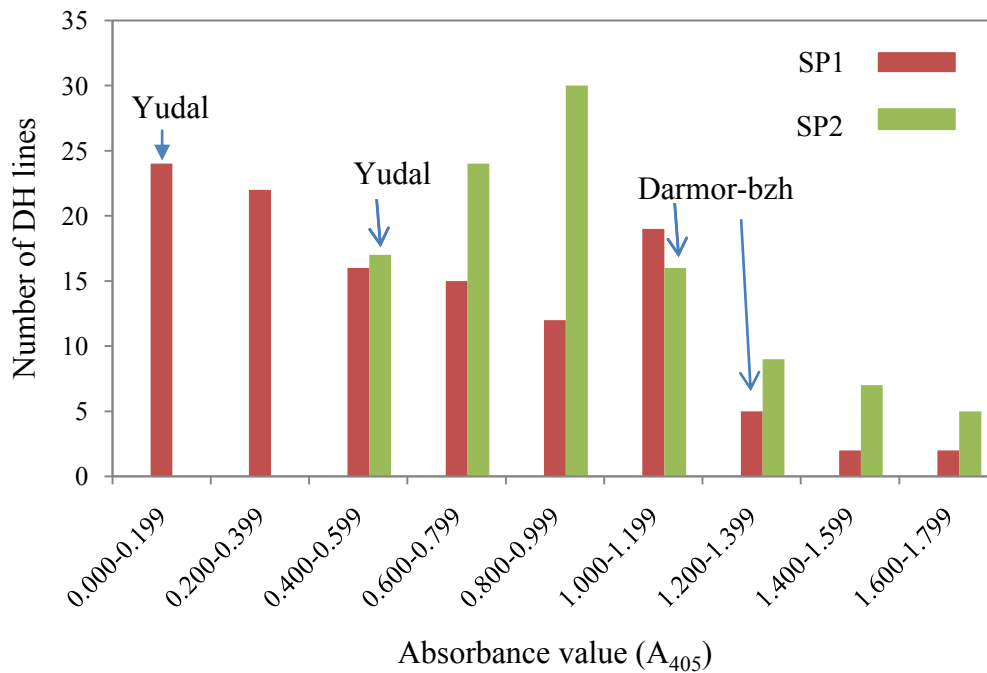


Figure 6.1 Frequency distribution of the Darmor-*bzh* x Yudal doubled haploid (DH) lines for partial resistance to *Turnip yellows virus* (TuYV) infection.

The histogram shows the range of absorbance values recorded in DH lines in populations SP1 (red) and SP2 (green) as determined by TAS-ELISA six weeks post inoculation with *Turnip yellows virus* (TuYV). The mean absorbance value of Darmor-*bzh* (susceptible parent) and Yudal (resistance parent) are indicated by the arrows.

There was a strong positive correlation in the titre levels (absorbance values) between SP1 and SP2 ($r = 0.475$; $P < 0.001$; $r^2 = 0.226$) (Figure 6.2), indicating that the pattern of virus titres of the DH lines in the two separate tests were similar.

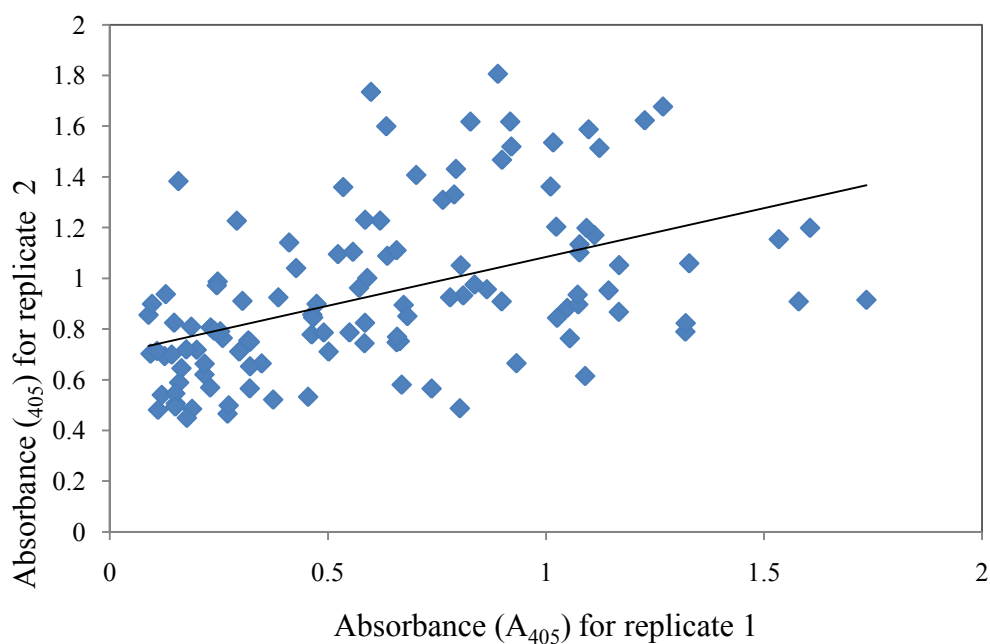


Figure 6.2 Correlations of the virus titre levels (A_{405}) between two DH lines subpopulations (SP1 and SP2) ($r = 0.475$; d.f. = 114; $P < 0.001$; $r^2 = 0.226$).

6.1.3.2 Genetic linkage analyses

Results of the QTL analyses of partial TuYV resistance in oilseed rape subpopulations SP1 and SP2 using R/qtl are shown in Figures 6.3 and 6.4 respectively. A significant QTL (LOD threshold = 2.94, $P < 0.05$) was detected on linkage group C4 (N14) (Figures 6.3 and 6.4). The QTL was linked to markers Tpi.2AB at position 0 cM (LOD = 12.237; $P < 0.05$) and FAD3.A at position 18 cM (LOD = 7.982; $P < 0.05$) in SP1. The same QTL and the linked markers were detected in SP2, but the LOD threshold at $P < 0.05$ was 2.88 LOD. The QTL was linked to markers Tpi.2AB at position 0 cM (LOD = 6.827; $P < 0.05$) and FAD3.A at position 18 cM (LOD = 5.732; $P < 0.05$) in SP2. A non-significant QTL was also observed on chromosome A6 (N06) (Figures 6.3 and 6.4).

When the DH line means were ranked based on the genotype at the QTL, it was observed that the QTL genotype inferring resistance were inherited from resistant parent, Yudal.

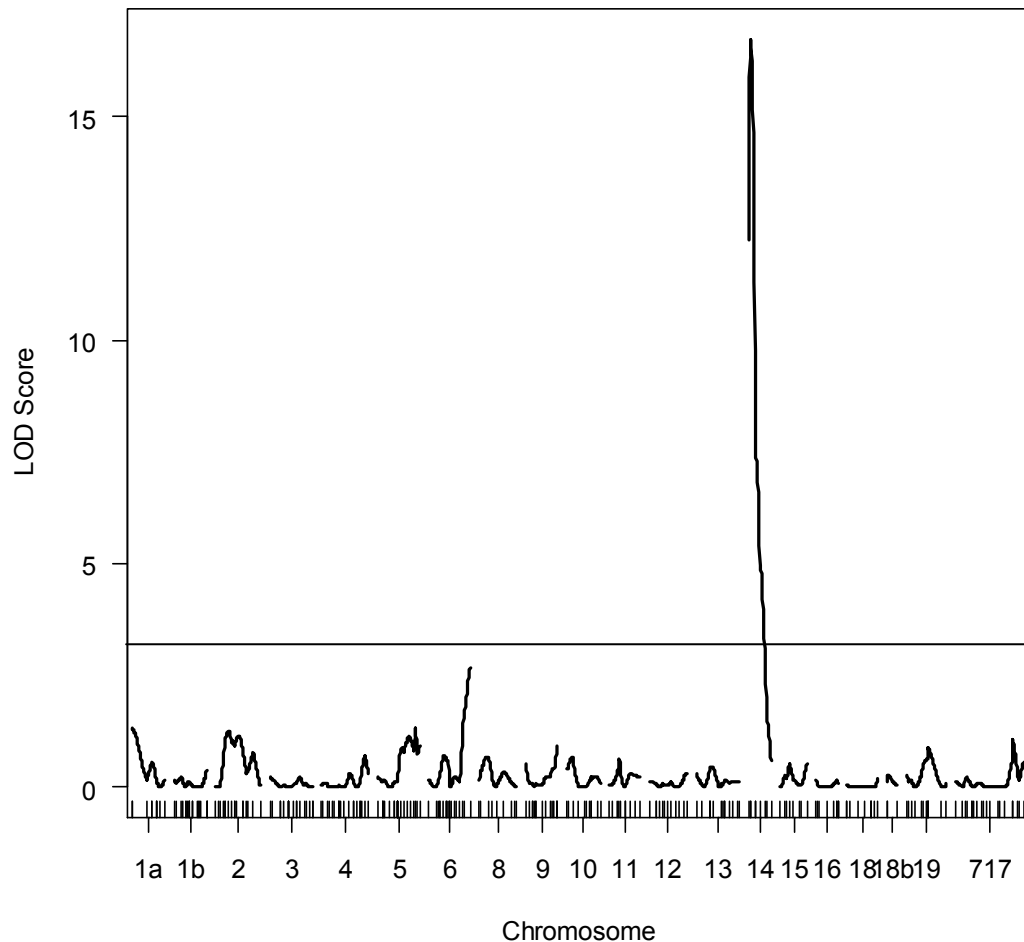


Figure 6.3 Detection of QTL for *Turnip yellows virus* (TuYV) resistance gene on chromosome 14 (i.e. N14) of *Brassica napus* in sub-population SP1.

A significant LOD score (2.94) was determined by permutation test (1000 times) at $P < 0.05$, and is indicated by horizontal line. R/qtl software (Broman *et al.*, 2003) was used in the QTL analysis based on ELISA absorbance values of 118 Yudal x Darmor DH lines (SP1). The markers and their corresponding positions flanking the QTL were Tpi.2Ab (0cM) - FAD3.A (18cM).

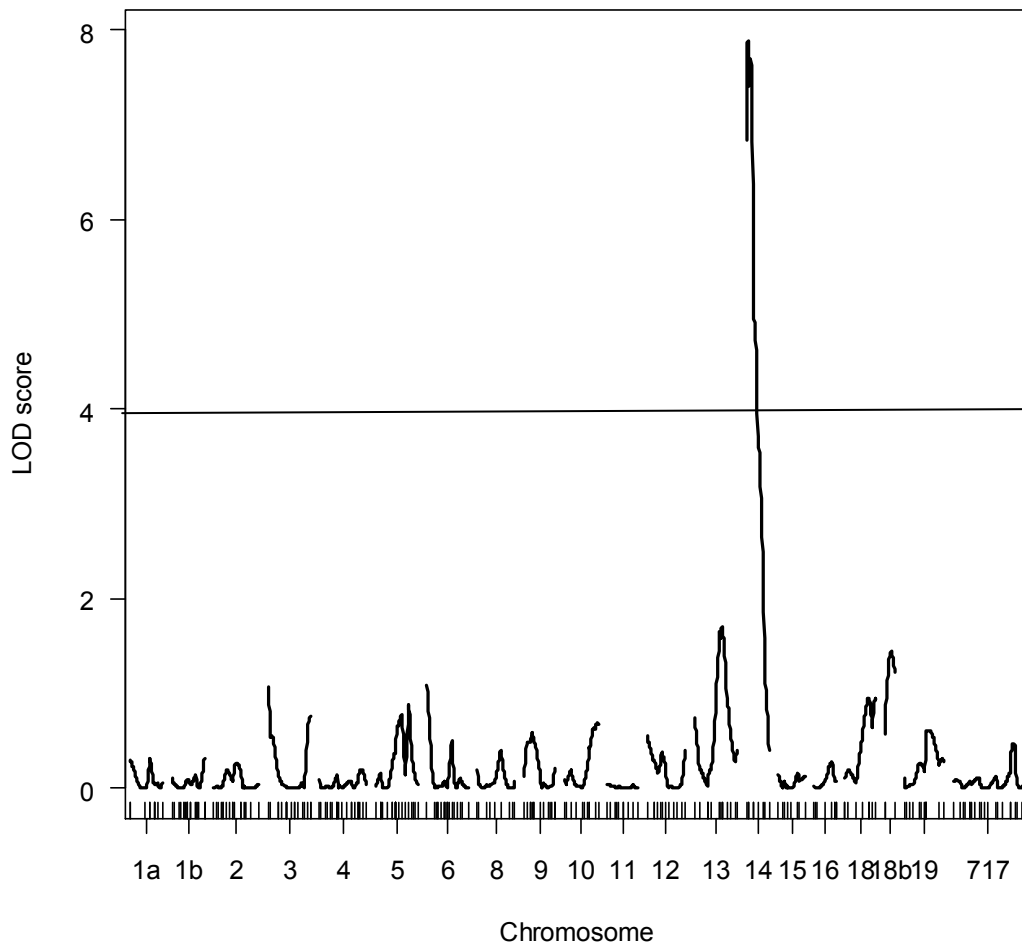


Figure 6.4 Detection of QTL for *Turnip yellows virus* (TuYV) resistance on chromosome 14 (i.e. N14) of *Brassica napus* in sub-population SP2. R/qtl software (Broman *et al.*, 2003) was used in the QTL analysis based on ELISA absorbance values of 115 Yudal x Darmor DH lines (SP2). LOD threshold at $P < 0.05$ and 1000 permutations was 2.88. The markers and their corresponding positions flanking the QTL were Tpi.2Ab (0 cM) - FAD3.A (18 cM).

The QTL analyses were repeated using MapQTL software (van Ooijen, 2009) and a significant QTL of large effect, explaining between 26.9% and 50.5% of the observed variance in the quantitative TuYV resistance was also located to linkage group C4 (N14) (Table 6.2). The non-significant QTL on linkage group A6 (N06) was not detected using MapQTL. When the mean absorbance values for replicate 1 (SP1) were used as phenotypic data, the QTL was detected at peak LOD of 18.02 at

position 6 cM (4 – 8 cM 95% confidence interval), flanked by markers Tpi.2AB at position 0 cM and FAD3.A at position 18 cM on the C4 (N14) linkage group. The QTL explained 50.5% of the total phenotypic variation observed in the study (Table 6.2; Figure 6.5).

Table 6.2 Details of quantitative trait locus (QTL) for *Turnip yellows virus* (TuYV) resistance detected on linkage group C4 (N14) of *Brassica napus* estimated from mean ELISA absorbance data in sub-populations of 115 – 118 Darmor-*bzh* x Yudal doubled-haploid lines.

Sub-pop. ^a	QTL detected	Peak Position (cM)	LOD at peak	CI interval (cM) ^b	% Exp ^c	Additive effect	Variance, R ²	Flanking markers ^d (position in cM)
SP1	1	6	18.02	4 - 8	50.5	0.228	0.049	Tpi.2Ab (0) - FAD3.A (18)
SP2	1	4	7.75	2 - 6	26.9	0.173	0.076	Tpi.2Ab (0) - /Pgd.1Ab (7) / I06.650 (7)
SP1 + SP2	1	6	18.76	4 - 8	50.3	0.226	0.049	Tpi.2Ab (0) - FAD3.A (18)

^a Sub-population: Sub-population1 (SP1) and sub-population 2 (SP2).

^b 1-, 2-LOD score confidence interval (CI).

^c % Explained: Proportion of the phenotypic variation explained by the QTL.

^d Closest markers linked to the QTL. Figure in parenthesis is the position of the flanking marker in the linkage group. The marker alleles associated with partial TuYV resistance were derived from the resistant parent Yudal.

When SP2 ELISA absorbance values were used as a phenotypic data, the QTL was detected at peak LOD of 7.75 at position 4 cM (2 – 6 cM 95% confidence interval), flanked by markers Tpi.2AB (8 cM) and Pgd.1Ab / I06.650 (7 cM) (Table 6.2; Figure 6.5). The QTL explained 26.9% of the total phenotypic variation observed.

When the mean data for both replicates was used as phenotype data, the QTL located to the same position as the QTL located for the mean data for SP1. The QTL was named *UoW_TuYV_N14.1* (Figure 6.5), following the nomenclature described at CropStore.DB (<http://www.cropstoredb.org/>).

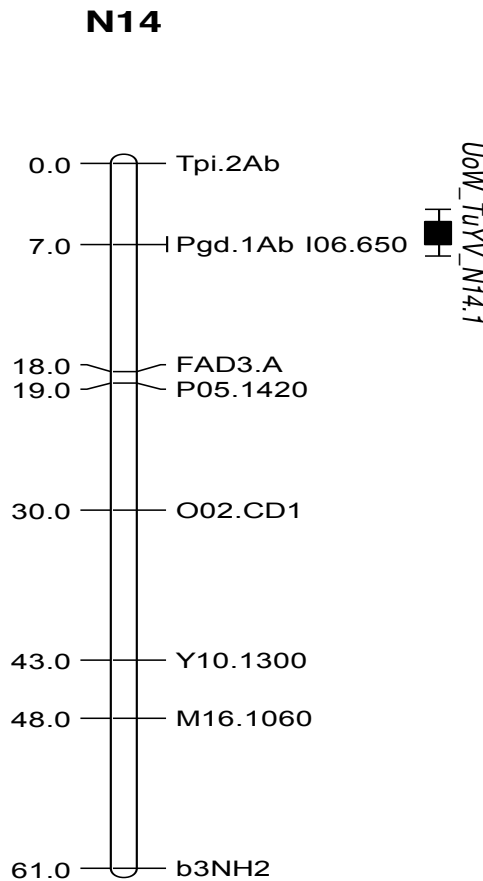


Figure 6.5 Linkage group C4 (N14) of *Brassica napus* (from the map DY0703b) showing markers and linked QTL involved in *Turnip yellows virus* (TuYV) resistance.

QTL “*UoW_TuYV_N14.1*” is drawn on right hand side as bar 1 LOD and sticks 2 LOD. The numbers on the left hand side of the linkage group represent the distance in centimorgans at named markers shown to the right.

When the DH line means were ranked based on the genotype at the QTL, it was observed that the QTL genotype inferring resistance were inherited from the parent Yudal which had displayed the original quantitative resistance.

6.1.4 Discussion

The reactions of the DH lines were fairly reproducible between the two experiments (SP1 and SP2), as shown by a strong correlation between them in terms of their mean absorbance values ($r = 0.475$; d.f. = 114; $P < 0.001$). However the average virus titres in experiment 2 (SP2) were higher than those in experiment 1 (SP1) even though they both showed continuous variation in the susceptibility to TuYV. Variation in such quantitative traits is often due to the effects of many multiple genetic loci as well as environmental factors (Broman and Sen, 2009). The variation in TuYV titre values observed in this study could be due to the different environmental conditions under which both experiments were conducted. Experiment 2 was conducted in a glasshouse under relatively warmer temperatures (up to 4°C higher daily temperature) and longer photoperiods (over 3 hours light) than that of experiment 1. There are several reports on the role of high temperature and other extreme environmental conditions in influencing multiplication of TuYV and / or possible breakdown of the TuYV-resistance in oilseed rape (Graichen, 1998; Dryer *et al.*, 2001; Juergens *et al.*, 2010). Generally, high light intensities and long days favour replication of plant viruses (Hull, 2002). High temperature is known to promote proliferation of virus. The resistance to TuYV in R54 is primarily determined by a dominant resistance gene but also influenced by environmental

factors, particularly temperature (Juergens *et al.*, 2010) and light intensity and duration.

The high absorbance values (higher virus titres) in the DH lines observed in the sub-population 2 (SP2) compared to that of sub-population 1 (SP1) clearly demonstrates that TuYV resistance in Yudal was not a complete type, as has been shown in chapter five of this thesis. Dreyer *et al.* (2001) and Juergens *et al.* (2010) have also pointed out that TuYV resistance in the resynthesised oilseed rape line, R54 is not a complete type of resistance but represents a quantitative reduction in virus titres.

Results obtained from interval mapping using R/qtl and MapQTL both showed the same significant QTL interval on linkage group C4 (N14) having association with TuYV resistance. This consistency is a clear demonstration of the reliability or true existence of the QTL *UoW_TuYV_N14.1* on chromosome C4 (N14) responsible for partial TuYV resistance in oilseed cv Yudal. Chromosome C4 (N14) is in the C-genome of *Brassica napus* (Parkin *et al.*, 2005). Comparison of common markers with the *B. napus* consensus map and the literature revealed that the detected QTL is on the same chromosome C4 (N14) that virus resistance was identified previously. A locus *TuRB02* which appeared to control the degree of susceptibility to *Turnip mosaic virus* (TuMV) isolate CHN 1 has been identified on the C-genome linkage group N14 (Walsh *et al.*, 1999). A major QTL for resistance against the fungal pathogen *Verticillium longisporum* in oilseed rape was also detected on C-genome linkage group N14 (Rygulla *et al.*, 2008).

It has been reported that the TuYV resistance in oilseed rape line R54 is controlled by a single major gene (Dreyer *et al.*, 2001; Juergens *et al.*, 2010). These reports agree with the findings of this current study where a major QTL for TuYV resistance was detected on chromosome C4 (N14) explaining up to 50.5% of the phenotypic variation, suggesting a possible single gene effect. The major QTL for TuYV resistance found by Dreyer *et al.* (2001) also explained 50% of the phenotypic variation. However, unlike *UoW_TuYV_N14.1*, the single major QTL for TuYV resistance found by Dreyer *et al.* (2001) and Juergens *et al.* (2010) was located on linkage group A4 (N04). Nevertheless, this work and that of Graichen (1998), Dreyer *et al.* (2001) and Juergens *et al.* (2010) have clearly demonstrated that TuYV-resistance can be controlled by single major gene along with additional contributing genes and influenced by environmental factors. It has also been reported that variation in quantitative traits is often due to the effects of multiple genetic loci as well as environmental factors (Broman and Sen, 2009).

It should be pointed out here that the QTL for TuYV-resistance described by the earlier workers (Graichen, 1998; Dreyer *et al.*, 2001; Juergens *et al.*, 2010) were based on the resynthesized oilseed rape line R54, derived from Chinese cabbage (Gland, 1980). Therefore, to my knowledge, this current study is the first report of genetic localization of genes conferring resistance to TuYV in oilseed rape derived from natural oilseed rape line (Yudal). It is also the first report of QTL for TuYV-resistance in oilseed rape located on C-genome linkage group C4 (N14). Flanking markers have been identified for this QTL within a genetic distance of 18 cM. This allows marker assisted selection (MAS) to be carried out by oilseed rape breeders by introgressing the resistance gene into current commercial oilseed rape cultivars in the

UK. In Germany, TuYV-resistance gene derived from resistant line R54 was transferred into modern oilseed rape breeding material (Graichen and Peterka, 1995; Graichen and Peterka, 1999).

Four markers, Tpi.2Ab, 1Pgd.1Ab, I06.650 and FAD3.A located on chromosome C4 (N14) were significant with both programmes. Interestingly, these markers revealed fragments inherited from the resistant parent (i.e. Yudal). These markers will be valuable in future MAS work. In contrast, within the support interval of 7 cM for the QTL detected by Dreyer *et al.* (2001), only one out of four markers revealed a fragment derived from the resistant parent, whilst the others were inherited from the non-resistant parent.

Although the QTL located on A6 (N6) was not significant (see Figure 6.3), it represents a chromosomal region of interest for future studies of TuYV resistance in oilseed rape. QTLs for resistance to other viruses and diseases have been found on linkage group A6 (N6). For example Walsh *et al.* (1999) detected resistance gene *TuRB01* on A6 (N6), conferring resistance to oilseed rape against TuYV. Rygulla *et al.* (2008) also detected QTL for resistance against *V. longisporum* in oilseed rape on A6 (N6). Further, QTL for blackleg resistance in oilseed rape has been detected on N6 (Pilet *et al.*, 1998). This non-significant QTL which was detected on A6 (N6) may be a weaker QTL that is influenced by environmental variation; therefore it is possible that this QTL may be significant if future replicates were carried out.

6.2 RESISTANCE OF OILSEED RAPE CV YUDAL TO *TURNIP YELLOWS VIRUS* (TuYV) ISOLATES OF DIFFERENT GENOTYPES

6.2.1 Introduction

Plant disease resistance can be classified into two categories: qualitative resistance conferred by a single resistance (*R*) genes and quantitative resistance (QR) mediated by multiple genes or quantitative trait loci (QTLs) with each providing a partial increase in resistance (Kou and Wang, 2010). Polygenic, quantitative resistance (and recessive resistance) that is pathogen species-non-specific or race-non-specific (Poland *et al.*, 2009; Wisser *et al.*, 2005) are regarded as more durable than qualitative, monogenic resistance (Harrison, 2002; Lindhout, 2002). Durable resistance refers to resistance that remains effective during its prolonged and widespread use in environments favourable to the pathogen or disease spread (Johnson, 1981). Durability is also favoured if the *R* gene is effective against the full range of variants of the virus occurring in the area of cultivation (Garcia-Arenal and McDonald, 2003) and when virulent strains are at a competitive disadvantage in the absence of a cognate resistance gene(s) (Bruening, 2006).

An important factor in deciding which resistance genes may be suitable for breeding into commercial crops would be their potential durability in the face of the extreme genetic plasticity of virus pathogens (Maule *et al.*, 2007). This is true for RNA viruses including TuYV, which have high mutation and recombination rates (Garcia-Arenal *et al.*, 2001) resulting in the evolution of resistance breaking pathotypes (Garcia-Arenal *et al.*, 2003). Broad-spectrum resistance and durable resistance to

diseases are desirable for crop improvement (Kou and Wang, 2010). Partial resistance to a virus, which reduces or prevents the normal development of the cycle of virus infection, is also valuable in the control of economically important plant viruses such as TuYV in oilseed rape.

A significant QTL *UoW_TuYV_N14.1* responsible for the partial resistance to TuYV infection in Yudal has been identified through the reaction of Yudal and other *B. napus* accessions with an isolate of TuYV (LAB) (see section 6.1). However, the previous work in determining the molecular diversity in TuYV infecting oilseed rape in England revealed three genetic groups (chapter 3). The LAB isolate belongs to one of the groups (genotype 3). It was therefore important to assess the reaction of Yudal to the different genetic groups of TuYV, to provide valuable information to oilseed rape breeders should they attempt to introgress the TuYV resistance in Yudal into current oilseed rape cultivars.

The objective of this experiment was to assess the reaction of Yudal to different isolates of TuYV belonging to different genetic groups with the aim of ascertaining the spectrum of the resistance.

6.2.2 Materials and methods

6.2.2.1 Plant materials

Oilseed rape cultivars Yudal (partial resistance to TuYV) and Darmor-*bzh* (TuYV-susceptible) which were the parents of the mapping population tested in section 6.1 above were used here.

6.2.2.2 Virus isolates

Seven TuYV isolates belonging to two different genetic groups were maintained separately on oilseed rape cv Mikado in an insect rearing room under 16 h photoperiod at $20 \pm 2^{\circ}\text{C}$ by serial transmission using *M. persicae*. The isolates were L1906 and LAB (genotype 1), L1808, L1843, L1851, L1876 and L1937 (genotype 3). Apart from LAB isolate which is a laboratory culture of TuYV isolate obtained from Brooms Barn, UK, the six other isolates were collected from oilseed rape crops from Lincolnshire, UK, in 2011 (see chapter four). TuYV isolates belonging to genotype 2 which comprised 3.1% of field isolates analysed in chapter 3, was not detected in the field in 2010 (see chapter 4). Hence it was not possible to include a genotype 2 isolate in this current experiment.

6.2.2.3 Plant cultivation

Seeds of oilseed rape cvs Yudal and Darmor-*bzh* were sown into M2 peat compost (Scotts Levington, UK) in FP9 pots and grown in an insect-proof air-conditioned glasshouse at 18°C for three weeks prior to inoculation. Four seeds were sown per pot in 112 pots and were later thinned out, leaving one plant per pot.

6.2.2.4 Inoculation procedure

The plants were challenged with TuYV when they were at 3 - 4 true leaf stage (3 weeks post planting) or left uninfected, as described in chapter 5, few modifications. Planting and inoculation of plants with different TuYV genotypes was done at different times in order to avoid cross-contamination.

6.2.2.5 Experimental design and layout

There were a total of 112 experimental units comprising two oilseed rape cultivars by seven TuYV isolates by eight replications; inoculated or mock-inoculated. The treatments were laid out in a Split plot design on a bench, with infected and uninfected plants facing north-south direction on a bench as the main plots. Oilseed rape cultivar (Yudal or Darmor-*bzh*) and the TuYV isolates formed the subplots.

6.2.2.6 Vernalisation of plants

Immediately after inoculation, the plants were vernalised in a cold room at 5 °C under natural light for 10 weeks to induce flowering. Vernalisation was necessary because Darmor-*bzh* is a winter oilseed rape and Yudal is a spring oilseed rape but behaves as an early-flowering winter type.

6.2.2.7 Cultural practice

Plants were watered when necessary. Fungicide (Thiovit at 2.0g/L) was sprayed to control powdery mildew infection in the glasshouse.

6.2.2.8 Test for relative virus infections

The relative amounts of the various virus isolates accumulating in both Yudal and Darmor-*bzh* were determined by TAS-ELISA six weeks post inoculation, according to the method described by D'Arcy *et al.* (1989). The ELISA was carried out as described in section 6.1.2.2. The primary antibody used was rabbit IgG (AS-0049), secondary antiserum was the mouse monoclonal antibody (AS-0049/1), and the tertiary alkaline phosphatase conjugated antiserum was rabbit anti-mouse (RAM-AP), all from DSMZ (Braunschweig, Germany).

Absorbance values ($A_{405\text{nm}}$) were measured with a Biochrom Anthos 2010 microplate reader (Biochrom Ltd., Cambridge, U.K.) as described in section 6.1.2.2. A portion of each leaf sample tested was stored at -80°C for further molecular studies.

6.2.2.9 Statistical analysis

A two-way ANOVA was carried out to assess the significance of cultivar and virus isolates on the virus accumulations in the plants. GenStat Release version 12.1 (Payne *et al.*, 2009) was used for the statistical analysis of the ELISA absorbance data.

6.2.3 Results

The susceptibilities of oilseed rape cvs. Darmor-*bzh* and Yudal to infections of different isolates of TuYV are presented in Table 6.3. Both cultivars were susceptible to the seven TuYV isolates, with absorbance values (levels of virus accumulation) ranging from 0.854 to 1.587 (in Darmor-*bzh*) and 0.308 to 1.334 (in Yudal).

ANOVA revealed that on the average the level of TuYV accumulated in Darmor-*bzh* was significantly higher than that in Yudal ($F_{1, 86} = 47.75$; $P < 0.001$), indication of the susceptibility of Darmor-*bzh* to TuYV infection than Yudal. There was also significant differences in the levels of accumulation of the different TuYV isolates irrespective of the host cultivar ($F_{6, 86} = 19.35$; $P < 0.001$), indicating that the isolates differ on their virulence levels or pathogenicity. Furthermore, the ANOVA showed a significant interaction between cultivar and isolates ($F_{6, 86} = 2.10$; $P < 0.05$),

suggesting that the levels of the various virus isolates accumulated varies between the two cultivars. For instance, L1843 accumulated to the highest levels in both Darmor-*bzh* ($A_{405} = 1.587$) and Yudal ($A_{405} = 1.334$) whereas the lowest mean virus accumulation in Darmor-*bzh* was isolate L1876 ($A_{405} = 0.854$) and isolate L1937 had the lowest level of accumulation in Yudal ($A_{405} = 0.131$).

There were no significant differences in the levels of virus accumulation between Darmor-*bzh* and Yudal infected with L1906, L1808 and L1843 ($P > 0.05$), indicating that these isolates were virulent/pathogenic to both cultivars, suggesting the isolates were able to overcome the partial resistance in Yudal.

The levels of the LAB, L1851, L1876 and L1937 isolates accumulated in Darmor-*bzh* were significantly higher than those in Yudal ($P < 0.05$), indicating that Darmor-*bzh* was more susceptible to these isolates than Yudal. Thus Yudal exhibited partial resistance to these isolates.

Table 6.3 Susceptibility of oilseed rape cultivars Darmor-*bzh* and Yudal to different isolates of *Turnip yellows virus* (TuYV)

<i>Turnip yellows virus</i>		ELISA Absorbance (A405)	
isolate	genotype	Darmor- <i>bzh</i>	Yudal
L1906	3	0.873	0.651 ^a
LAB	3	1.281	0.534*
L1808	1	1.405	1.259
L1843	1	1.587	1.334
L1851	1	1.518	1.025*
L1876	1	0.854	0.308*
L1937	1	0.891	0.131*
Mean		1.201	0.749 ^b

*The difference in mean accumulation of a TuYV isolate between Darmor-*bzh* and Yudal was significant ($P < 0.05$).

^a Least significant difference (LSD) for comparing mean absorbance values between Darmor-*bzh* and Yudal infected with a TuYV isolate was 0.3443 at 86 degrees of freedom.

^b LSD for comparing the overall mean absorbance values between Darmor-*bzh* and Yudal was 0.1301 at 86 degrees of freedom.

6.2.4 Discussion

In the previous experiments (chapter five), Darmor-*bzh* was found to be more susceptible to infection by some TuYV isolates than Yudal, confirming that Yudal has partial resistance to TuYV infection. The levels of TuYV accumulating in Darmor-*bzh* were significantly higher than that in Yudal for four isolates (LAB, L1851, L1876, L1937).

There were higher levels of accumulation of all the seven TuYV isolates in Darmor-*bzh* than Yudal, although the differences were not significant for all isolates. Significant differences between the levels of LAB, L1851, L1876 and L1937

accumulating in Darmor-*bzh* and Yudal were seen. This finding is consistent with the concept of partial or quantitative resistance put forward by Do Vale *et al.* (2001) as resistance that varies in a continuous way between the various phenotypes of the host population, from almost imperceptible (only a slight reduction in the growth of the pathogen) to quite strong (little growth of the pathogen). Partial resistances have also been found in other poleroviruses. For example in the USA, a number of sugar beet lines have been developed that have partial resistance to BWYV (Stevens *et al.*, 2005). Of the 600 accessions of *Beta* species screened, 22 were identified as having partial resistance to BMYV, whilst three accessions showed partial resistance to *Beet chlorosis virus* (BChV) (Asher *et al.*, 2001). Partial resistance of potato to PLRV has also been reported (Barker *et al.*, 1994; Derrick and Barker, 1997).

Results from previous work (chapter 3 and 4) showed that these seven isolates belonged to two different genetic groups, which are common in oilseed rape in England. This quantitative or partial resistance in Yudal is controlled by a single major gene and is heritable (section 6.1) as were observed by Graichen (1998), Dreyer *et al.* (2001) and Juergens *et al.* (2010) for TuYV-resistance in oilseed rape derived from the resynthesised resistant oilseed rape line, R54. TuYV-resistance in Yudal can therefore be transferred into current oilseed rape cultivars by plant breeders or the cultivar can be used as planting material.

It was observed that the partial resistance in Yudal was overcome by three isolates (L1906, L1808 and L1843) belonging to the two different genetic groups. There were no significant differences in the levels of TuYV accumulation between Darmor-*bzh* and Yudal infected with these isolates. This suggests that Yudal does

not possess resistance against broad-spectrum of TuYV genotypes or strains. If Yudal is not resistant to some isolates belonging to the two main genetic groups of TuYV identified in Lincolnshire, Warwickshire and Yorkshire, the use of Yudal as a planting material or a resistance cultivar based on the partial resistance in Yudal will not be effective in controlling TuYV in these regions. Further research should be conducted in search for a broad-spectrum and durable resistance to TuYV as broad-spectrum resistance and durable resistance to diseases are desirable for crop improvement (Kou and Wang, 2010).

CHAPTER 7:

GENERAL DISCUSSION

The increased production of oilseed rape in the UK due to an increasing demand for the crop both as a healthy edible oil and as a renewable source of biodiesel and oleochemicals has resulted in a high disease pressure for major pathogens including viruses. *Turnip yellows virus* (TuYV) is the most important virus disease in oilseed rape which can cause yield losses of up to 45% (Stevens and Clark, 2009; Stevens *et al.*, 2008). Control of this disease is necessary to improve the yields of the crop.

The first aim of this project was to determine the incidence and distribution of TuYV infecting oilseed rape in three regions of England. This study has significantly improved the understanding of the incidence, prevalence, mode of infection and spread of TuYV within oilseed rape crops. The lack of impact of control measures adopted by oilseed rape farmers against TuYV infection has been clear to see from this study. Incidences of TuYV infection were determined in 27 fields in Lincolnshire, Warwickshire and Yorkshire in both autumn and spring of the 2007-8, 2008-9 and 2009-10 crop seasons. TuYV was prevalent in the 26 of the 27 oilseed rape fields sampled from the three regions, with incidences of infection ranging from 0 to 100%, indicating that the virus is prevalent in oilseed rape crops in England. Previous reports have also indicated the widespread incidence of TuYV in oilseed crops in the UK with infected crops identified from the North, West, East, South and central parts of England as well as from Scotland and Wales (Smith and Hinckes, 1985; Hill *et al.*, 1989; Hardwick *et al.*, 1994; Bayer CropScience, 2007, Stevens *et al.*, 2008; Clark and Stevens, 2009).

Most of the fields (18 of the 25 analysed) showed a slightly aggregated pattern of distribution when sampled in autumn, but spring sampling revealed a slightly more random pattern (13 of the 26 fields analysed). This suggests that there were increased infections in most of the fields during spring after initial primary infection in autumn. It is therefore important to control the *Myzus persicae* vector during late autumn (in December) if numbers of the vector increase at this time. This is necessary to prevent secondary spread or re-infection, because the seed treatment can only offer protection for the crops for up to 10 weeks after sowing (Dewar *et al.*, 2011).

The Modesto chemical constituents and Chinook chemical constituents treated seeds and the foliar insecticide application which were mainly pyrethroids (cypermethin, deltamethrin and bifenthrin) used in the oilseed rape crops sampled in my study were not effective in controlling TuYV. There was a significant relationship between cumulative numbers of *M. persicae* caught in the Rothamsted Insect Survey suction traps closest to the fields that were sampled between August and November and the mean percentage TuYV incidence in oilseed rape crops in the autumn of each year in the three regions. This suggests that whenever the aphid population was high, TuYV incidence was also high irrespective of seed treatment or foliar spray. This may explain the high levels of incidences of TuYV infections (up to 100% incidence) recorded in 2009-10 crop season compared to the lowest levels of infection recorded in the previous crop season of 2008-9.

A striking revelation from this study was the fact that oilseed rape farmers are still planting Chinook treated seed instead of the Modesto or Cruiser OSR treated seeds

which are supposed to offer better protection than Chinook. In field plot trials carried out by Dewar *et al.* (2011), Cruiser OSR (thiamethoxam + fludioxinil + mefenoxam) and Modesto gave significantly better control of *M. persicae* for up to 10 weeks after sowing compared to Chinook which was significantly poorer and less persistent. Field trials carried out at Brooms Barn with Modesto gave a 0.4 t/ha yield increase in 2007 and 0.7 t/ha in 2008, and was more persistent than the previous standard Chinook (Blake, 2009). Farmers should therefore be encouraged to plant Modesto- or Cruiser OSR-treated seeds instead of Chinook. Nevertheless, TuYV incidence in oilseed rape crops planted with Modesto treated seeds in August 2009, followed by cypermethrin (e.g. Lincolnshire field 3 of the 2009-10 crop season) were still very high (96%) when sampled in December 2009. This observation is further confirmation of the ineffectiveness of both seed treatment and insecticide spray in controlling TuYV infection when infection pressures are high. With infection levels of up to 100% recorded in this study and yield losses of up to 45% reported by Stevens and Clark (2009), it is very important to urgently find the most effective means of controlling TuYV infection in order to increase oilseed rape yields and improve food security.

A more effective control strategy against TuYV infection would be the introgression of resistances into current breeding lines (Dreyer *et al.*, 2001). However, broad-spectrum resistance and durable resistance to diseases are preferable for crop improvement (Kou and Wang, 2010). Durability is also favoured if the resistance gene(s) is / are effective against the full range of variants of the virus occurring in the area of cultivation (Garcia-Arenal and McDonald, 2003). A resistant oilseed rape variety should preferably be resistant to all strains of TuYV present in the oilseed

rape producing regions of Europe. However, prior to this study nothing was known about the molecular variability of TuYV infecting oilseed rape in England. The second aim of this project was to determine the molecular diversity and genetic structure in TuYV infecting oilseed rape in England based on sequence comparisons of P0 and P3 genes of the virus. Two main genetic groups and a recombinant group were detected in the oilseed rape crops surveyed in the regions (Lincolnshire, Warwickshire and Yorkshire) between 2007 and 2010. These genetic groups occurred in all the regions, over the three crop seasons. Genetic group 1 was the most common in the three regions, comprising 86.3% of the total number of the field isolates analysed. This was followed by genotype 3 (13.7%) and then the group 2 (the recombinants), comprising only 3.1% of the total number of field isolates analysed. Therefore for more durable control of TuYV in these regions, oilseed rape breeders should develop a variety which is resistant to these three genetic groups of TuYV or at least the two major genetic groups.

The study also showed that TuYV has high rates of mutation and frequent recombination events resulting in genetic variation. Mutation and recombination are the two main types of errors which bring about genetic variation in viruses (Garcia-Arenal *et al.*, 2003). It is therefore important for plant virologists and oilseed rape breeders to periodically assess the effectiveness of any introduced TuYV-resistant oilseed rape variety in controlling the TuYV infection since according to Garcia-Arenal *et al.* (2001) an evolution of resistance breaking pathotypes will render the control measures ineffective. There was more variability in the P0 gene of TuYV than the P3 gene. Hence for the P0 gene sequence dataset was selected for the analysis of spatial and temporal genetic structure of TuYV infecting oilseed rape in

the three regions surveyed. It has been reported that the P0 gene of poleroviruses is more variable than the coat protein gene (Hauser *et al.*, 2000a).

Despite the greater number of mutations, high haplotype diversity and frequent recombination events, the genetic diversity detected was low. This suggests that most mutations observed were not of adaptive nature but eliminated by purifying selection. This confirms the assertion that analysed populations of plant viruses are relatively genetically stable and this is so regardless of the many haplotypes that may occur in the population (Garcia-Arenal *et al.*, 2001). The coat protein was found to be under stronger purifying selection than the P0 protein. This explains the higher diversity of the P0 gene than the coat protein gene.

The study identified high haplotype diversity of TuYV in Lincolnshire, Warwickshire and Yorkshire. The TuYV populations were highly structured between these regions with only a limited gene flow between them. Of the 174 haplotypes identified, only three were shared between the three regions, an indication of limited spread of the virus between the regions. This suggests that a regional based control strategy may be more effective against TuYV infection of oilseed rape. On the other hand, any control strategy developed should be evaluated for effectiveness in these oilseed rape growing regions, before recommending to farmers.

It will be necessary for virologists and plant breeders to evaluate the effectiveness of control measures such as growing TuYV-resistant oilseed rape varieties against TuYV isolates belonging to the three genetic groups identified in my earlier study. It was therefore important to isolate and identify these genotypes from oilseed rape

crops and then propagate pure cultures for subsequent plant resistance tests. The third aim of this project was to develop a RT-PCR-based method for rapid detection and differentiation of the TuYV isolates belonging to different genetic groups. PCR-based methods have been developed for detection and discrimination of members of the family *Luteoviridae* (Chomic *et al.*, 2010; Chomic *et al.*, 2010b; Chomic *et al.*, 2010b; Mayo and D'Arcy, 1999) but there was no protocol for differentiating the genotypes of TuYV. Three RT-PCR assays based on genotype-specific primers have been developed for the discrimination of the three TuYV genotypes. These assays successfully detected and differentiated isolates belonging to the two main genetic groups (groups 1 and 3) from oilseed rape samples collected from Lincolnshire. Isolates belonging to the smallest genetic group (i.e. the recombinants) was not detected and so future work should aim at detecting and isolating it in the field for subsequent resistant tests. Efforts should also be made to identify the genetic diversity and structure of TuYV infecting oilseed rape in other parts of Europe.

The fourth aim of this study was to identify *B. napus* lines with resistance to TuYV infection. This was achieved by screening 27 accessions of a *B. napus* diversity fixed foundation set with a laboratory isolate of TuYV (LAB) belonging to genetic group 3 (the only isolate available at the time of the experiment). The accessions showed continuous variation in susceptibility to TuYV infection, as measured by ELISA absorbance, indicating that they were all infected with TuYV. This confirmed the report of Stevens (2010) which states that there is currently no known varietal resistance to TuYV. However, one accession (Yudal) showed a low level of virus accumulation, suggesting that it had partial resistance to TuYV infection. The TuYV resistance in Yudal represented a quantitative reduction in virus accumulation as

found in TuYV resistance in a resynthesised oilseed rape line, R54 (Juergens *et al.*, 2010) and in resistance to other luteoviruses such as *Barley yellow dwarf virus* (Niks *et al.*, 2004).

The fifth aim of the study was to determine the effect of TuYV on the growth and yield fitness of Yudal and four other oilseed rape lines (i.e. Castille, Rafal, Tapidor, and Westar). Because oilseed rape is an annual plant, the effect of TuYV infection over its lifetime fitness could be estimated by total seed yield, as was done by Kover and Schaal (2002) when studying genetic variation for disease resistance and tolerance in *A. thaliana*. Yield losses of up to 44.7% were recorded for the susceptible variety (i.e. Castille), comparable to the yield losses of up to 45% reported by Stevens and Clark (2009). This suggests that TuYV is a serious threat to oilseed rape production in England. The results also confirmed the partial resistance of Yudal to TuYV infection described in chapter five (section 5.1). TuYV infection did not have a significant effect on the growth and seed yield of Yudal, indicating that Yudal possesses a gene, or genes which can reduce the impact of TuYV on its fitness. It has been reported that pathogens usually affect fitness-related trait, and hence have important economical effects on crops by reducing growth and yield (Wolfe, 2000). To my knowledge, this is the first report of an oilseed rape variety in England having some degree of resistance to TuYV infection.

Because the resistance identified in Yudal was partial or quantitative, the sixth aim was to seek qualitative or complete resistance to TuYV in *A. thaliana*. Consequently, 20 *A. thaliana* accessions were screened for resistance to TuYV. Unfortunately, all the accessions were very susceptible to TuYV, even though they varied in their

degree of susceptibility to TuYV infection as measured by ELISA absorbance values (chapter 5.3).

Since none of the *A. thaliana* lines was resistant to TuYV infection, it was decided to conduct further studies into the partial resistance identified in Yudal. Although quantitative traits (such as the partial resistance to TuYV in Yudal) confer only moderate resistance, it may nevertheless be extremely valuable within an agronomic context, since improvement in crop yield of only few per cent can provide the difference between profit and loss (Maule *et al.*, 2007). Partial resistance can be more durable than monogenic qualitative resistance especially in the face of RNA viruses such as TuYV with high genetic variation. The seventh aim of the studies was to identify the QTL involved in the partial resistance of Yudal to TuYV infection and to define a set of linked markers to position the gene on a *B. napus* linkage map. A major QTL for TuYV resistance was detected on chromosome C4 (N14) explaining up to 50.5% of the phenotypic variation, suggesting a possible single gene effect. An interesting feature of this work was that the markers linked to the QTL revealed fragments inherited from the resistant parent (i.e. Yudal). These markers will be valuable in future marker assisted selection (MAS) work. This work and that of Graichen (1998), Dreyer *et al.* (2001) and Juergens *et al.* (2010) have clearly demonstrated that TuYV-resistance is controlled by single major gene. The partial resistance to TuYV identified in Yudal and the subsequent QTL for TuYV resistance identified in the Darmor-*bzh* x Yudal DH population were identified using one TuYV isolate, (LAB, belonging to genetic group 3). Since the resistance identified in Yudal could potentially be introgressed into current oilseed rape cultivars, it was important to assess this resistance against different TuYV isolates of

the same and different genetic groups to that of the LAB isolate. Consequently, the study assessed the spectrum of TuYV resistance in Yudal by challenging it with the seven different isolates belonging to two different genetic groups, previously isolated and maintained as pure cultures in an insectary. Yudal showed partial resistance to only four isolates (LAB, L1851, L1876, L1937) but the resistance was overcome by three isolates (L1906, L1808 and L1843), suggesting that the partial resistance in Yudal was not broad-spectrum type. Quantitative resistance is characterised by partial and durable effect of resistance that is generally pathogen species non-specific or race non-specific (Kou and Wang, 2010; Wisser *et al.*, 2005).

This study has made considerable advances in the knowledge of genetic diversity and population structure and evolutionary dynamics of TuYV infecting oilseed rape in three regions of England. It has also broadened the understanding on the host – TuYV interaction patterns and the problems associated with control of the virus disease. This provides a framework necessary for future oilseed rape breeding programmes and other strategies for effective management of TuYV in oilseed rape crops.

CONCLUSIONS

This study has led to the following conclusions:

1. TuYV infection was prevalent in the three regions surveyed; the virus was detected in 26 of the 27 fields sampled.
2. There were large differences in the incidences of TuYV in the various fields, counties and crop seasons surveyed, ranging from 0% (recorded in autumn

and spring in Warwickshire in 2008-9) to 100% (recorded in Lincolnshire in the autumn of 2009).

3. The levels TuYV infection of winter oilseed rape in both autumn and spring sampling times were highest in Lincolnshire, followed by Warwickshire whilst Yorkshire had the lowest. Lincolnshire is thus a hot spot of TuYV infection.
4. The highest cumulative (August to November) trap catches of *M. persicae* during the three crop seasons occurred in Lincolnshire and the lowest in Yorkshire; catches in the 2009-10 were the highest and those in 2008-9 were lowest.
5. There was a close relationship between the cumulative flight activities of *M. persicae* between August and November and the autumn incidence of TuYV. Thus incidence of TuYV infection was high when the flight activities of the *M. persicae* vector were high, irrespective of insecticidal seed treatments or sprays applied.
6. Most of the oilseed rape fields showed a slightly aggregated pattern of distribution when sampled in autumn, but spring sampling revealed a slightly more random pattern. This indicated that there were increased infections in some of the fields during spring after initial primary infection in autumn.
7. Phylogenetic analysis of both nucleotide and amino acid sequences showed that TuYV isolates could be divided into two (for P3 gene) or three (for P0 gene) genetic groups (evolutionary divergent lineages) irrespective of the geographical origin or year of sampling. The P0 gene was more diverse than the P3 gene. Even though both P0 and P3 proteins were under purifying

(negative) selection, the P3 protein was under stronger selective constraint than the P0 protein.

8. The mean rate of nucleotide substitution among all isolates of TuYV ranged from 9.4×10^{-4} to 9.6×10^{-4} subs/site/year and from 1.8×10^{-3} to 2.1×10^{-3} subs/site/year for P0 and P3 genes respectively.
9. TuYV populations in the three regions Lincolnshire, Warwickshire and Yorkshire, were highly structured with limited movement of populations between them. Thus most of the variation observed occurred within population at a particular locality or field.
10. Three RT-PCR assays developed were useful for rapid and reliable detection and differentiation of different genotypes of TuYV identified in the study. This is the first record of a PCR-based method for differentiation of TuYV genotypes using genotype specific-primers.
11. All the 27 accessions of the *B. napus* DFFS were susceptible to TuYV but varied in the degree of susceptibility to TuYV infection. However, one accession, Yudal showed partial or quantitative resistance to TuYV infection in terms of virus accumulation. The virus caused yield losses of up to 44.7% in a susceptible cultivar (Castille).
12. All 20 accessions of *A. thaliana* were severely infected with TuYV, indicating that *A. thaliana* is a good host of the virus.
13. Even though TuYV infection reduced the plant fitness in oilseed rape and *A. thaliana* accessions (seed production/seed yield/growth), the levels of virus accumulation was not correlated with the amount of fitness loss (i.e. yield loss).

14. A major QTL for TuYV resistance was detected on chromosome C4 (N14) of oilseed rape cultivar Yudal, explaining up to 50.5% of the phenotypic variation, suggesting a possible single gene effect. The TuYV-resistance in Yudal appears to be controlled by a single major gene along with additional contributing genes.

FUTURE WORK

Suggested further work includes:

1. Identification of TuYV isolates belonging to genetic group 2 (i.e. the recombinants) from oilseed rape crops in at least one of the three regions sampled by using the RT-PCR assay with genotype specific primers and assessing their infection of Yudal.
2. Further determination of the genetic diversity of TuYV infecting oilseed rape in the UK and the rest of Europe by sequence analysis of the P0 and P3 genes of TuYV isolates from all the major oilseed rape growing regions.
3. Identification of different sources of TuYV resistance by screening more accessions of the *B. napus* diversity fixed foundation sets and other brassica crops.
4. Introgression of the resistance in Yudal into current oilseed rape cultivars using marker-assisted selection. Back crossing TuYV-resistant DH lines with the susceptible parent (Darmor-*bzh*) will allow further dissection of the QTL detected.

REFERENCES

a. Refereed references

A'Brook, J. (1973). The effect of plant spacing on the number of aphids trapped over cookfoot and kale crops. *Annals of Applied Biology*, 74, 279-285.

Agnew, P., Koella, J. C. and Michalakis, Y. (2000). Host life-history responses to parasitism. *Microbes and Infections*, 8, 891-896.

Akaike, H. (1974). A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, 19, 716-723

Akbar, M. A. (1989). Chromosomal stability and performance of resynthesised *Brassica napus* produced for gain in earliness and short day response. *Hereditas*, 111, 247-253.

Ala-Poikela, M., Svensson, E., Rojas, A., Horko, T., Paulin, L., Valkonen, J. P. T. and Kvarnheden, A. (2005). Genetic diversity and mixed infections of begomoviruses infecting tomato, pepper and cucurbit crops in Nicaragua. *Plant Pathology*, 54, 448-459.

Armitage, P. and Berry, G. (1994). Statistical methods in medical research (Oxford: Blackwell Science).

Asher, M. J. C., Luterbacher, M. and Frese, L. (2001). Wild *Beta* species as a source of resistance to sugar-beet pests and diseases. *International Sugar Journal*, 103, 447-451.

Balaji, B., Bucholtz, D. D. and Anderson, J. M. (2003). Barley yellow dwarf virus and Cereal yellow dwarf virus quantification by real-time polymerase chain reaction in resistance and susceptible plants. *Phytopathology*, 93, 1386-1392.

Barker, H., Solomon-Blackman, R. M., McNicol, J. W. and Bradshaw, J. E. (1994). Resistance to *Potato leafroll virus* multiplication in potato is under major gene control. *Theoretical and Applied Genetics*, 88, 754-758.

Barker, H. and Harrison, B. D. (1985). Restricted multiplication of *Potato leafroll virus* in resistant potato genotypes. *Annals of Applied Biology*, 107, 205-212.

Barker, H. and Harrison, B. D. (1984). *Polygenic resistance to Potato leafroll virus (PLRV)*. In: Annual Report Scottish Crop Research Institute. 194-195.

- Barr, J. N. and Fearn, R. (2010). How RNA viruses maintain their genome integrity. *Journal of General Virology*, 91, 1373 - 1387.
- Basu, P. K., Lin, C. S. and Binns, M. R. (1977). A comparison of sampling methods for surveying alfalfa foliage diseases. *Canadian Journal of Plant Science*, 57, 1091-1097.
- Beachy, R. N., Abel, P. P. and Oliver, M. J. (1986). Potential for applying genetic information to studies of virus pathogenesis and cross protection. In *Biotechnology in Plant Science: Relevance to Agriculture in the Eighties*, pp. 265-275. Edited by M. Zaitlin, P. Day and S. Hollaender. Orlando, FL: Academic Press.
- Beachy, R. N., Loesch-Fries, S. and Tumer, N. E. (1990). Coat protein-mediated resistance against virus infection. *Annual Review of Phytopathology*, 28, 451-474.
- Berry, P. M. and Spink, J. (2006). A physiological analysis of oilseed rape yields: Past and future. *Journal of Agricultural Science*, 144, 381-392
- Birch, R. G. (1997). Plant transformation: Problems and strategies for practical application. *Annual Review of Plant Physiology and Plant Molecular Biology*, 48, 297-326.
- Bittner-Eddy, P. D., Crute, I. R., Holub, E. B. and Benyon, J. (2000). *RPP13* is a simple locus in *Arabidopsis thaliana* for alleles that specify downy mildew resistance to different avirulence determinants in *Peronospora parasitica*. *Plant Journal*, 21, 177-188.
- Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P. and Ziegler-Graff, V. (2007). The Pulerovirus F Box Protein P0 Targets ARGONAUTE1 to Suppress RNA Silencing. *Current Biology*, 17, 1615-1621.
- Bourdon, J. J. (1987). *Diseases and plant population biology*. In: Cambridge, UK, Cambridge, UK: Cambridge University Press.
- Braut, V., Perigon, S., Reinbold, C., Erdinger, M., Scheidecker, D., Herrbach, E., Richards, K. and Ziegler-Graff, V. (2005). The polerovirus minor capsid protein determines vector specificity and intestinal tropism in the aphid. *Journal of Virology*, 79, 9685-9693.
- Broman, K. W. and Sen, S. (2009). *A guide to to QTL mapping with R/qtl: Statistics for biology and health*. In: New York, 396. New York: Springer.
- Broman, K. W., Wu, H., Sen, S. and Churchill, G. A. (2003). R/qtl: QTL mapping in experimental crosses. *Bioinformatics*, 19, 889-890.
- Bruening, G. (2006). Resistance to infection, pp. 211-240. Edited by G. Loebenstein and J. P. Carr. The Netherlands: Springer.

- Buckland, S. T., Anderson, D.R., Burnham, K.P., Laake, J.L., Borchers, D.L. and Thomas, L. (2001). Introduction to Distance Sampling (Oxford: Oxford University Press).
- Burland, T. G. (2000). DNASTAR's Lasergene sequence analysis software. *Methods in Molecular Biology*, 132, 71-91.
- Castle, S. J., Mowry, T. M. and Berger, P. H. (2008). Differential settling by *Myzus persicae* (Homoptera; Aphididae) on various virus infected host plants. *Annals of Entomological Society of America*, 9, 661-667.
- Christie, P., Richner, H. and Oppliger, A. (1996). Begging, food provisioning, and nestling competition in great tit broods infested with ectoparasites. *Behavioural Ecology*, 7, 127-131.
- Chare, E. R. and Holmes, E. C. (2006). A phylogenetic survey of recombination frequency in plant RNA viruses. *Archives of Virology*, 151, 933-946.
- Chomic, A., Pearson, M. N., Clover, G. R. G., Farreyrol, K., Saul, D., Hampton, J. G. and Armstrong, K. F. (2010a). A generic RT-PCR assay for the detection of Luteoviridae. *Plant Pathology*, 59, 429-442.
- Chomic, A., Winder, L., Armstrong, K. F., Pearson, M. N. and Hampton, J. G. (2010b). Detection and discrimination of members of the family *Luteoviridae* by real-time PCR and SYBR GreenER melting curve analysis. *Journal of Virological Methods*, 171, 46-52.
- Clark, B. and Stevens, M. (2009). Strategies to reduce the impact of *Turnip yellows virus* on oilseed rape production in the UK. In: *HGCA Research and Development, Annual Report, 2009 results*. Stoneleigh Park, Kenilworth, UK: Home-Grown Cereal Authority.
- Clarke, J., Wynn, S., Twining, S., Berry, P., Cook, S., Ellis, S. and Gladders, P. (2009). *Pesticide availability for cereals and oilseeds following revision of Directive 91/414/EEC; effects of losses and new research priorities*. In: HGCA Research Review No. 70. March 2009. ADAS Boxworth, Cambridge, ADAS Boxworth, Cambridge.
- Cliff, A. D. and Ord, J. K. (1969). *The problem of spatial autocorrelation*. In: London Papers in Regional Science. London, 25-55. London: Pion.
- Coutts, B. A. and Jones, R. A. C. (2000). Viruses infecting canola (*Bassica napus*) in south-west Australia: incidence, distribution, spread, and infection reservoir in wild radish (*Raphanus raphanistrum*). *Australian Journal of Agricultural Research*, 51, 925-936.
- Crasta, O. R., Franki, M. G., Bucholtz, D. B., Sharma, H. C., Zhang, J., Wang, R. C., Ohm, H. W. and Anderson, J. M. (2000). Identification and characterisation of wheat-wheatgrass translocation lines and localisation of *Barley yellow dwarf virus* resistance. *Genome*, 43, 698-706.

Crute, I. R. and Pink, D. A. C. (1996). Genetics and utilisation of pathogen resistance in plants. *The Plant Cell*, 8, 1747-1755.

Daniells, J. W., Geering, A. D. W., Bryde, N. J. and Thomas, J. E. (2001). The effect of *Banana streak virus* on the growth and yield of dessert bananas in tropical Australia. *Annals of Applied Biology*, 139, 51-60.

D'Arcy, C. A. and Domier, L. L. (2005). *Luteoviridae*. In *Virus Taxonomy: Eight Report of the International Committee on Taxonomy of Viruses*, p. 1259. Edited by M.A. Mayo, C.M. Fauquet, J. U, Desselberger, L.A. Ball. Oxford, UK: Elsevier Inc.

D'Arcy, C. J., Tolerance, L. and Martin, R. R. (1989). Discrimination among luteoviruses and their strains by monoclonal antibodies and identification of common epitopes. *Phytopathology*, 79, 869-873.

Dawson, W. O. and Hilf, M. E. (1992). Host-range determinants of plant viruses. *Annual Review of Plant Physiology and Plant Molecular Biology*, 43, 527-555.

Degenhart, D. F. and Kondra, Z. P. (1984). Relationships between seed yield and growth characters, yield components, seed quality of summer-type oilseed rape (*Brassica napus* L.). *Euphytica*, 33, 885-889.

de Miranda, J. R., Stevens, M., de Bruyne, E., Smith, H. G., Bird, C. and Hull, R. (1995). Beet luteovirus coat protein sequence variation. *Annals of Applied Biology*, 127, 113-124.

Derrick, P. M. and Barker, H. (1997). Short and long distance spread of *Potato leafroll luteovirus*: effects of host genes and transgenes conferring resistance to virus accumulation in potato. *Journal of General Virology*, 78, 243-251.

Dewar, A. M., Tait, M. F. and Stevens, M. (2011). Efficacy of thiamethoxam seed treatment against aphids and *Turnip yellows virus* in oilseed rape. *Aspects of Applied Biology 106, Crop Protection in Southern Britain*, 106, 195-202.

Diedrichsen, E. and Sacristan, M. D. (1991). *Resynthesis of amphidiploid Brassica species and their clubroot disease reactions. Proceeding of GCIRC 1991 Congress*, 1, 274-279.

Dixon, G. R. (2007). Vegetable brassicas and related crucifers (Wallingford, Oxfordshire, UK: CABI).

Dogimont, C., Bussemakers, A., Martin, J., Slama, S., Lecoq, H. and Pitrat, M. (1997). Two complementary recessive genes conferring resistance to *Cucurbit aphid borne yellows luteovirus* in an Indian melon line (*Cucumis melo* L.). *Euphytica*, 96, 391-395.

Domingo, E. and Holland, J. J. (1997). RNA virus mutations and fitness for survival. *Annual Review of Microbiology*, 51, 151-178.

- Domingo, E. and Holland, J. J. (1994). Mutation rates and rapid evolution of RNA viruses. In *The evolutionary biology of viruses*, pp. 161-184. Edited by S. S. Morse. New York, NY: Raven Press, Ltd.
- Donovan, G. M. (2000). *Characterisation of pathotypes of Bean common mosaic virus (BCMV)*. In: Doctor of Philosophy, University of Birmingham.
- Do Vale, F. X. R., Parlevliet, J. E. and Zambolim, L. (2001). Concepts in plant disease resistance. *Fitopatologia Brasileira*, 26, 577-589.
- Drake, J. W. and Holland, J. J. (1999). *Mutation rates amongs among RNA viruses*. USA, 96, 13910-13913. National Academy of Science, USA.
- Drake, J. W., Charlesworth, B., Charlesworth, D. and Crow, J. F. (1998). Rates of spontaneous mutation. *Genetics*, 148, 1667-1686.
- Dreyer, F., Graichen, K. and Jung C. (2001). A major quantitative trait locus for resistance to *Turnip yellows virus* (TuYV, syn. *Beet western yellows virus*, BWYV) in rapeseed. *Plant Breeding*, 120, 457-462.
- Drijfhout, E. (1978). *Genetic interaction between Phaseolus vulgaris L. and bean common mosaic virus with implications for strain identification and breeding for resistance* In: Cent. Agric. Publ. Doc. Pudoc, Wageningen, Netherlands, Pudoc, Wageningen, Netherlands: Agricultural Research Rep. 872,.
- Drummond, A. J. and Rambaut, A. (2007). BEAST:Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, 7, 214.
- Duffus, J. E. (1961). Economic significance of *Beet western yellows virus* (Radish yellows) on sugar beet. *Phytopathology*, 120, 605-607.
- Duffus, J. E. and Russell, G. E. (1970). Serological and host range evidence for the occurrence of *Beet western yellows virus* in Europe. *Phytopathology*, 60, 1199-1202.
- Duffy, S., Shackelton, L. A. and Holmes, E. C. (2008). Rates of evolutionary change in viruses: patterns and determinants. *Nature Reviews Genetics*, 9, 267-276.
- Eckel, R. V. W., Lambert, E.P. (1993). Spatial and temporal analysis of tobacco etch virus distribution and its relationship to aphid (Homoptera:Aphidae) vectors in flue-cured tobacco. *Journal of Economic Entomology*, 86, 1534-1545.
- Eigen, M., McCaskill, J. and Schuster, P. (1988). Molecular quasi-species. *Journal of Physical Chemical*, 92, 6881-6891.
- Excoffier, L., Laval, G. and Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, 1, 47-50.
- Falk, A., Feys, B. J., Frost, L. N., Jones, J. D., Daniels, M. J. and Parker, J. E. (1999). EDS1, an essential component of R gene-mediated disease resistance in

Arabidopsis has homology to eukaryotic lipases. *Proceedings of National Academy of Science, USA*, 96, 3292-7.

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annual Review of Phytopathology*, 9, 275-296.

Foisset, N., Delourme, R., Barret, P. and Renard, M. (1995). Molecular tagging of the dwarf *Breizh* (*Bzh*) gene in *Brassica napus*. *Theoretical and Applied Genetics*, 91, 756-761.

Foisset, N., Delourme, R., Lucas, M.-O. and Renard, M. (1997). In vitro androgenesis and segregation distortion in *Brassica napus* L.: spontaneous versus colchicine-doubled lines. *Plant Cell Reports*, 16, 464-468.

Foster, S. P., Devine, G. and Devonshire, A. L. (2007). Insecticide Resistance. In *Aphids as crop pests*, p. 717. Edited by H. F. van Emden and R. Harrington. Wallingford, Oxfordshire, UK: CAB International.

Fraser, R. S. S. (1992). The genetics of plant-virus interactions: implications for plant breeding. *Euphytica*, 63, 175-185.

Fraser, R. S. S. (1990). The genetics of resistance to plant viruses. *Annual Review of Phytopathology*, 28, 179-200.

Fuchs, M. and Gonsalves, D. (1995). Resistance of transgenic hybrid squash ZW-20 expressing coat protein genes of *Zucchini yellow mosaic virus* and *Watermelon mosaic virus 2* to mixed infections by both potyviruses. *Nature Biotechnology*, 13, 1466-1473.

Fu, Y.-X. and Li, W.-H. (1993). Statistical tests of neutrality of mutations. *Genetics*, 133, 693-709.

Gal-On, A. and Shibolet, Y. M. (2006). Cross-Protection. In *Natural resistance mechanisms of plants to viruses*, pp. 261-288. Edited by G. Loebenstein and J. P. Carr. The Netherlands: Springer.

Garcia-Arenal, F. and McDonald, B. A. (2003). An analysis of the durability of resistance to plant viruses. *Phytopathology*, 93, 941-952.

Garcia-Arenal, F., Fraile, A. and Malpica, J. M. (2001). Variability and genetic structure of plant virus populations. *Annual Review Phytopathology*, 39, 157-186.

Gibbs, M. and C Gibbs, A. J., Fargette, D., Garcia-Arenal, F. and Gibbs, M. J. (2010). Time - the emerging dimension of plant virus studies. *Journal of General Virology*, 91, 13-22.

Gibbs, A., Gibbs, M., Ohshima, K., García-Arenal, F., Domingo, E., Parrish, C. R. and Holland, J. J. (2008). More About Plant Virus Evolution: Past, Present, and Future. In *Origin and Evolution of Viruses (Second Edition)*, pp. 229-250. Edited by. London: Academic Press.

- Gibbs, M. and Cooper, J. (1995). A recombinant event in the history of luteoviruses probably induced by base-pairing between genomes of two distinct viruses. *Virology*, 206, 4.
- Gibbs, M. (1995). *The luteovirus supergroup: rampant recombination and persistent partnerships*. In: Molecular Basis of Virus Evolution. Cambridge, UK, pp. 351-368. Cambridge, UK: Cambridge University Press.
- Gibson, R. W. (1983). *The ability of different pyrethroids to control spread of potato viruses by aphids*. In: 10th International Congress of Plant Protection 1983. Brighton, UK, 31, 1192.
- Gildow, F. E. (1999). Luteovirus transmission and mechanisms regulating vector specificity, H. G. Smith and H. Baker, eds. (Oxford: CAB International).
- Gilligan, C. A., Pechan, P.M., Day, R., and Hill, S.A. (1980). *Beet western yellows virus* on oilseed rape. *Plant Pathology*, 29, 53.
- Gladders, P. (2009). *Relevance of verticillium wilt (Verticillium longisporum) in winter oilseed rape in the UK*. In: HGCA Research Review 2009. Wallingford, Oxfordshire, UK, 39. Wallingford, Oxfordshire, UK:
- Gland, A. (1980). *Glucosinatgehalt und-muster in in den Samen resynthetisierter Rapsformen*. In: Gottingen, Germany, Gottingen, Germany: Georg-August Univ.
- Gland, A., Robbelen, G. and Thies, W. (1981). Variation of alkenyl glucosinolates in seeds of *Brassica* species. *Z Pflanzenzuchtg*, 87, 96-110.
- Graichen, K. (1998). Einfluß von Umweltbedingungen auf die Symptom- und Resistenzausprägung von Winterraps nach Inokulation mit dem *Turnip yellows virus*. *Mitteilungen aus der Biologische Bundesanstalt*, 357, 291-292.
- Graichen, K. (1994). Nachweis von Resistenz gegenüber dem *Turnip yellows luteovirus* (TuYV) in Winterraps und verwandten. *Vortr Pflanzenzuchtung*, 30, 132-143.
- Graichen, K. and Peterka, H. (1999). *Generation of oilseed rape with resistance to turnip yellows luteovirus*. In: 10th International Rapeseed Congress. Camberra, ACT, 1-13. International Consultative Group for Rapeseed Research.
- Graichen, K. and Peterka, H. (1995). Evidence of resistance to *Beet western yellows virus* in oilseed rape. *Züchtungsforsch. Berichte Bundesanstalt Züchtungsforsch. Kulturpflanzen*, 1, 87-90.
- Graichen, K., Rabeinstein, F. and Kurtz, E. (2000). The occurrence of *Turnip yellows virus* in winter oilseed rape in Austria. *Pflanzenschutzberichte*, 59, 35-46.
- Graichen, K. and Rabenstein, F. (1996). European isolates of *Beet western yellows virus* (BWYV) from oilseed rape (*Brassica napus* L. ssp. *napus*) are non-pathogenic

on sugar beet (*Beta vulgaris* L. *altissima*) but represent isolates of *Turnip yellows virus* (TuYV). *Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection*, 103, 233-245.

Graichen, K. and Schliephake, E. (1999). *Infestation of winter oilseed rape by Turnip yellows luteovirus and its effect on yield in Germany*. In: 10th International Rapeseed Congress. Camberra, ACT,31-136. International Consultative Group for Rapeseed Research.

Grant, M. R., Godiard, L., Straube, E., Ashfield, T. and Lewald, J. (1995). Structure of the *Arabidopsis RPM1* gene enabling dual specificity disease resistance. *Science*, 269, 843-846.

Gray, S. M. and Gildow, F. E. (2003). Luteovirus-aphid interactions. *Annual Review of Phytopathology.*, 41, 539-566.

Grimmer, M. K., Bean, K. M. R., Luterbacher, M. C., Stevens, M. and Ashser, M. J. C. (2008). *Beet mild yellowing virus* resistance derived from wild and cultivated Beta germplasm. *Plant Breeding*, 127, 315-318.

Guo, D.-P., Guo, Y.-P., Zhao, J.-P., Liu, H., Pen, Y., Wang, Q.-M., Chen, J.-S. and Rao, G.-Z. (2004). Photosynthetic rate and chlorophyll fluorescence in leaves of stem mustard (*Brassica juncea* var. tsatsai) after *Turnip mosaic virus* infection. *Plant Science*, 168, 57-63.

Gutierrez-Aguirre, I., Mehle, N., Delic, D., Gruden, K., Mumford, R. and Ravnkar, M. (2009). Real-time quantitative PCR based sensitive detection and genotype discrimination of *Pepino mosaic virus*. *Journal of Virological Methods*, 162, 46-55

Hahn, S. K. (1979). Effects of viruses (SPVD) on growth and yield of sweet potato. *Experimental Agriculture*, 15, 253-256.

Hall, T. (2005). *Bioedit*. CA, Carlsland, CA: Ibis Theurapeutics.

Hall, G. S. and Little, D. P. (2007). Relative quantification of virus population size in mixed genotype infections using sequencing chromatograms. *Journal of Virological Methods*, 146, 22-28.

Hardwick, N. V., Davies, J. M. and Wright, D. M. (1994). The incidence of three virus diseases in England and Wales in the 1991/92 and 1992/93 seasons. *Plant Pathology*, 43, 1045-1049.

Hardwick, N. V., Davies, J. M. L., Gladders, J. H. and Slawson, D. D. (1989). Incidence and severity of fungal diseases of winter oilseed rape in England and Wales, 1986-1988. In *Aspects of Applied Biology 23, Poroduction and Protection of Oilseed Rape and other Brassica Crops*, pp. 383-400. Edited by M. F. B. Dale, A. M. Dewar, R. J. Froud-Williams, T. J. Hocking and B. L. Rea. Wellesbourne, Warwick, UK: The Association of Applied Biologists.

- Harrison, B. D. (2002). Virus variation in relation to resistance-breaking in plants. *Euphytica*, 124, 181-192.
- Hauser, S., Stevens, M., Mougél, C., Smith, H. G., Fritsch, C., Herrbach, E. and Lemaire, O. (2000a). Biological, Serological, and Molecular Variability Suggests Three Distinct Polerovirus Species Infecting Beet or Rape. *Phytopathology*, 90, 460-466.
- Hauser, S., Weber, C., Vetter, G., Stevens, M., Beuve, M. and Lemaire, O. (2000b). Improved detection and differentiation of poleroviruses infecting beet or rape by multiplex RT-PCR. *Journal of Virological Methods*, 89, 11-21.
- Hasegawa, M., Kishino, H. and Yano, T. (1985). "Dating of human-ape splitting by a molecular clock of mitochondrial DNA". *Journal of Molecular Evolution*, 22, 160-174.
- Hill, S. A., Lane, A. and Hardwick, N.V. (1989). The incidence and importance of Beet western yellows virus in oilseed rape. *Aspects of Applied Biology*, 23, 311-318.
- Hodgson, D. J., Hitchman, R. B., Vanbergen, A. J., Hails, R. S., Possee, R. D. and Cory, J. S. (2004). Host ecology determines the relative fitness of virus genotypes in mixed-genotype nucleopolyhedrovirus infection. *Journal of Evolutionary Biology*, 17, 1018-1025.
- Hodgson, D. J., Vanbergen, A. J., Watt, A. D., Hails, R. S. and Cory, J. S. (2001). Phenotypic variation between naturally co-existing genotypes of a Lepidopteran baculovirus. *Evolution and Ecology Research*, 3, 687-701.
- Hoffmann, K., Verbeek, M., Romano, A., Dullemans, A. M., van den Heuvel, J. F. J. M. and van der Wilk, F. (2001). Mechanical transmission of poleroviruses. *Journal of Virological Methods*, 91, 197-201.
- Holmes, E. C. (2003). Error thresholds and the constraints to RNA virus evolution. *Trends in Microbiology*, 11, 543-546.
- Home-Grown Cereals Authority (2009). *Strategies to reduce the impact of Turnip yellows virus on oilseed rape production in the UK*. In: HGCA Research and Development, Annual Project Report, 2009 results. Stoneleigh Park, Kenilworth, Stoneleigh Park, Kenilworth: Home-Grown Cereals Authority.
- Hudson, R. R. (2000). A new statistic for detecting genetic differentiation. *Genetics*, 155, 2011-2014.
- Hudson, R. R., Slatkin, M. and Maddison, W. P. (1992). Estimation of levels of gene flow from DNA sequence data. *Genetics*, 132, 583-589.
- Hull, R. (2009). *Comparative Plant Virology*, 2 Edition (London: Elsevier Academic Press).

Hull, R. (2002). *Matthews' Plant Pathology*, 4th Edition (London, UK: Elsevier Academic Press).

Hughes, S. L. (2001). *Interaction of Turnip mosaic virus (TuMV) with members of Brassicaceae*. In: School of Biosciences, Faculty of Science. Birmingham, Doctor of Philosophy, 176. Birmingham: The University of Birmingham.

Hunter, P. J., Jones, J. E. and Walsh, J. A. (2002). Involvement of *Beet western yellows*, *Cauliflower mosaic virus* and *Turnip mosaic virus* in internal disorders of stored white cabbage. *Phytopathology*, 92, 816-826.

ICTVdB Management (2006). *Polerovirus*. In: ICTV dB-The Universal Virus Database. New York, USA, version 4, New York, USA: Columbia University.

Innan, H., Terauchi, R. and Miyashita, N. T. (1997). Microsatellite polymorphism in natural populations of the wild plant *Arabidopsis thaliana*. *Genetics*, 146, 1441-1452.

Janssen, D., Velasco, L., Martin, G., Segundo, E. and Cuadrado, I. M. (2007). Low genetic diversity among *Cucumber vein yellowing virus* isolates from Spain. *Virus Genes*, 34, 367-371.

Jasnic, S. and Bagi, F. (2007). Viral diseases of oilseed rape. *Plant Doctor*, 35, 458-461.

Jay, C. N., Rossall, S. and Smith, H. G. (1999). Effects of beet western yellows virus on growth and yield of oilseed rape (*Brassica napus*). *Journal of Agricultural Science*, 133, 131-139.

Jenkins, G. M., Rambaut, A., Pybus, O. G. and Holmes, E. C. (2002). Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. *Journal of Molecular Biology and Evolution*, 54, 156-165.

Johnson, R. (1981). Durable resistance: definition of, genetic control, and attainment in plant breeding. *Phytopathology*, 71, 567-568.

Jones, R. A. C., Coutts, B. A. and Hawkes, J. (2007). Yield-limiting potential of *Beet western yellows virus* in *Brassica napus*. *Australian Journal of Agricultural Research*, 58, 788-801.

Jones, D., Taylor, W. and Thornton, J. M. (1991). The rapid generation of mutation data matrices from protein sequences. *Computer Application in the Biosciences*, 8, 170-179.

Juergens, M., Paetsch, C., Kramer, I., Zahn, M., Rabenstein, F., Schondelmaier, J., Schliephake, E., Snowdon, R., Friedt, W. and Ordon, F. (2010). Genetic analyses of the host-pathogen system *Turnip yellows virus* (TuYV)-rapeseed (*Brassica napus* L.) and development of molecular markers for TuYV-resistance. *Theoretical and Applied Genetics*, 120, 735-744.

Kang, B.-C., Yeam, I. and Jahn, M. (2005). Genetics of plant virus resistance. *Annual Review of Phytopathology*, 43, 581-621.

Kassanis, B. and Schwabe, W. W. (1961). The effect of paracrinkle virus on the growth of King Edward potato at different temperatures and daylengths. *Annals of Applied Biology*, 49, 616-620.

Kasschau, K. D., Xie, Z., Allen, E., Llave, C., Chapman, E. J., Kirzan, K. A. and Carrington, J. C. (2003). P1/HC-Pro, a viral suppressor of RNA silencing interferes with Arabidopsis development and miRNA function. *Developmental Cell*, 4, 205-217.

Keller, K. E., Johansen, I. E., Martin, R. R. and Hampton, R. O. (1998). Potyvirus genome-linked protein (VPg) determines pea seed-borne mosaic virus pathotype-specific virulence in *Pisum sativum*. *Molecular Plant-Microbe Interactions*, 11, 124-130.

Kerlan, C. (1991). Les viroses, une etude de longue haleine. *Le dossier, Oleoscop no.5, septembre*, 91, 6-7.

Kessman, H., Staub, T., Hofmann, C., Maetzke, Herzog, J., Ward, E., Uknes, S. and Ryals, J. (1994). Induction of systemic acquired disease resistance in plants by chemicals. *Annual Review of Phytopathology*, 32, 439-459.

Kimura, M. (1983). *The Neutral Theory of Molecular Evolution* (Cambridge, MA: Cambridge University Press).

King, G., Nienhuis, D. and Hussey, C. (1993). Genetic similarity among ecotypes of *Arabidopsis thaliana* estimated by analysis of restriction fragment length polymorphisms. *Theoretical and Applied Genetics*, 86, 1028-1032.

Knight, B. and Turner, R. (2009). *Topic No 1-Agrochemical Resistance Fungicides in combinable crops in the UK*. In: Report on Economic, Financial and Market Studies to demonstrate the economic value of research carried out by Rothamsted Research. Burwell, Cambridgeshire, 1-27. Burwell, Cambridgeshire: Innovation Management.

Koella, J. C. and Agnew, P. (1999). A correlated response of a parasite's virulence and life cycle to selection on its host's life history. *Journal of Evolutionary Biology*, 12, 70-79.

Koev, G., Mohan, B. R., Dinesh-Kumar, S. P., Somers, D. A. and Miller, W. A. (1998). Extreme reduction of disease in oats transformed with with 5' half of the Barley yellows dwarf virus-PAV genome. *Phytopathology*, 88, 1013-1019.

Kosakovsky, P. S. L. and Frost, S. D. W. (2005b). Not so different after all: a comparison of methods for detecting amino acid sites under selection. *Molecular Biology and Evolution*, 21, 1208-1222.

- Kosakovsky, P. S. L., Frost, S. D. W. and Muse, S. V. (2005a). HyPHY: hypothesis testing using phylogenetics. *Bioinformatics*, 21.
- Kou, Y. and Wang, S. (2010). Broad-spectrum and durability: understanding of quantitative disease resistance. *Current Opinion in Plant Biology*, 13, 1-5.
- Kover, P. X. and Schaal, B. A. (2002). Genetic variation for disease resistance and tolerance among *Arabidopsis thaliana* accessions. *PNAS*, 99, 11270-11274.
- Kover, P. X., Wolf, J. B., Kunkel, B. N. and Cheverud, J. M. (2005). Genetic architecture of *Arabidopsis thaliana* response to infection by *Pseudomonas syringae*. *Heredity*, 94, 507-517.
- Lacey, M. E., Rawlinson, C. J. and McCartney, H. A. (1987). First record of the natural occurrence in England of the teleomorph of *Pyrenopeziza brassicae* on oilseed rape. *Transactions of the British Mycological Society*, 89, 135-140.
- Lafforge, G., Sardanyes, J. and Elena, S.F. (2011). Differences in accumulation and virulence determine the outcome of competition during *Tobacco etch virus* coinfection. *PLoS ONE* 6(3): e17917. doi: 10.1371/journal.pone.0017917
- Leisner, R. M., Ziegler-Graff, V., Reuteneuer, A., Herrbach, E., Lemaire, O., Guilley, H., Richards, K. and Jonard, G. (1992). *Agroinfection as an alternative to insects for infecting plants with Beet western yellows luteovirus*. USA, 89, 9136-9140. National Academy of Science.
- Li, H. Y. and Roossinck, M. J. (2004). Genetic bottlenecks reduce population variation in an experimental RNA virus population. *Journal of Virology*, 78, 10582-10587.
- Librado, P. and Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451-1452.
- Lindhout, P. (2002). The perspectives of polygenic resistance in breeding for durable disease resistance. *Euphytica*, 124, 217-226.
- Luterbacher, M. C., Asher, M. J. C., De Ambrogio, E., Biancardi, E., Stevenato, P. and Frese, L. (2004). Sources of resistance to diseases of sugar beet in related *Beta* germplasm: I. Foliar diseases. *Euphytica*, 139, 105-121.
- Lynch, M. and Crease, T. J. (1990). The analysis of population survey data on DNA sequence variation. *Molecular Biology Evolution*, 7, 377-394.
- Mahajan, S. K., Chisholm, S. T., Witham, S. A. and Carrington, J. C. (1998). Identification and characterisation of a locus (*RTM 1*) that restricts long distance movement of tobacco etch virus in *Arabidopsis thaliana*. *The Plant Journal*, 14, 177-186.
- Marczewski, W., Flis, B., Syller, J., Schafer-Pregl, R. and Gebhardt, C. (2001). A major quantitative trait locus for resistance to *Potato leafroll virus* is located in a

resistance hotspot on potato chromosome XI and is tightly linked to N-gene-like markers. *Molecular Plant and Microbe Interactions*, 14, 1420-5.

Martin, D. P., Lemey, P., Lott, M., Moulton, V., Posada, D. and Lefevvre, P. (2010). RDP3: a flexible and fast computer programme for analysing recombination. *Bioinformatics*, 26, 2462-2463.

Martinez-Herrera, D., Romero, J., Martinez-Zapater, J. M. and Ponz, F. (1999). Suitability of *Arabidopsis thaliana* as a system for the study of plant-virus interactions. *Fitopatologia*, 29, 132-136.

Maule, A. J., Caranta, C. and Boulton, M. I. (2007). Sources of natural resistance to plant viruses: status and prospects. *Molecular Plant Pathology*, 8, 223-231.

Mauricio, R. and Rausher, M. (1997). Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. *Evolution*, 51, 1435-1444.

Mayo, M. A. (2002). ICTV at the Paris ICV: results of the preliminary session and the binomial ballot. *Archives of Virology*, 147, 2254-2260.

Mayo, M. A. (1999). Development in plant virus taxonomy since the publication of the 6th ICTV Report. *Archives of Virology*, 188, 1059-1666.

Mayo, M. A. and D'Arcy, C. J. (1999). Family Luteoviridae: a reclassification of luteoviruses. In *The Luteoviridae*, pp. 15-22. Edited by H. G. Smith and H. Baker. Wallingford, UK: CABI Publishing.

Mayo, M. A. and Ziegler-Graff, V. (1996). Molecular biology of Luteoviruses. *Advances in Virus Research*, 56, 413-460.

Meyerowitz, E. M. and Pruitt, R. E. (1985). *Arabidopsis thaliana* and plant molecular genetics. *Science*, 229, 1214-1218.

Michalakos, Y. and Hochberg, M. E. (1994). Parasitic effects on host's life history traits: a review of recent studies. *Parasite*, 1, 291-294.

Miller, W. A., Dinesh-Kumar, S. P. and Paul, C. P. (1995). Luteovirus gene expression. *Critical Reviews in Plant Sciences*, 14, 179-211.

Miralles, R., Ferrer, R., Sole, R. V., Moya, A. and Elena, S. V. (2001). Multiple infection dynamics has pronounced effects on the fitness of RNA viruses. *Journal of Evolutionary Biology*, 14, 654-662.

Moran, N. A. and Whitham, T. G. (1990). Differential colonisation of resistant and susceptible host plants: *Pemphigus* and *Populus*. *Ecology*, 71, 1059-1067.

Muse, S. V. and Gaut, B. S. (1994). A likelihood approach for comparing synonymous and non-synonymous nucleotide substitution rates, with application to the chloroplast genome. *Molecular Biology and Evolution*, 11, 715-724.

- Nagarajan, K., Govier, D. A. and Cockbain (1987). Diseases of break crops. Oilseed rape. Viruses. *Report of Rothamsted Experimental for 1986:115*.
- Nelder, J. A. and Wedderburn, R. W. M. (1972). Generalised linear models. *Journal of Royal Statistical Society, Series A*, 135, 370-384.
- Niks, R. E., HabekuB, A., Bekele, B. and Ordon, F. (2004). A novel major gene on chromosome 6H for resistance of barley against the *Barley yellow dwarf virus*. *Theories of Applied Genetics*, 109, 1536-1543.
- Njuguna, J. G., Govier, D.A. and Cockbain, A.J. (1986). *Viruses of oilseed rape*. In: Report of Rothamsted Experimental for 1985. 123,
- Olsson, G. (1960). Species crosses within the genus Brassica II. Artificial *Brassica napus* L. *Hereditas*, 46, 351-396.
- Ozer, H., Oral, E. and Dogru, U. (1999). Relationships between yield and yield components on currently improved spring rapeseed cultivars. *Turkish Journal of Agriculture and Forestry*, 23, 603-609.
- Pagan, I., Alonso-Blanco, C. and Garcia-Arenal, F. (2008). Host responses in life-history traits and tolerance to virus infection in *Arabidopsis thaliana*. *PLoS Pathogens*, 4, 1-10.
- Pagan, I. and Holmes, E. C. (2010). Long-term evolution of the *Luteoviridae*: time scale and mode of virus speciation. *Journal of Virology*, 84, 6177-6187.
- Pagan, I., Fraille, A., Fernandez-Fueyo, E., Montes, N., Alonso-Blanco, C. and Garcia-Arenal, F. (2010). *Arabidopsis thaliana* as a model for the study of plant-virus co-evolution. *Transactions of The Royal Society Biological*, 365, 1983-1995.
- Parker, J. E., Coleman, M. J., Szabo, V., Frost, L. N., Schmidt, R., van der Biezen, E. A., Moores, T., Dean, C., Daniels, M. J. and Jones, J. D. G. (1997). The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the toll and interleukin-1 receptors with N and L6. *Plant Cell*, 9, 879-894.
- Parkin, I. A. P., Sigrun, M. G., Sharpe, A. G., Lukens, L., Trick, M., Osborn, T. C. and Lydiate, D. J. (2005). Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. *Genetics*, 171, 765-781.
- Patterson, H. D. and Williams, E. R. (1976). A new class of resolvable incomplete block design. *Biometrika*, 63, 83-92.
- Patterson, H. D. and Thompson, R. (1971). Recovery of inter-block information when block sizes are unequal. *Biometrika*, 58, 545-554.
- Payne, R. W., Murray, D. A., Harding, S. A., Baird, D. B. and Soutar, D. M. (2009). *Genstat for Windows: Introduction*. In: Hemel Hempstead, Hemel Hempstead: VSN International.

Pazhouhandeh, E., Dieterle, M., Marrocco, K., Lechner, E., Berry, B., Brault, V., Hemmer, O., Kretsch, T., Richard, K. E., Genschik, P. and Ziegler-Graff, V. (2006). *F-box like domain in the polerovirus protein PO is required for silencing function*. USA, Pro. Natl. Acad. Sci. Jan 30.

Pazhouhandeh, M. (2007). *The mechanism of action of Polerovirus PO in RNA SILENCING*. In: Faculty of Life Science. Strasbourg, France, PhD, 127. Strasbourg, France: Louis Pasteur University.

Pfeffer, S., Dunoyer, F., Heim, F., Richards, K. E., Jonard, G. and Ziegler-Graff, V. (2002). PO of *Beet western yellows virus* is a suppressor of posttranscriptional gene silencing. *Journal of Virology*, 76, 6815-6824.

Pilet, M. L., Delourme, R., Foisset, N. and Renard, M. (1998). Identification of QTL involved in field resistance to light leaf spot and blackleg (*Pyrenopezia brassicae*) and blackleg resistance (*Leptosphaeria maculans*) in winter rapeseed (*Brassica napus* L.). *Theories of Applied Genetics*, 97, 398-406.

Pink, D. and Puddephat, I. (1999). Deployment of disease resistance genes by plant transformation- a "mix and match " approach. *Trends in Plant Sciences*, 4, 71-75.

Pink, D. A. C., Walkey, D. G. A. and McClement, S. J. (1991). Genetics of resistance to *Beet westen yellows virus* in lettuce. *Plant Pathology*, 40, 542-545.

Pol, D. (2004). Empirical problems of Hierachical Likelihood ratio test for model selection. *System Biology*, 53, 949-962.

Polak, J. and Majkova, L. (1992). Winter oilseed rape as a likely source and reservoir of *Beet western yellows virus*. *Ochrana Rostlin-UVTIZ*, 28, 191-196.

Poland, J. A., Balint-Kurti, P. J., Wisser, R. J., Pratt, R. C. and Nelson, R. J. (2009). Shades of gray: the world of quantitative disease resistance. *Trends in Plant Sciences*, 14, 21-29.

Pond, D.L.K., Frost, S.D,W. and Muse, S.V. (2005). HyPhy: hypothesis testing using phylogenetics. *Bioinformatics*, 21 (5), 676-679.

Powell-Abdel, P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T. and Beachy, R. N. (1986). Delay of disease development in transgenic plants that express the *Tobacco mosaic virus* coat protein gene. *Science*, 232, 738-743.

Posada, D. and Crandall, K. A. (1998). MODEL TEST: testing the model of DNA substitution. *Bioinformatics*, 14, 817-818.

Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M. and Tepfer, M. (2008). Review: Strategies for antiviral resistance in transgenic plants. *Molecular Plant Pathology*, 9, 73-83.

- Rabenstein, F., Graichen, K., Proll, E., Herrbach, E. and Lemaire, O. (1995). Detection of a second distinct strain of *Beet western yellows luteovirus* in oilseed rape using monoclonal antibodies. *Ber. Bundesanst. Zuchtungsforsch. Kulturpflanzen*, 1, 137-140.
- Rawlinson, C. J. and Muthyalu, G. (1979). Diseases of winter oilseed rape: occurrence, effects and control. *Journal of Agricultural Science*, 107, 299-305.
- Raybould, A. F., Maskell, L. C., Edwards, M. L., Cooper, J. I. and Gray, A. J. (1999). The prevalence and spatial distribution of viruses in natural populations of *Brassica oleracea*. *New Phytologist*, 141, 265-275.
- Read, M. and Hewson, R. T. (1988). Prevention of *Beet western yellows virus* (BWYV) in winter oilseed rape by control of aphid vectors with deltamethrin. Brighton Crop Protection Conference-pest and diseases, pp 989-997.
- Read, A. F. and Taylor, L. H. (2001). The ecology of genetically diverse infections. *Science*, 292, 1099-1102.
- Reinbold, C., Herrbach, E. and Brault, V. (2003). Posterior midgut and hindgut are both sites of acquisition of *Cucurbit aphid-borne virus* in *Myzus persicae* and *Aphis gossypii*. *Journal of General Virology*, 84, 3473-3484.
- Retenauer, A., Ziegler-Graff, V., Lott, H., Scheidecker, D., Guilley, H., Richards, K. and Jonard, G. (1993). Identification of *Beet western yellows virus* genes implicated in viral replication and particle morphogenesis. *Virology*, 195, 692-699.
- Ribeiro, S. G., Ambrozevicius, L. P., Avila, A. C., Bezerra, I. C., Calegario, R. F., Fernandes, J. J., Lima, M. F., de Mello, R. N., Roche, H. and Zerbini, F. M. (2003). Distribution and genetic diversity of tomato infecting begomoviruses in Brazil. *Archives of Virology*, 148, 281-295.
- Rich, T. C. G. (1991). Crucifers of Great Britain and Ireland. B.S.B.I. Handbook No. 6 (London: Botanical Society of the British Isles).
- Riley, J. R., Reynolds, D. R., Mukhopadhyay, S., Ghosh, M. R. and Sarkar, T. K. (1995). Long-distance migration of aphids and other small insects in northeast India. *European Journal of Entomology*, 92, 639-653.
- Robinson, D. J. (1996). Environmental risk assessment of releases of transgenic plants containing virus-derived inserts. *Transgenic Research*, 5, 359-362.
- Roossinck, M. and Ali, A. (2007). *Mechanisms of plant virus evolution and identification of genetic bottlenecks: impact on disease management*. In: Biotechnology and plant disease management. Wallingford, United Kingdom, 109-124. Wallingford, United Kingdom: CABI Publishing.

- Rusholme, R. L., Higgins, E. E., Walsh, J. A. and Lydiate, D. L. (2007). Genetic control of broad-spectrum resistance to *Turnip mosaic virus* in *Brassica rapa* (Chinese cabbage). *Journal of General Virology*, 88, 3177-3186.
- Russell, G. E. (1972). Inherited resistance to virus yellows in sugar beet. *Proceedings of the Royal Society of London, Series B.*, 181, 267-279.
- Russell, G. E. (1969). Effects of sucrose sprays and darkness on aphid colonisation of sugar beet and on aphid transmission of yellowing viruses. *Annals of Applied Biology*, 63.
- Russell, G. E. (1966). Breeding for resistance to infection with yellowing viruses in sugar beet. Resistance in virus-tolerant breeding material. *Annals of Applied Biology*, 57, 311-320.
- Russell, G. E. (1958). Sugar beet yellows: a preliminary study of the distribution and interrelationships of viruses and virus strains found in East Anglia. *Annals of Applied Biology*, 46, 393-398.
- Rygulla, W., Snowdon, R. J., Friedt, W., Happstadius, I., Cheung, W. Y. and Chen, D. (2008). Identification of quantitative trait loci for resistance against *Verticillium longisporum* in oilseed rape (*Brassica napus*). *Phytopathology*, 98, 215-221.
- Saldana, J., Elena, S. F. and Sole, R. V. (2003). Coinfection and superinfection in RNA virus populations: a selection-mutation model. *Mathematical Bioscience*, 183, 135-160.
- Schurch, S. and Roy, B. A. (2004). Comparing single- vs. mixed-genotype infections of *Mycopharella graminicola* on wheat: effects on pathogen virulence and host tolerance. *Evolutionary Ecology*, 18, 1-14.
- Sanford, J. C. and Johnston, S. A. (1985). The concept of parasite derived resistance. *Journal of Theoretical Biology*, 113, 395-405.
- Sassen, B. (1983). The effect of two pyrethroids on the feeding behaviour of three aphid species and on transmission of two different viruses. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz*, 90, 119-126.
- Schliephake, E., Graichen, K. and Rabenstein, F. (2000). Investigations on the vector transmission of the *Beet mild yellowing virus* (BMV) and the *Turnip yellows virus* (TuYV). *Zeitschrift Für Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection*, 107, 81-87.
- Schroder, M. (1994). Investigations on the susceptibility of oilseed rape (*Brassica napus* L. ssp. *napus*) to different virus diseases. *Zeitschrift Für Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection*, 101, 576-589.

- Schubert, J., Rabeinstein, F., Graichen, K. and Richter, K. (1998). Comparison of the 5'-end nucleotide sequences of luteoviruses from oilseed rape and sugar beet. *Archives of Phytopathology and Plant Protection*, 31 (6), 519-530.
- Scottish Agricultural College (2009). *Winter oilseed rape pests and diseases. Technical Note TN620*. In: Edinburgh, UK, Edinburgh, UK: Scottish Agricultural College.
- Shahraeen, N., Farzadfar, S. and Lesemann, D. E. (2003). Incidence of viruses infecting winter oilseed rape (*Brassica napus* ssp. *oleifera*) in Iran. *Journal of Phytopathology*, 151, 614-616.
- Sharma, H. C., Ohm, H. W. and Perry, K. L. (1997). Registration of *Barley yellow dwarf virus* resistant wheat germplasm line P29. *Crop Science*, 37, 1032-1033.
- Sheng, J., Lartey, R., Ghoshroy, S. and Citovsky, V. (1998). An *Arabidopsis thaliana* mutant with virus-inducible phenotype. *Virology*, 249, 119-128.
- Simmonds, P. and Smith, D. B. (1999). Structural constraints on RNA virus evolution. *Journal of Virology*, 73, 5787-5794.
- Smith, H. G. and Baker, H. (1999). The *Luteoviridae* (Oxon, United Kingdom: CAB International).
- Smith, H. G. and Hinckes, J. A. (1985). Studies on *Beet western yellows virus* in oilseed rape (*Brassica napus* ssp. *oleifera*) and sugar beet (*Beta vulgaris*). *Annals of Applied Biology*, 107, 473-484.
- Song, K. M. and Osborn, T. C. (1992). Polyphyletic origins of *Brassica napus*: new evidence based on organelle and nuclear RFLP analyses. *Genome*, 35, 992-1001.
- Sorci, G., Morand, S. and Hugot, J.-P. (1997). Host-parasite coevolution: comparative evidence for covariation of life history traits in primates and oxyurid parasites. *Proceedings of the Royal Society of London*, 264, 285-289.
- Stevens, M. and Clark, W. (2009). The impact of *Turnip yellows virus* (TuYV) on winter oilseed rape- do we really appreciate the risk? *Aspects of Applied Biology*, 91, 109-114.
- Stevens, M., McGrann, G., Clark, B. (2008). *Turnip yellows virus (syn Beet western yellows virus): an emerging threat to European oilseed rape production?* In: Research Review No. 69. Suffolk, UK, 36. Suffolk, UK: Broom's Barn Research Centre, Higham, Bury St Edmunds.
- Stevens, M., Freeman, B., Liu, H.-Y., Herrbach, E. and Lemaire, O. (2005). Beet poleroviruses: close friends or distant relatives? *Molecular Plant Pathology*, 6, 1-9.

- Stevens, M., Smith, H. G. and Hallsworth, P. B. (1995). Detection of the luteoviruses, beet mild yellowing virus and beet western yellows virus, in aphids caught in sugar-beet and oilseed rape crops, 1990-1993. *Annals of Applied Biology*, 127, 309-320.
- Syngenta (2010). *Cruiser OSR. No. 1 in the UK*. In: Cambridge, Cambridge: Syngenta Crop Protection UK Ltd.
- Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123, 585-595.
- Tally, A., Oostensdorp, M., Lawton, K., Staub, T. and Bassi, B. (1999). Commercial development of elicitors of induced resistance to pathogens. In *Induced Plant Defenses Against Pathogens and Herbivores*, pp. 357-369. Edited by A. Agrawal, S. Tuzun and E. Bent. St Paul, MN, USA: APS Press.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA 5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, doi:10.1093/molbev/msr121.
- Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24, 1596-1599.
- Tamura, K., Nei, M. and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbour-joining method. *Proceedings National Academy of Science, USA*, 101, 11030-11035.
- Taylor, L. R. (1975). Longevity, fecundity and size; control of reproductive potential in a polymorphic migrant, *Aphis fabae*. *Journal of Animal Ecology*, 44, 135-163.
- Thomas, P. E., Pike, K.S. and Reed, G.L. (1997). Role of Green Peach Aphid Flights in the Epidemiology of Potato Leaf Roll Disease in the Columbia Basin. *Plant Disease*, 81, 1311-1316.
- Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673-4680.
- Thresh, J. M. (1976). Gradients of plant virus diseases. *Annals of Applied Biology*, 82, 381-406.
- Torres, M. W., Correa, R. L. and Schrago, C. G. (2005). Analysis of differential selective forces acting on the coat protein (P3) of plant virus family Luteoviridae. *Genetics and Molecular Research*, 4, 790-802.

Tuncturk, M. and Ciftci, V. (2007). Relationships between yield and some yield components in rapeseed (*Brassica napus* ssp. *oleifera* L.) cultivars by using correlation and path analysis. *Pakistan Journal of Botany*, 39, 81-84.

U, N. (1935). Genome analysis in Brassica with special reference to the experimental formation of *Brassica napus* and peculiar mode of fertilisation. *Japanese Journal of Botany*, 7, 389-452.

United States Department of Agriculture (2011). *Oilseeds: World Markets and Trade*. In: Circular Series, FOP 03-11. USA,1-33. USA:

van der Wilk, F., Verbeek, M., Dullemans, A. M. and van den Heuvel, J. F. J. M. (1997). The genome-linked protein of potato leafroll virus is located downstream of the putative protease domain of the ORF1 product. *Virology*, 234, 300-303.

van den Heuvel, J. F. J. M. (1999). Fate of luteovirus in the haemolymph of an aphid, H. G. Smith and A. H. Baker, ed. (Oxon, UK: CAB International).

Van Dongen, S., Molenberghs, G. and Mattysen, E. (1999). The statistical analysis of fluctuating asymmetry: REML estimation of a mixed regression model. *Journal of Evolutionary Biology*, 12, 94-102.

van Molken, T. and Stuefer, J. F. (2011). The potential of plant viruses to promote genotypic diversity via genotype x environment interactions. *Annals of Botany*, 107, 1391-1397.

Van Ooijen, J. W. (2009). *MapQTL 6: Software for the mapping of quantitative trait loci in experimental populations of diploid species*. In: Wageningen, Netherlands, Wageningen, Netherlands: Kyazma B.V.

Veidt, I., Lot, H., Leiser, M., Scheidecker, D., Guilley, H., Richards, K. and Jonard, G. (1988). Nucleotide sequence of *Beet western yellows virus* RNA. *Nucleic Acids Research*, 16, 9917-9931.

Velasquez, A. C., Mihovilovich, E. and Bonierbale, M. (2007). Genetic characterisation and mapping of major gene resistance to potato leafroll virus in *Solanum tuberosum* ssp. *andigena*. *Theoretical and Applied Genetics*, 114, 1051-8.

Voorrips, R. E. (2002). MapChart: Software for the graphical presentation of linkage maps and QTLs. *The Journal of Heredity*, 93, 77-78.

Walkey, D. G. A. (1985). *Applied Plant Virology* (London, UK: William Heinemann Ltd).

Walkey, D. G. A. and Pink, D. A. C. (1990). Studies on resistance to *Beet western yellows virus* in lettuce (*Lactuca sativa*) and the occurrence of field sources of the virus. *Plant Pathology*, 39, 141-155.

- Walsh, J. A. (1986). *Viral diseases of oilseed rape and their control*. British Crop Protection Conference-Pest and diseases, pp 737-743.
- Walsh, J. A., Sharpe, A. G., Jenner, C. E. and Lydiate, D. J. (1999). Characterisation of resistance to *Turnip mosaic virus* in oilseed rape (*Brassica napus*) and genetic mapping of TuRB01. *Theoretical and Applied Genetics*, 99, 1149-1154.
- Walsh, J. A., Perrin, R. M., Miller, A. and Laycock, D. S. (1989). Studies on Beet western yellows virus in winter oilseed rape (*Brassica napus* ssp *oleifera*) and the effect of insecticidal treatment on its spread. *Crop Protection*, 8, 137-143.
- Walsh, J. A. and Tomlinson, J. A. (1985). Viruses infecting winter oilseed rape (*Brassica napus* spp *oleifera*). *Annals of Applied Biology*, 107, 485-495.
- Wang, M. B., Abbott, D. C. and Waterhouse, P. M. (2000). A simple copy of virus-derived transgene encoding hairpin RNA gives immunity to *Barley yellow dwarf virus*. *Molecular Plant Pathology*, 1, 347-356.
- Watson, M. A. (1952). *Beet yellows viruses and other yellowing virus diseases of sugar beet*. In: Report of Rothamsted Experimental Research. 1951, 157-167.
- Watson, D. J. and Wilson, J. H. (1956). An analysis of the effects of infection with leaf-roll virus on the growth and yield of potato plants, and of its interactions with nutrient supply and shading. *Annals of Applied Biology*, 44, 390-409.
- Watt, A. D. and Dixon, A. F. G. (1981). The role of cereal growth stages and crowding in the induction of alatae in *Sitobion avenae* and its consequences for population growth. *Ecological Entomology*, 6, 441-447.
- Warwick, S. I., Francis, A. and Al-Shehbaz, I. A. (2006). *Brassicaceae*: species checklist and database on CD-Rom. *Plant Syst Evolution*, 259, 249-258.
- Waterhouse, P. M., Gildow, F. E. and Johnstone, G. R. (1998). *Description of plant viruses: Luteovirus group*. In: Show DPV. 1-16. Association of Applied Biologists.
- Wei, T. Y., Yang, J. G., Liao, F. L., Gao, F. L., Lu, L. M., Zhang, X. T., Li, F., Wu, Z. J., Lin, Q. Y., Xie, L. H. and Lin, H. X. (2009). Genetic diversity and population structure of rice stripe virus in China. *Journal of General Virology*, 90, 1025-1034.
- Wiangjun, H. and Anderson, J. M. (2004). The basis for *Thinopyrum*-derived resistance to *Cereal yellow dwarf virus*. *Phytopathology*, 94, 1102-1106.
- Wisser, R. J., Sun, Q., Hubert, S. H., Kresovich, S. and Nelson, R. J. (2005). Identification and characterisation of regions of the rice genome associated with broad-spectrum, quantitative disease resistance. *Genetics*, 169, 2277-2293.
- Wolfe, M. (2000). Crop strength through diversity. *Nature*, 406, 681-682.

Workman, P. L. and Niswander, J. D. (1970). Population studies on southwestern Indian tribes. *American Journal of Human Genetics*, 22, 24-29.

Young, N. D. (1996). QTL mapping and quantitative disease resistance in plants. *Annual Review of Phytopathology*, 34, 479-501.

Yount, D. J., Martin, J. M., Carroll, T. W. and Zaske, S. K. (1985). Effects of *Barley yellow dwarf virus* on growth and yield of small grains in Montana. *Plant Disease*, 69, 487-491.

Zwart, R. S., Bansul, U. K., Thompson, J. P., Williamson, P. M. and Bariana, H. S. (2008). *QTL mapping of multiple disease resistance traits in a synthetic hexaploid x bread wheat population*. The University of Sydney, 1, Sydney University Press.

b. Electronic references

Abram M. (2010) Cereals 2010: Seed treatment may help OSR *Tolerate turnip yellows virus*. *Farmers Weeekly Interactive* [Online]. Available: <http://www.fwi.co.uk/Articles/2010/06/11/121746/Cereals-2010-Seed-treatments-may-help-OSR-tolerate-turnip-yellows.htm> [Accessed 19 April 2011].

Anon. (2011) Aphid bulletin: Bulletin Archives. Available: <http://www.rothamsted.bbsrc.ac.uk/insect-survey/STAphidBulletinArchive.php?Year=2011> [Accessed August 2011].

Bayer CropScience (2010) Modesto. Available: <http://www.bayercropscience.co.uk/product/seed-treatments/modesto/> [Accessed 18 August 2011].

Bayer CropScience (2007a) Oilseed rape. Global statistics. Available: <http://bayer.dbt.co.uk/content.output/1538/1561/Crop%20Centre/Oilseed%20Rape/Global%20Statistics.msp> [Accessed 12 May 2011].

Bayer CropScience (2007b) Overcoming threats. Aphids and *Turnip yellows virus*. Available: <http://www.septoria.com/content.output/1543/1548/Crop%20Centre/Overcoming%20Threats/Aphids%20and%20TuYV.msp> [Accessed 19th April 2011].

Blake A. (2009) *Turnip yellows virus threatens oilseed rape* [Online]. Reed Business Information. Available: <http://www.fwi.co.uk/Articles/2009/06/04/115896/Turnip-yellow-virus-threatens-oilseed-rape.htm> [Accessed 19th April 2011].

Department for Environment Food and Rural Affairs (2010) National Statistics. Joint Announcement by the Agricultural Departments of the United Kingdom and Cereals and Oilseed rape Production Estimates: 2009 Harvest United Kingdom-Final Results. Available: <http://archive.defra.gov.uk/evidence/statistics/foodfarm/food/cereals/documents/cps-osr-mincrop-final.pdf> [Accessed 1st August 2010]

Collier, R. (2008). *Archive of aphid summaries in 2008*. In: HDC Pest Bulletin. Wellesbourne, <http://www2.warwick.ac.uk/fac/sci/lifesci/wcc/hdcpestbulletin/archive/>. Wellesbourne: School of Life Sciences, University of Warwick.

Collier, R. (2009). *Insecticide resistance in *Myzus persicae**. *HDC Pest Bulletin. Wellesbourne, UK*. Available: http://www2.warwick.ac.uk/fac/sci/lifesci/wcc/hdcpestbulletin/lettuce/aphids/resistance_myzus/. Wellesbourne, Wellesbourne:

Collier, R. (2010). *What effect will the cold winter of 2009-10 have on vegetable pests? HDC Pest Bulletin. Wellesbourne, UK*. In: <http://www2.warwick.ac.uk/fac/sci/lifesci/wcc/hdcpestbulletin/coldwinter/>.

Department for Environment Food and Rural Affairs (2010) National Statistics. Joint Announcement by the Agricultural Departments of the United Kingdom and Cereals and Oilseed rape Production Estimates: 2009 Harvest United Kingdom-Final Results. Available: <http://archive.defra.gov.uk/evidence/statistics/foodfarm/food/cereals/documents/cps-osr-mincrop-final.pdf> [Accessed 1st August 2010].

Impey L. (2010) Diseases and pests: Project reveals true incidence of *Turnip yellows virus* in rape. *AgroNews* [Online]. Available: <http://news.agropages.com/Feature/FeatureDetail---245.htm> [Accessed 24 June 2011].

Insecticide Resistance Action Group-UK (2008) Guidelines for controlling aphids in brassica crops and managing insecticide resistance in the peach-potato aphid, *Myzus persicae*. Available: http://www.pesticides.gov.uk/uploadedfiles/Web_Assets/RAGs/Brassica%20Doc_7%20July_08.pdf [Accessed 11 May 2011].

King G. (2009) brassica.info:Diversity set. Available: http://www.brassica.info/resource/plants/diversity_sets.php [Accessed 24th June, 2011]

Stevens M. (2010) *Turnip yellows virus*. Better understanding of a forgotten virus of oilseed rape-turnip yellows-could help growers increase yield. *Farmers Weekly Interactive* [Online]. Available: <http://www.fwi.co.uk/academy/article/121591/turnip-yellows-virus.html>

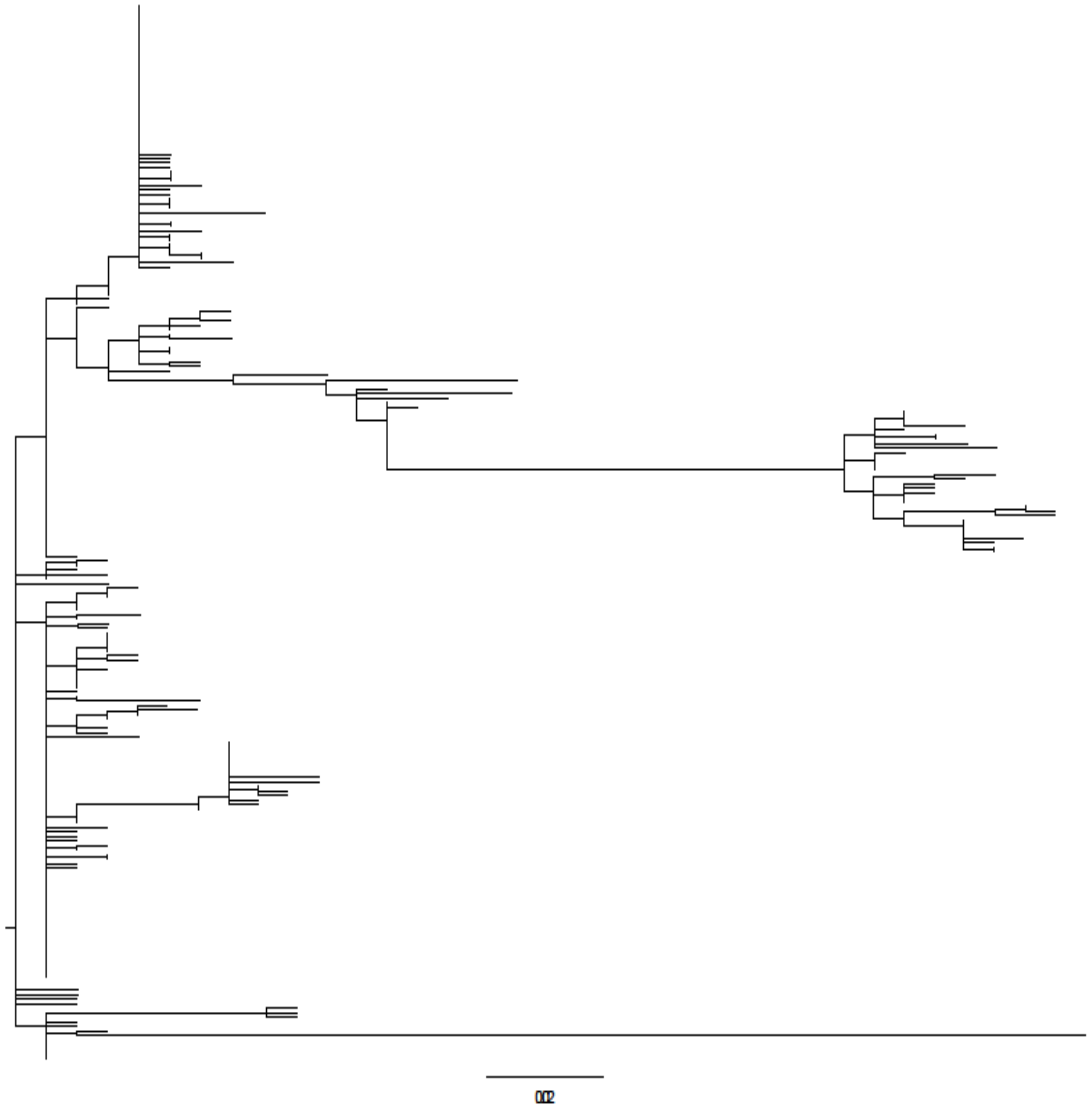
Taylor, M. and Harrington, R. (2010). *Aphid News 10th March 2010. The late late show. Potato, sugar beet and brassica aphid forecasts 2010. HDC Pest Bulletin*. In:

http://www2.warwick.ac.uk/fac/sci/lifesci/wcc/hdcpestbulletin/2010_rothamsted_predictions.pdf.

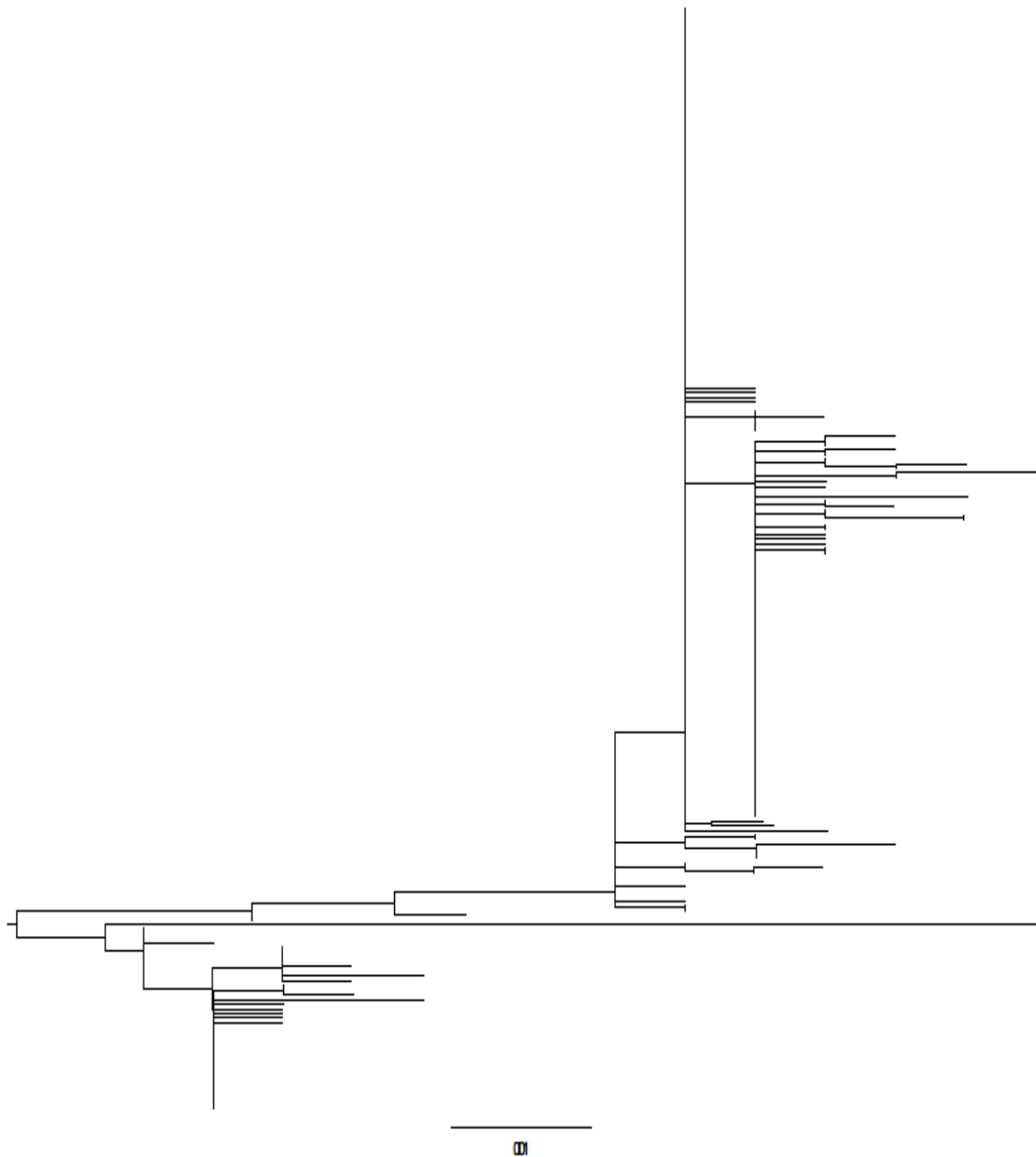
Teakle G. (2009) OREGIN *B. napus* diversity set. Available:
http://www.oregin.info/stakeholders/meetings/16nov2010/02_TEAKLE_OREGIN_SHF8_Nov2010_BnaDFFS.pdf [Accessed 27 July 2011].

UK Agriculture (2010) Oiled rape-its role the UK. Available:
http://www.ukagriculture.com/crops/oil_seed_rape.cfm [Accessed 5 May 2011].

APPENDICES



Appendix 3.1 Maximum likelihood tree of amino acid sequence of P0 of *Turnip yellows virus* isolates



Appendix 3.2 Maximum likelihood tree of amino acid sequence of P3 of *Turnip yellows virus* isolates

L300 ATGCAATTTGTTGCTCACGA AACTTTACACTCTAGAAAGTCAGGAAAGTCAGTACCTCCAGCAAGTAAAGCTT 80
 W300 ATGCAATTTGTTGCTCACGA AACTTTACACTCTAGAAAGTCAGGAAAGTCAGTACCTCCATCAAGTAAAGCTT 80
 Y990 ATGCAATTTGTTGCTCACGA AACTTTACACTCTAGAAAGTCAGGAAAGTCAGTACCTCCAGCAAGTAAAGCTT 53
 L188 ATGCAATTTGTTGCTCACGACAACCTTTACACTCTAGAAAGTCAGGAAAGTCAGTACCTCCATTCAGGACAAGTAAAGCTT 80
 W1088b ATGCAATTTGTTGCTCACGACAACCTTTACACTCTAGAAAGTCAGGAAAGTCAGTACCTCCATTCAGGACAAGTAAAGCTT 53
 Y1266 ATGCAATTTGTTGCTCACGACAACCTTTACACTCTAGAAAGTCAGGAAAGTCAGTACCTCCATTCAGGACAAGTAAAGCTT 80
 L288 ATGCAATTTGTTGCTCACGACAACCTTTACACTCTAGAAAGTCAGGAAAGTCAGTACCTCCATTCAGGACAAGTAAAGCTT 80
 W903 ATGCAATTTGTTGCTCACGACAACCTTTACACTCTAGAAAGTCAGGAAAGTCAGTACCTCCATTCAGGACAAGTAAAGCTT 53
 Y72 ATGCAATTTGTTGCTCACGACAACCTTTACACTCTAGAAAGTCAGGAAAGTCAGTACCTCCATTCAGGACAAGTAAAGCTT 80

L300 TCTTTTAGCAGGCTTTGGCTTAACATAACAATTTGTAAAAGCAATCAAAGGCGCAACAATGAATTCAAAAATGATA 160
 W300 TCTTTTAGCAGGCTTTGGCTTAACATAACAATTTGTAAAAGCAATCAAAGGCGCAACAATGAATTCAAAAATGATA 160
 Y990 TCTTTTAGCAGGCTTTGGCTTAACATAACAATTTGTAAAAGCAATCAAAGGCGCAACAATGAATTCAAAAATGATA 160
 L188 TCTTTTAGCAGGCTTTGGCTTAACATCGAACAAATCGTAAAAGCAATCAAAGGCGCAACAATGAATTCAAAAATGATA 133
 W1088b TCTTTTAGCAGGCTTTGGCTTAACATCGAACAAATCGTAAAAGCAATCAAAGGCGCAACAATGAATTCAAAAATGATA 133
 Y1266 TCTTTTAGCAGGCTTTGGCTTAACATCGAACAAATCGTAAAAGCAATCAAAGGCGCAACAATGAATTCAAAAATGATA 160
 L288 TCTTTTAGCAGGCTTTGGCTTAACATCGAACAAATCGTAAAAGCAATCAAAGGCGCAACAATGAATTCAAAAATGATA 160
 W903 TCTTTTAGCAGGCTTTGGCTTAACATCGAACAAATCGTAAAAGCAATCAAAGGCGCAACAATGAATTCAAAAATGATA 133
 Y72 TCTTTTAGCAGGCTTTGGCTTAACATCGAACAAATCGTAAAAGCAATCAAAGGCGCAACAATGAATTCAAAAATGATA 160

L300 TTTTCTTTCGCTCTCTGCTCTATCAGCTTCTCTCCACTCGGAGACCCTTCCAGCAAGTAGGAAAGTCCATACTT 240
 W300 TTTTCTTTCGCTCTCTGCTCTATCAGCTTCTCTCCACTCGGAGACCCTTCCAGCATGATAGGAAAGTCCATACTT 240
 Y990 TTTTCTTTCGCTCTCTGCTCTATCAGCTTCTCTCCACTCGGAGACCCTTCCAGCATGATAGGAAAGTCCATACTT 213
 L188 TTTTCTTTCGCTCTCTGCTCTATCAGCTTCTCTCCACTCGGAGACCCTTCCAGCATGATAGGAAAGTCCATACTT 240
 W1088b TTTTCTTTCGCTCTCTGCTCTATCAGCTTCTCTCCACTCGGAGACCCTTCCAGCATGATAGGAAAGTCCATACTT 213
 Y1266 TTTTCTTTCGCTCTCTGCTCTATCAGCTTCTCTCCACTCGGAGACCCTTCCAGCATGATAGGAAAGTCCATACTT 240
 L288 TTTTCTTTCGCTCTCTGCTCTATCAGCTTCTCTCCACTCGGAGACCCTTCCAGCATGATAGGAAAGTCCATACTT 240
 W903 TTTTCTTTCGCTCTCTGCTCTATCAGCTTCTCTCCACTCGGAGACCCTTCCAGCATGATAGGAAAGTCCATACTT 213
 Y72 TTTTCTTTCGCTCTCTGCTCTATCAGCTTCTCTCCACTCGGAGACCCTTCCAGCATGATAGGAAAGTCCATACTT 240

L300 GCCC GAACCAAGAGCTTTGTGCTGGTCTCTTTACAAAACGGGATATGCTCCGCTCCACTCAGGCCGTGTTAACTT 320
 W300 GCCC GAACCAAGAGCTTTGTGCTGGTCTCTTTACAAAACGGGATATGCTCCGCTCCACTCAGGCCGTGTTAACTT 320
 Y990 GCCC GAACCAAGAGCTTTGTGCTGGTCTCTTTACAAAACGGGATATGCTCCGCTCCACTCAGGCCGTGTTAACTT 293
 L188 GTCCC GAACCAAGAGCTTTGTGCTGGTCTCTTTACAAAACGGGATATGCTCCGCTCCACTCAGGCCGTGTTAACTT 320
 W1088b GCCC GAACCAAGAGCTTTGTGCTGGTCTCTTTACAAAACGGGATATGCTCCGCTCCACTCAGGCCGTGTTAACTT 293
 Y1266 GTCCC GAACCAAGAGCTTTGTGCTGGTCTCTTTACAAAACGGGATATGCTCCGCTCCACTCAGGCCGTGTTAACTT 320
 L288 GTCCC GAACCAAGAGCTTTGTGCTGGTCTCTTTACAAAACGGGATATGCTCCGCTCCACTCAGGCCGTGTTAACTT 320
 W903 GTCCC GAACCAAGAGCTTTGTGCTGGTCTCTTTACAAAACGGGATATGCTCCGCTCCACTCAGGCCGTGTTAACTT 293
 Y72 GTCCC GAACCAAGAGCTTTGTGCTGGTCTCTTTACAAAACGGGATATGCTCCGCTCCACTCAGGCCGTGTTAACTT 320

L300 AACGTGCCAGGAACCAAGACCTCTCGC GAAGAATCATACAACGATCTTTTGGAGCAATTTCTCAGAAAAGCTCAAG 400
 W300 AACGTGCCAGGAACCAAGACCTCTCGC GAAGAATCATACAACGATCTTTTGGAGCAATTTCTCAGAAAAGCTCAAG 400
 Y990 AACGTGCCAGGAACCAAGACCTCTCGC GAAGAATCATACAACGATCTTTTGGAGCAATTTCTCAGAAAAGCTCAAG 373
 L188 AACGTGCCAGGAACCAAGACCTCTCGC GAAGAATCATACAACGATCTTTTGGAGCAATTTCTCAGAAAAGCTCAAG 400
 W1088b AACGTGCCAGGAACCAAGACCTCTCGC GAAGAATCATACAACGATCTTTTGGAGCAATTTCTCAGAAAAGCTCAAG 373
 Y1266 AACGTGCCAGGAACCAAGACCTCTCGC GAAGAATCATACAACGATCTTTTGGAGCAATTTCTCAGAAAAGCTCAAG 400
 L288 AACGTGCCAGGAACCAAGACCTCTCGC GAAGAATCATACAACGATCTTTTGGAGCAATTTCTCAGAAAAGCTCAAG 400
 W903 AACGTGCCAGGAACCAAGACCTCTCGC GAAGAATCATACAACGATCTTTTGGAGCAATTTCTCAGAAAAGCTCAAG 373
 Y72 AACGTGCCAGGAACCAAGACCTCTCGC GAAGAATCATACAACGATCTTTTGGAGCAATTTCTCAGAAAAGCTCAAG 400

L300 GATTTCCAGAAATGCCTATTCCGTTAGCCTTGGATTTCCAGCGATTTCTATCAACATGGAATAAAGAGCTTGAAGAGCT 480
 W300 GATTTCCAGAAATGCCTATTCCGTTAGCCTTGGATTTCCAGCGATTTCTATCAACATGGAATAAAGAGCTTGAAGAGCT 480
 Y990 GATTTCCAGAAATGCCTATTCCGTTAGCCTTGGATTTCCAGCGATTTCTATCAACATGGAATAAAGAGCTTGAAGAGCT 453
 L188 GATTTCCAGAAATGCCTATTCCGTTAGCCTTGGATTTCCAGCGATTTCTATCAACATGGAATAAAGAGCTTGAAGAGCT 480
 W1088b GATTTCCAGAAATGCCTATTCCGTTAGCCTTGGATTTCCAGCGATTTCTATCAACATGGAATAAAGAGCTTGAAGAGCT 453
 Y1266 GATTTCCAGAAATGCCTATTCCGTTAGCCTTGGATTTCCAGCGATTTCTATCAACATGGAATAAAGAGCTTGAAGAGCT 480
 L288 GATTTCCAGAAATGCCTATTCCGTTAGCCTTGGATTTCCAGCGATTTCTATCAACATGGAATAAAGAGCTTGAAGAGCT 480
 W903 GATTTCCAGAAATGCCTATTCCGTTAGCCTTGGATTTCCAGCGATTTCTATCAACATGGAATAAAGAGCTTGAAGAGCT 453
 Y72 GATTTCCAGAAATGCCTATTCCGTTAGCCTTGGATTTCCAGCGATTTCTATCAACATGGAATAAAGAGCTTGAAGAGCT 480

L300 ATCTTTTCTCGCTGTGAGAAAATTCCTTGGGGTCTGACACGCTTGGAGCTCGCTAATCTTGGCGAGCTTCTCCGCT 560
 W300 ATCTTTTCTCGCTGTGAGAAAATTCCTTGGGGTCTGACACGCTTGGAGCTCGCTAATCTTGGCGAGCTTCTCCGCT 560
 Y990 ATCTTTTCTCGCTGTGAGAAAATTCCTTGGGGTCTGACACGCTTGGAGCTCGCTAATCTTGGCGAGCTTCTCCGCT 533
 L188 ATCTTTTCTCGCTGTGAGAAAATTCCTTGGGGTCTGACACGCTTGGAGCTCGCTAATCTTGGCGAGCTTCTCCGCT 560
 W1088b ATCTTTTCTCGCTGTGAGAAAATTCCTTGGGGTCTGACACGCTTGGAGCTCGCTAATCTTGGCGAGCTTCTCCGCT 533
 Y1266 ATCTTTTCTCGCTGTGAGAAAATTCCTTGGGGTCTGACACGCTTGGAGCTCGCTAATCTTGGCGAGCTTCTCCGCT 560
 L288 ATCTTTTCTCGCTGTGAGAAAATTCCTTGGGGTCTGACACGCTTGGAGCTCGCTAATCTTGGCGAGCTTCTCCGCT 560
 W903 ATCTTTTCTCGCTGTGAGAAAATTCCTTGGGGTCTGACACGCTTGGAGCTCGCTAATCTTGGCGAGCTTCTCCGCT 533
 Y72 ATCTTTTCTCGCTGTGAGAAAATTCCTTGGGGTCTGACACGCTTGGAGCTCGCTAATCTTGGCGAGCTTCTCCGCT 560

L300 TATGGTGGTGGTGAAGCAATTTACAACTCCCCTCTCTGTCTGCGCTTGTGTACACTGTACAAAGATTTATGGTGAAG 640
 W300 TATGGTGGTGGTGAAGCAATTTACAACTCCCCTCTCTGTCTGCGCTTGTGTACACTGTACAAAGATTTATGGTGAAG 640
 Y990 TATGGTGGTGGTGAAGCAATTTACAACTCCCCTCTCTGTCTGCGCTTGTGTACACTGTACAAAGATTTATGGTGAAG 613
 L188 TATGGTGGTGGTGAAGCAATTTACAACTCCCCTCTCTGTCTGCGCTTGTGTACACTGTACAAAGATTTATGGTGAAG 640
 W1088b TATGGTGGTGGTGAAGCAATTTACAACTCCCCTCTCTGTCTGCGCTTGTGTACACTGTACAAAGATTTATGGTGAAG 613
 Y1266 TATGGTGGTGGTGAAGCAATTTACAACTCCCCTCTCTGTCTGCGCTTGTGTACACTGTACAAAGATTTATGGTGAAG 640
 L288 TATGGTGGTGGTGAAGCAATTTACAACTCCCCTCTCTGTCTGCGCTTGTGTACACTGTACAAAGATTTATGGTGAAG 640
 W903 TATGGTGGTGGTGAAGCAATTTACAACTCCCCTCTCTGTCTGCGCTTGTGTACACTGTACAAAGATTTATGGTGAAG 613
 Y72 TATGGTGGTGGTGAAGCAATTTACAACTCCCCTCTCTGTCTGCGCTTGTGTACACTGTACAAAGATTTATGGTGAAG 640

L300 ACGGTTTCATTTCTTTTGGAGGATGCCAACTCGGATCATTCGATTGCTTTCTCACTCCTGAAGAAATCCTTTTCAGC 720
 W300 ACGGTTTCATTTCTTTTGGAGGATGCCAACTCGGATCATTCGATTGCTTTCTCACTCCTGAAGAAATCCTTTTCAGC 720
 Y990 ACGGTTTCATTTCTTTTGGAGGATGCCAACTCGGATCATTCGATTGCTTTCTCACTCCTGAAGAAATCCTTTTCAGC 693
 L188 ACGGTTTCATTTCTTTTGGAGGATGCCAACTCGGATCATTCGATTGCTTTCTCACTCCTGAAGAAATCCTTTTCAGC 720
 W1088b ACGGTTTCATTTCTTTTGGAGGATGCCAACTCGGATCATTCGATTGCTTTCTCACTCCTGAAGAAATCCTTTTCAGC 693
 Y1266 ACGGTTTCATTTCTTTTGGAGGATGCCAACTCGGATCATTCGATTGCTTTCTCACTCCTGAAGAAATCCTTTTCAGC 720
 L288 ACGGTTTCATTTCTTTTGGAGGATGCCAACTCGGATCATTCGATTGCTTTCTCACTCCTGAAGAAATCCTTTTCAGC 720
 W903 ACGGTTTCATTTCTTTTGGAGGATGCCAACTCGGATCATTCGATTGCTTTCTCACTCCTGAAGAAATCCTTTTCAGC 693
 Y72 ACGGTTTCATTTCTTTTGGAGGATGCCAACTCGGATCATTCGATTGCTTTCTCACTCCTGAAGAAATCCTTTTCAGC 720

L300 TCTTCCG 726
 W300 TCTTCCG 726
 Y990 TCTTCCG 699
 L188 TCTTCCG 726
 W1088b TCTTCCG 699
 Y1266 TCTTCCG 726
 L288 TCTTCCG 726
 W903 TCTTCCG 699
 Y72 TCTTCCG 726

Appendix 3.3 Nucleotide sequence alignment of P0 gene of TuYV isolates of the three genetic groups (clades)

L9	ATGAATACGGTCGTGGGTAG	AGAACAATCAATGGAAGAAGACGACCACGGAGGCAA	CACGACGCGCTC	70
W71a	ATGAATACGGTCGTGGGTAG	AGAACAATCAATGGAAGAAGACGACCACGGAGGCAA	CACGACGCGCTC	70
Y63	ATGAATACGGTCGTGGGTAG	AGAACAATCAATGGAAGAAGACGACCACGGAGGCAA	CACGACGCGCTC	70
L44	ATGAATACGGTCGTGGGTAG	AGAACAATCAATGGAAGAAGACGACCACGGAGGCAA	CACGACGCGCTC	70
W6	ATGAATACGGTCGTGGGTAG	AGAACAATCAATGGAAGAAGACGACCACGGAGGCAA	CACGACGCGCTC	70
Y45	ATGAATACGGTCGTGGGTAG	AGAACAATCAATGGAAGAAGACGACCACGGAGGCAA	CACGACGCGCTC	70
L9	AGCGCTCTCAGCCAGTGGTGTGGTCCAA	CCTCTCGGCAACACAACGCCGACC	AGACGACGACGAAG	140
W71a	AGCGCTCTCAGCCAGTGGTGTGGTCCAA	CCTCTCGGCAACACAACGCCGACC	AGACGACGACGAAG	140
Y63	AGCGCTCTCAGCCAGTGGTGTGGTCCAA	CCTCTCGGCAACACAACGCCGACC	AGACGACGACGAAG	140
L44	AGCGCTCTCAGCCAGTGGTGTGGTCCAA	CCTCTCGGCAACACAACGCCGACC	AGACGACGACGAAG	140
W6	AGCGCTCTCAGCCAGTGGTGTGGTCCAA	CCTCTCGGCAACACAACGCCGACC	AGACGACGACGAAG	140
Y45	AGCGCTCTCAGCCAGTGGTGTGGTCCAA	CCTCTCGGCAACACAACGCCGACC	AGACGACGACGAAG	140
L9	AGGTAATAACCGGACAAGA	GAACTGTTCTACCAGAGGAGCAGG	TCAGCGAGACATTTGTTTTCTC	210
W71a	AGGTAATAACCGGACAAGA	GAACTGTTCTACCAGAGGAGCAGG	TCAGCGAGACATTTGTTTTCTC	210
Y63	AGGTAATAACCGGACAAGA	GAACTGTTCTACCAGAGGAGCAGG	TCAGCGAGACATTTGTTTTCTC	210
L44	AGGTAATAACCGGACAAGA	GAACTGTTCTACCAGAGGAGCAGG	TCAGCGAGACATTTGTTTTCTC	210
W6	AGGTAATAACCGGACAAGA	GAACTGTTCTACCAGAGGAGCAGG	TCAGCGAGACATTTGTTTTCTC	210
Y45	AGGTAATAACCGGACAAGA	GAACTGTTCTACCAGAGGAGCAGG	TCAGCGAGACATTTGTTTTCTC	210
L9	AAAGACAATCTCGCGGGAAGT	CCAGCGGAGCAATCAGGTCGGGCCGAGTCTATCAGACTGCC	GCAT	280
W71a	AAAGACAATCTCGCGGGAAGT	CCAGCGGAGCAATCAGGTCGGGCCGAGTCTATCAGACTGCC	GCAT	280
Y63	AAAGACAATCTCGCGGGAAGT	CCAGCGGAGCAATCAGGTCGGGCCGAGTCTATCAGACTGCC	GCAT	280
L44	AAAGACAATCTCGCGGGAAGT	CCAGCGGAGCAATCAGGTCGGGCCGAGTCTATCAGACTGCC	GCAT	280
W6	AAAGACAATCTCGCGGGAAGT	CCAGCGGAGCAATCAGGTCGGGCCGAGTCTATCAGACTGCC	GCAT	280
Y45	AAAGACAATCTCGCGGGAAGT	CCAGCGGAGCAATCAGGTCGGGCCGAGTCTATCAGACTGCC	GCAT	280
L9	TCCTAATGGAATCTCAAGGCCTACCATGAGTATAAAATCTC	ATGGTCATTTTGGAGTT	CGTCTCCGA	350
W71a	TCCTAATGGAATCTCAAGGCCTACCATGAGTATAAAATCTC	ATGGTCATTTTGGAGTT	CGTCTCCGA	350
Y63	TCCTAATGGAATCTCAAGGCCTACCATGAGTATAAAATCTC	ATGGTCATTTTGGAGTT	CGTCTCCGA	350
L44	TCCTAATGGAATCTCAAGGCCTACCATGAGTATAAAATCTC	ATGGTCATTTTGGAGTT	CGTCTCCGA	350
W6	TCCTAATGGAATCTCAAGGCCTACCATGAGTATAAAATCTC	ATGGTCATTTTGGAGTT	CGTCTCCGA	350
Y45	TCCTAATGGAATCTCAAGGCCTACCATGAGTATAAAATCTC	ATGGTCATTTTGGAGTT	CGTCTCCGA	350
L9	AGCCTCTTCCCAAAA	TCCGGTCCATCGCTTACGAGCTGGACCCCACTGTA	AACTCAACTCCCTTTCC	420
W71a	AGCCTCTTCCCAAAA	TCCGGTCCATCGCTTACGAGCTGGACCCCACTGTA	AACTCAACTCCCTTTCC	420
Y63	AGCCTCTTCCCAAAA	TCCGGTCCATCGCTTACGAGCTGGACCCCACTGTA	AACTCAACTCCCTTTCC	420
L44	AGCCTCTTCCCAAAA	TCCGGTCCATCGCTTACGAGCTGGACCCCACTGTA	AACTCAACTCCCTTTCC	420
W6	AGCCTCTTCCCAAAA	TCCGGTCCATCGCTTACGAGCTGGACCCCACTGTA	AACTCAACTCCCTTTCC	420
Y45	AGCCTCTTCCCAAAA	TCCGGTCCATCGCTTACGAGCTGGACCCCACTGTA	AACTCAACTCCCTTTCC	420
L9	TCAACTATCAACAA	TTCCGGATCACAAAGCCCGGGA	GAGGCGTTTACAGCGTCTTACATCAACGG	A 490
W71a	TCAACTATCAACAA	TTCCGGATCACAAAGCCCGGGA	GAGGCGTTTACAGCGTCTTACATCAACGG	A 490
Y63	TCAACTATCAACAA	TTCCGGATCACAAAGCCCGGGA	GAGGCGTTTACAGCGTCTTACATCAACGG	A 490
L44	TCAACTATCAACAA	TTCCGGATCACAAAGCCCGGGA	GAGGCGTTTACAGCGTCTTACATCAACGG	A 490
W6	TCAACTATCAACAA	TTCCGGATCACAAAGCCCGGGA	GAGGCGTTTACAGCGTCTTACATCAACGG	A 490
Y45	TCAACTATCAACAA	TTCCGGATCACAAAGCCCGGGA	GAGGCGTTTACAGCGTCTTACATCAACGG	A 490
L9	AGGAATGGCAGGACGTTGCCGAGGACCAATT	CAGGATCCTCTACAAAGGCAATGGTCTT	CATCGATAGC	560
W71a	AGGAATGGCAGGACGTTGCCGAGGACCAATT	CAGGATCCTCTACAAAGGCAATGGTCTT	CATCGATAGC	560
Y63	AGGAATGGCAGGACGTTGCCGAGGACCAATT	CAGGATCCTCTACAAAGGCAATGGTCTT	CATCGATAGC	560
L44	AGGAATGGCAGGACGTTGCCGAGGACCAATT	CAGGATCCTCTACAAAGGCAATGGTCTT	CATCGATAGC	560
W6	AGGAATGGCAGGACGTTGCCGAGGACCAATT	CAGGATCCTCTACAAAGGCAATGGTCTT	CATCGATAGC	560
Y45	AGGAATGGCAGGACGTTGCCGAGGACCAATT	CAGGATCCTCTACAAAGGCAATGGTCTT	CATCGATAGC	560
L9	TGGTCTTT	AGAATCACCAT	AAGTGCCAATTCACAATCCCAAATAG	609
W71a	TGGTCTTT	AGAATCACCAT	AAGTGCCAATTCACAATCCCAAATAG	609
Y63	TGGTCTTT	AGAATCACCAT	AAGTGCCAATTCACAATCCCAAATAG	609
L44	TGGTCTTT	AGAATCACCAT	AAGTGCCAATTCACAATCCCAAATAG	609
W6	TGGTCTTT	AGAATCACCAT	AAGTGCCAATTCACAATCCCAAATAG	609
Y45	TGGTCTTT	AGAATCACCAT	AAGTGCCAATTCACAATCCCAAATAG	609

Decoration 'Decoration #1': Shade (with dark green at 50% fill) residues that differ from the Consensus.

Appendix 3.4 Nucleotide sequence alignment of P3 gene of TuYV isolates of the two genetic groups (clades).

Hap_15

CACACTTTAGAAGTCAGGAAAGTCAGATACCTCCATCCAAAGCAAGTAACGTTTCTTTTAGCAGGTCTATTGCTT
AACATTAACAATTTGTAAAAGCAATCAACGAGCGCAACAATGAATTCAAAAGTATATTTTCTTCGCTCTCTG
CTCTATCAGCTTCTCTCCACCTCGGAGACCACATCCACGATGATGTCAGGAAGTCCATACTTGCTCCTGAACCA
GAGCTTTGTGCTGGTTCTCTTTACAAACGGGATATGCTCCCGCCTCCACCTCAGGCCGTGTTAACTTACACGTG
CCAGGAACCAAGACCTCTCGCGAAGAATCATAACGATCTCTTGGGAGCGATTTCTCAGAAAAGCTCAAGCGA
TTTCCAGAATGCCTATTCCGTAGCCTTGAGTATTTCCAGCGATTTCTATCAACATGGACTAAAGACGTTGAAAGA
CGTATCTTTTCTAGCTGTGAGAAATCTCTCTGGGGTCTGACACGCTTATGGAGCTCGCTAATCTTGGCGAGCTT
CTCCGCGTTATGGTGGTTGGTGAGCAATTTCAACTCCCCTCTTCTGTCTCGCCTTGCTGTACACTGTTACAAG
ATTTATGGTGAAGACGTTTCATTTCTTTTTGGAGGATTGCCAATCTGGATCATCTCGATTGCTTTCTCACTCCT
GAAGAAATCCTTTTCAGCTCTTCG

Hap_31

CACACTTTAGAAGTCAGGAAAGTCAGATACCTCCACCCAAAGCAAGTAACGTTTCTTCTAGCAGGTCTATTGCTT
AACATTAACAATTTGTCAAAGCAATCAAAGAGCGCAACAATGAATTCAAAAGTATATTTTCTTCGCTCTCTG
CTCTATCAGCTTCTCTCCACCTCGGAGACCACATCCACGATGATGTCAGGAAGTCCATACTTGCCCTGAACCA
GAGCTTTGTGCTGGTTCTCTTTACAAACGGGATATGCTCCCGCCTCCACCTCAGGCCGTGTTAACTTATACGTG
CCAGGAACCAAGACCTCTCGCGAAGAATCTTACAACGATCTTTTGGGAGCGATTTCTCAGAAAAGCTCAAGCGA
TTTCCAGAATGCCTATTCCGTAGCCTTGAGTATTTCCAGCGATTTCTATCAACATGGACTAAAGACGTTGAAAGA
CGTATCTTTTCTCGCTGTGAGAAATCCCTCTGGGGTCTGACACGCTTGTGGAGCTCGCTAATCTTGGCGAGCTT
CTCCGCGTTATGGTGGTTGGTGAGCAATTTCAACTCCCCTCTTCTGTCTCGCCTTGCTGTACACTGTTACAAG
ATTTATGGTGAAGACGTTTCATTTCTTTTTGGAGGATTGCCAATCTGGATCATTTGATGCTTTCTCACTCCT
GAAGAAATCCTTTTCAGCTCTTCG

Hap_42

CACACTCTAGAAGTCAGGAAAGTCAGATACCTCCACCCAAAGCAAGTAACGTTTCTTTTAGCAGGTCTATTGCTT
AACATCAAACAATTTGTAAAAGCAATCAAAGAGCGCAACAATGAGTTCAAAAGTATATTTTCTTCGCTCTCTG
CTCTATCAGCTTCTCTCCACCTCGGAGACCACATCCACGATGATGTCAGGAAGTCCATACTTGCTCCTGAACCA
GAGCTTTGTGCTGGTTCTCTTTACAAACGGGATATGCTCCCGCCTCCACCTCAGGCCGTGTTAACTTACACGTG
CCAGGAACCAAGACCTCTCGCGAAGAATCATAACGATCTTTTGGGAGCAATTTCTCAGAAAAGCTCAAGAGA
TTTCCAGAATGCCTATTCCGTAGCCTTGAGTATTTCCAGCGATTTCTATCAACATGGACTAAAGACGTTGAAAGA
CGTATCTTTTCTCGCTGTGAGAAATCCTTTGGGGTCTGACACGCTTATGGAGCTCGCTAATCTTGGCGAGCTT
CTCCGCGTTATGGTGGTTGGTGAGCAATTTCAACTCCCCTCTTCTGTCTCGCCTTGCTGTACACTGTTACAAG
ATTTATGGTGAAGACGTTTCATTTCTTTTTGGAGGATTGCCAATCTGGATCATTTGATGCTTTCTCACTCCT
GAAGAAATCCTTTTCAGCTCTTCG

Appendix 3.5 Haplotypes (Hap 15, Hap 31, Hap 42) of *Turnip yellows virus* shared occurring in all three regions of Lincolnshire, Warwickshire and Yorkshire