Resistance to Turnip mosaic virus (TuMV) in *Brassica juncea* and introgression of resistance from *Brassica rapa*, *Brassica napus* and *Brassica nigra* into *Brassica juncea*

by

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Declarations

I declare that the work presented in this thesis was conducted by myself except otherwise stated. This thesis was written by myself and has not been previously submitted for any other degree. It is presented in accordance with the regulations for the degree of Doctor of Philosophy.

Tongtong Wang

Abstract

Turnip mosaic virus (TuMV, family *Potyviridae,* genus *Potyvirus*) has the widest host range amongst potyviruses. Globally it was said to be the second most important virus infecting field vegetables. *Brassica juncea* (Oriental mustard, family *Brassicaceae*), is an amphidiploid plant species with the genome AABB, comprising the genomes of the two diploid species, *Brassica rapa* (AA) and *Brassica nigra* (BB). It is widely grown and has various uses including as a leaf, stem, or root vegetable, oilseed crop, forage crop, condiment and biofumigant. Most *B. juncea* cultivars are very susceptible to TuMV, resulting in severe losses. Research on TuMV resistance and the mapping and identification of natural resistance genes would be very useful in order to speed up breeding resistant crops through marker-assisted selection.

Sources of resistance to TuMV have been identified in *B. juncea*. The specificity of the resistances has been determined. A *B. juncea* DH line for which there is genomic information has been challenged with TuMV and found to be susceptible. This line has been used as a susceptible parent in crosses with resistant plants derived from different sources to develop segregating populations for mapping the resistance gene(s). Two BC_1 populations (222 plants and 205 plants) and one F_2 population (159 plants) have been phenotyped and segregation ratios were not significantly different from a Mendelian model based on the action of two recessive genes.

Parental lines and selected plants in the two BC_1 populations have been analysed by SNPs genotyping using the Illumina Infinium Chip. Genetic linkage maps have been constructed and QTLs have been mapped. Additionally, attempts are being made to identify a dominant TuMV resistance gene present in both *Brassica napus* and *B. rapa*. Inter-specific crosses have been made in order to introgress this gene into *B. juncea*. Resynthesised *B. juncea* plants possessing this dominant resistance have been produced through embryo rescue and polyploidy induction of F_1 plants from crosses between resistant *B. rapa* and susceptible *B. nigra* plants. BC_2 plants have also been developed by crossing *B. rapa* and *B. napus* plants possessing the dominant TuMV resistance with a susceptible *B. juncea* plant line.

Abbreviations

Virus Abbreviations

ClYVV	Clover yellow vein virus
CMV	Cucumber mosaic virus
LMV	Lettuce mosaic virus
PPV	Plum pox virus
PVMV	Pepper veinal mottle virus
PVY	Potato virus Y
TMV	Tobacco mosaic virus
TuMV	Turnip mosaic virus

Common Abbreviations

aa	amino acid
AFLP	Amplified fragment length polymorphism
APAF-1	Apoptotic protease-activating factor 1
bp	base pair
CAPS	Cleaved amplified polymorphic sequences
CC	Coiled coil motif
cDNA	complementary deoxyribonucleic acid
CDS	Coding DNA sequence
CED-4	Cell death protein 4
CP	Coat protein
dCAPS	derived cleaved amplified polymorphic sequences
ddH_2O	double-distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
eIF4E	eukaryotic translation initiation factor 4E
eIF(iso)4E	eukaryotic translation initiation factor isoform 4E
ELISA	Enzyme-linked immunosorbent assay
EM algorithm	Expectation maximisation algorithm
ETI	Effector-triggered immunity

ETS	Effector-triggered susceptibility
gDNA	genomic deoxyribonucleic acid
HR	Hypersensitive response
LRR	Leucine-rich repeat domain
mRNA	messenger ribonucleic acid
NB	Nucleotide binding domain
NGS	Next generation sequencing
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PTI	PAMP-triggered immunity
QTL	Quantitative trait loci
RFLP	Restriction fragment length polymorphism
RLK	Receptor-like kinase
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
STK	Serine/threonine kinase
TIR	Toll/interleukin 1 receptor domain
TM domain	Transmembrane domain
UTR	Untranslated region
VPg	Viral protein genome-linked

Chapter 1 General Introduction

1.1 *Brassicaceae*

Brassicaceae (or *Cruciferae*/mustard family) is a large angiosperm dicot family distributed worldwide on all continents except Antarctica. According to "The Plant List (2013)", it comprises 372 genera and 4060 accepted species. Flowers of *Brassicaceae* plants have four petals forming a cross or sometimes reduced, six stamens with the outer two being shorter than the inner four (Anjum *et al.*, 2012). The family contains many economically important species such as broccoli, cabbage, rapeseed, cauliflower, Chinese cabbage, radish, turnip, etc. The well-known model organism, *Arabidopsis thaliana*, belongs to *Brassicaceae* and is significantly important to plant research (Anjum *et al*., 2012).

1.1.1 *Brassica* genus

The *Brassica* genus is a monophyletic group within the family *Brassicaceae* including about 35 species of mainly annual herbs, with some perennial herbs and small shrubs. It contains a large proportion of economically important plant species. There are three diploid species [*Brassica rapa* (genome AA, 2n=20), *B. nigra* (BB, 2n=16), *B. oleracea* (CC, 2n=18)] and three allotetraploid species [*Brassica juncea* (AABB, 2n=36), *B. napus* (AACC, 2n=38), *B. carinata* (BBCC, 2n=34)] (Fig. 1.1) which were derived by natural hybridisation and polyploidisation from two of the diploid species (Snowdon *et al.*, 1997). The botanical and genomic relationship between these six interrelated species is usually represented as the triangle of U (U, 1935) (Fig. 1.1). In addition to vegetable uses, *Brassica* plants are also the third most important source of vegetable oil in the world, after palm and soybean (Anjum *et al.*, 2012). Arabidopsis is a well-known model plant that has revolutionised knowledge in many fields of modern plant biology.

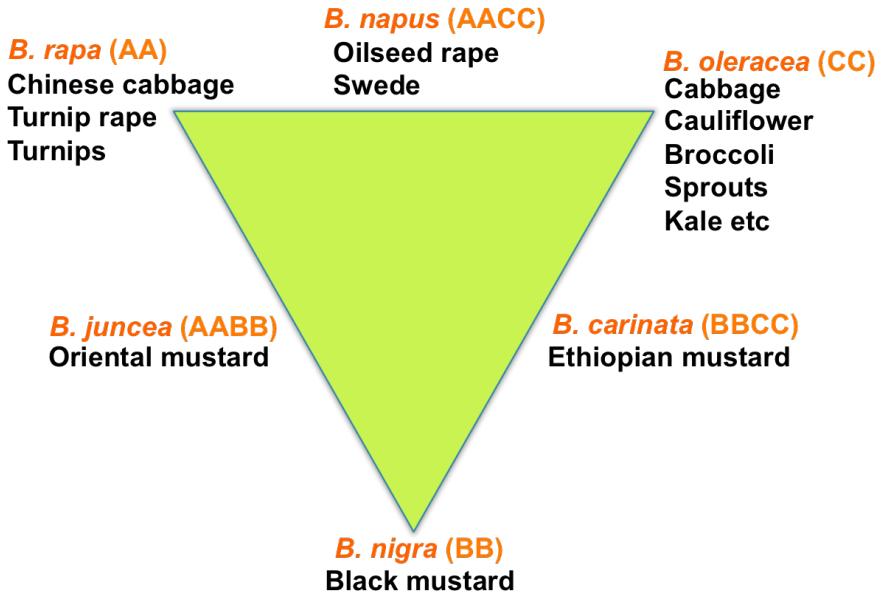


Figure 1.1 − The "Triangle of U" representing genomic relationships amongst *Brassica* species (U, 1935).

1.1.2 *Brassica juncea*

Brassica juncea (family *Brassicaceae*, genus *Brassica*), commonly known as oriental mustard, is a natural allotetraploid plant species with the genome AABB, comprising the genomes of the two diploid progenitor species, *Brassica rapa* (2n=20, AA) and *Brassica nigra* (2n=16, BB) (U, 1935). Archaeological remains of *B. juncea* from 2000 BC have been identified in the Indus valley (Spect and Diederichsen, 2001). There are various types of *B. juncea* with different morphology characteristics and uses. The largest diversity is found in West and Central China and Central Asia (Hammer *et al.*, 2013). *B. juncea* can be classified into the following four sub-species: 1. ssp. *integrifolia*, used as a common leaf vegetable in East and Southeast Asia; 2. ssp. *juncea*, cultivated mainly for the seeds which are processed into oil or condiments and occasionally cultivated as a forage crop; 3. ssp. *napiformis*, a type of tuberous root vegetable, largely used as a pickle in East Asia; 4. ssp. *taisai*, stems and leafs are used as vegetables in China. Zha cai, pickled *B. juncea* ssp. *taisai* stem originated from Sichuan, is a highly popular vegetable product in China (Hammer *et al.*, 2013).

As there are various types of *B. juncea*, it is difficult to pinpoint the center of origin. However, cytogenetic, biochemical and molecular studies have indicated the polyphyletic origin of *B. juncea* and the different varieties of parent species, *B. rapa*

and *B. nigra* contributed to the evolution of distinct morphotypes of *B. juncea* (Gómez-Campo and Prakash, 1999). Based on 99 SSR (Simple Sequence Repeat) markers, Chen *et al*. (2013) studied the molecular genetic diversity of 119 *B. juncea* varieties collected from China, India, Europe and Australia. Two distinct groups were identified, which occurred in overlapping regions of China and India. This supported the polyphyletic origin of *B. juncea*, and was consistent with the classification of two major geographic races, the Indian race and Oriental race (Vaughan, 1977). Meanwhile, it also supported the proposal made by Vavilov (1951) that China and India are the secondary centers of genetic diversity of *B. juncea*. There might have been two independent migrations of *B. juncea* from its center of origin in the Middle East and adjacent regions where distributions of *B. rapa* and *B. nigra* overlap, to China and India along trade routes, followed by regional domestication/adaptation (Chen *et al*. 2013).

B. juncea is an economically important crop and has various uses. It is widely cultivated as an economically important oilseed crop (Burton *et al.*, 2008). It is the predominant oilseed crop in India and has been very important to Indian agriculture (Chen *et al.*, 2013). In comparison with the other oilseed crops (*B. napus* and *B. rapa*), *B. juncea* is more tolerant to drought and heat stress (Woods *et al.*, 1991). Low erucic acid and low glucosinolate varieties have been developed for canola quality oil (Potts *et al*., 1999). The oil extracted is not only used as an edible vegetable oil, but also as a biofuel source which is economically and environmentally friendly (Dorado *et al.*, 2006). It has a variety of vegetable uses and is widely eaten. According to "PROTAbase" on *B. juncea*, the leaves are eaten in Africa and many parts of Asia and are often served as a side dish with the staple food. Young tender leaves, called 'mustard greens' are used in salads, mixed with other salad greens. The stem and root types of *B. juncea* are popularly used as pickle in China and other Asian countries. In addition to oil extraction, seed of *B. juncea* has also been largely used in the production of condiment mustard. In North America, oilseed *B. juncea* is largely grown as a condiment crop. *B. juncea* is also grown as a forage crop and is useful in crop rotations (Si and Walton, 2004). Recently, *B. juncea* has been developed as a biofumigant as it can play an important role in suppressing soil-borne pathogens and pests, such as potato cyst nematodes (Ngala *et al.*, 2014). In addition, several investigations have supported the use of *B.*

juncea in phytoremediation as it tolerates high concentrations of heavy metals and accumulates them in its cells (Mohamed *et al.*, 2012; Sharma *et al.*, 2015).

1.2 Plant viruses

Historically, plants were the first recognized hosts for viruses (Beijerinck, 1898) and there have been much research on plant viruses for nearly 120 years. There are nearly 1000 species of plant viruses according to the report of the International Committee for the Taxonomy of Viruses (King *et al*., 2012). The vast majorities of these plant viruses have been found in crop plants and have caused significant losses to the yield.

As with all other viruses, plant viruses are obligate intracellular parasites that lack the molecular machinery to replicate without a host. They consist of two components, capsid, which is a protein acting as a protective shell and nucleic acid. Based on the nature of the genome, plant viruses can be classified into six major groups, positive sense single-stranded RNA (ssRNA+), negative sense single-stranded RNA (ssRNA-), double-stranded RNA (dsRNA), single stranded DNA (ssDNA), double-stranded DNA (dsDNA) and reverse-transcribing viruses (Nellist, 2013).

1.2.1 *Potyviridae* and *Potyvirus*

The family *Potyviridae* contains the largest numbers of species of all plant virus families, comprising eight genera: *Brambyvirus*, *Bymovirus*, *Ipomovirus*, *Macluravirus*, *Poacevirus*, *Potyvirus*, *Rymovirus* and *Tritimovirus* (King *et al*., 2012). A common feature of the *Potyviridae* family is that all members induce the formation of virus inclusion bodies called cylindrical inclusions in their obligate hosts (López-Moya *et al.*, 2009).

Potyvirus is a genus of plant viruses in the family *Potyviridae*. It is the largest genus in this family, containing almost 90% of its species. *Potyvirus* is also one of the two largest genera of all plant viruses, with the other one *Begomovirus* (Gibbs and Ohshima, 2010). Potato virus Y (PVY) is the type species of the *Potyvirus* genus.

Many viruses in this genus cause severe damage in agricultural crops and infect an extensive range of mono- and dicotyledonous plant species worldwide. The *Potyvirus* virion is non-enveloped with a flexuous and filamentous nucleocapsid, 700-750 nm long. It contains a single copy of the genome, which is a positive sense, single-stranded RNA (ssRNA+) molecule of approximately 10,000 nucleotides. The genome contains untranslated regions (UTR) flanking both ends of the open reading frame (Gibbs and Ohshima, 2010).

1.3 Turnip mosaic virus (TuMV)

Turnip mosaic virus (TuMV) belongs to the genus *Potyvirus* and was first described in 1921 in the USA in host plant *Brassica rapa* (Gardner and Kendrick, 1921; Schultz, 1921). It was ranked as the second most important virus infecting field vegetables in a survey of virus diseases in 28 countries and regions, second only to Cucumber mosaic virus (CMV) (Tomlinson, 1987).

1.3.1 Host range and symptoms

The host range of TuMV is cosmopolitan and it has been isolated from an extensive range of crop and weed plant species. It has the broadest host range of any virus in the *Potyvirus* genus and is recognised to infect at least 318 species in more than 43 dicot families, including *Cruciferae*, *Compositae*, *Chenopodiaceae*, *Leguminosae* and *Caryophyllaceae*, and is also known to infect monocots (Walsh and Jenner, 2002). TuMV is particularly damaging to brassicas in parts of Asia, Europe and North America. TuMV is rated as the most important virus infecting brassicas in many Asian countries (Yoon *et al*., 1993). Additionally, TuMV significantly affects many non-brassica crops and ornamentals including radish, lettuce, escarole, endive, horseradish, pea, courgette, rhubarb, statice and stock (Walsh, 1997).

TuMV causes various leaf symptoms in infected plants, such as classical systemic mosaic, leaf curling, chlorotic lesions, chlorotic mottling, vein clearing, necrotic lesions and stunted growth. Symptom variation largely depends on the virulence of the virus pathotypes and on the pattern of susceptibility or resistance of the host. Apart from symptoms on leaves, the number and size of seed pods of infected

plants can be reduced, sometimes malformed and seedless, according to the survey on TuMV infecting oilseed rape (*Brassica napus* ssp. *oleifera*); seed size, yield and seed viability can be affected as well (Walsh and Tomlinson, 1985).

1.3.2 Physical properties and genome organization

As with other members of the genus *Potyvirus*, the virion of TuMV is non-enveloped with a flexuous, filamentous, rod-shaped particle (Fig. 1.2). It is approximately 720 nm long and 15-20 nm in diameter. The virions consist of 95% coat protein (CP) and 5% RNA (Walsh and Jenner, 2002).

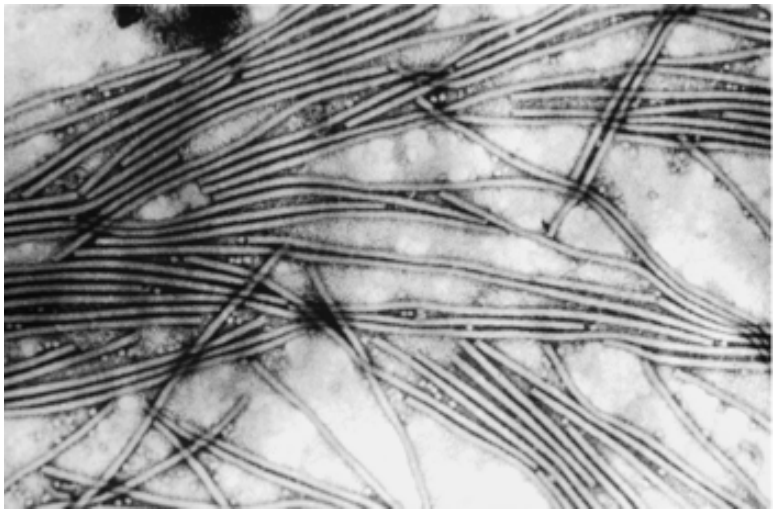


Figure 1.2 − Electron micrograph of TuMV virions with methylamine tungstate staining (Colin Clay, University of Warwick).

The TuMV genome is composed of a positive sense, single-stranded RNA molecule of approximately 9830 nucleotides (nt), with a polyA tail of variable length at the 3' terminus and a 22 kDa virus-encoded VPg (virus protein genome-linked) attached at the 5' terminus (Fig. 1.3). Not only is the RNA a template for replication, the genomic RNA is also the messenger RNA (mRNA) for protein synthesis. Flanked by two untranslated regions (UTRs), the large open reading frame (ORF) is translated into a single polyprotein, which is co- and post-translationally cleaved by three virus-encoded proteases. The proteins after processing include the 40 kDa P1, 52 kDa helper component protease (HC-Pro), 40 kDa P3, 6 kDa 6K1 (might be attached to P3), 72 kDa cytoplasmic/cylindrical inclusion (Cl) protein, 6 kDa 6K2,

22 kDa VPg, 27 kDa nuclear inclusion protein a (NIa, which might be attached to VPg), 60 kDa nuclear inclusion protein b (NIb) and 33 kDa coat protein (CP) (Table 1.1) (Walsh and Jenner, 2002).

As conventional gene-hunting software is not sufficient for detecting short overlapping coding sequences (CDSs), Chung *et al*. (2008) applied MLOGD to the TuMV genome and found an overlapping ORF embedded within the TuMV P3 cistron but translated in the +2 reading-frame. This overlapping ORF was named *pipo* (Pretty Interesting *Potyviridae* ORF) and was confirmed by bioinformatic evidence and experimental verification. It was indicated that the PIPO protein was expressed as a fusion protein with the N-terminal of P3 (P3N-PIPO) (Chung *et al*., 2008). Furthermore, the P3N-PIPO protein of TuMV was recognised as a plasmodesmata (PD)-located protein that physically interacts with the CI protein *in planta* (Wei *et al*., 2010). PD-associated structures are essential for intercellular transport of potyviruses, coordinated by the CI protein and P3N-PIPO complex (Wei *et al*., 2010).

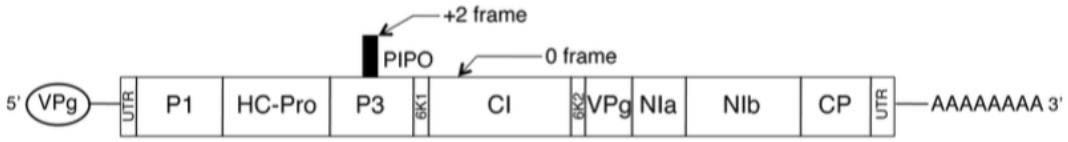


Figure 1.3 − Schematic representation of Turnip mosaic virus genome, showing the position of the overlapping coding region within the P3 cistron, the viral protein genome-linked (VPg) covalently attached to the 5' end of the genome and the polyadenylated tail at the 3' end (Nellist, 2013).

Table 1.1 − Functions of mature potyviral proteins (Nellist, 2013).

Name	Function	References
P1	Proteinase	Carrington *et al*., 1990
	Genome amplification	Verchot and Carrington, 1995
	Suppressor of gene silencing	Anandalakshmi *et al*., 1998
HC-Pro	Aphid transmission	Pirone and Thornbury, 1984
	Proteinase	Carrington *et al*., 1989
	Systemic movement	Rojas *et al*., 1997
	Suppressor of gene silencing	Anandalakshmi *et al*., 1998
P3	Genome amplification	Rodríguez-Cerezo *et al*., 1993
	Avirulence gene	Jenner *et al*., 2003
6K1	RNA replication	Riechmann *et al*., 1992
CI	ATPase/RNA helicase	Laín *et al*., 1990
	Cell-to-cell movement	Carrington *et al*., 1998
	Avirulence gene	Jenner *et al*., 2000
6K2	Virus replication	Restrepo-Hartwig and Carrington, 1994
	Long-distance movement	Rajamäki and Valkonen, 1999
VPg	Interaction with eIF4E and eIF(iso)4E	Léonard *et al*., 2000
	Replication	Schaad *et al*., 1997
	Cell-to-cell and systemic movement	Schaad *et al*., 1997
NIa	Proteinase	Dougherty *et al*. 1989
NIb	RNA-dependent RNA polymerase	Hong and Hunt, 1996
CP	Encapsidation	Shukla and Ward, 1989
	Aphid transmission	Atreya *et al*., 1990
	Cell-to-cell and systemic movement	Dolja *et al*., 1994
	Virus assembly	Dolja *et al*., 1994
	Genome amplification	Mahajan *et al*., 1996
PIPO	Virus movement	Wei *et al*., 2010

1.3.3 Transmission, infection and replication

TuMV is transmitted by aphids in the non-persistent stylet-borne manner. It is non-specifically transmitted by a large number of aphid species. At least 89 aphid species are reported to be able to transmit TuMV, including the well known *Myzus persicae* and *Brevicoryne brassicae* (Edwardson and Christie, 1986). Aphids do not need a long-feeding period to take up non-persistent viruses but only need brief probes of seconds to minutes. Such viruses have a short retention time in aphids and are lost readily during probing a healthy plant (Hooks *et al.*, 2007).

As for TuMV replication, the virus particle is uncoated and the genome replicated, once it enters a plant cell. Genome replication takes place in the cytoplasm close to membrane surfaces (Carrington *et al*., 1996). Because host RNA dependent RNA polymerase (RdRp) produces only short molecules, being a + strand RNA virus, TuMV has to encode its own RdRp, which is done by the NIb protein. The CI protein has helicase activity in replication, which is necessary to remove template secondary structures and to unwind the duplex formed between template and the freshly developed RNA strand. The 6K2 and VPg proteins are also engaged in replication complexes on cytoplasmic membranes (Walsh and Jenner, 2002). Template specificity is reported to dwell in the sequence of 3' UTR, which is to prevent the virus amplifying host mRNAs (Dreher, 1999).

1.3.4 Diversity and mutation

There have been various studies on the classification of TuMV strains and pathotypes. Early researchers differentiated strains of TuMV according to host range and symptomatology of test plants. Pound and Walker (1945) investigated TuMV isolates by inoculating *Brassica* and *Nicotiana* species. Yoshii (1963) distinguished two strains based on symptom types in *B. oleracea* and *N. glutinosa*. McDonald and Hiebert (1975) divided TuMV into two main groups based on whether all *Brasscia* species are infected or not. Liu *et al*. (1990) defined seven TuMV pathotypes (Tu1-Tu7) using a variety of *Brassica* species.

Other differentiation schemes are based on the symptomatology of differential lines of a single *Brassica* species. Six TuMV strains (C1-C6) have been distinguished based on the symptom types of differential lines of *B. rapa* (Provvidenti, 1980, strains C1-C4; Green and Deng, 1985, C5; Stobbs and Shattuck, 1989, C6). A collection of 124 isolates of TuMV from around the world were characterised by inoculation onto four *B. napus* differential lines, as a result, twelve TuMV pathotypes (1-12) were differentiated (Jenner and Walsh, 1996; Walsh, 1989; Table 1.2). The *B. napus* differential system is considered more comprehensive not only because of the large scale of TuMV isolates surveyed, but also it described the gene-for-gene interactions between TuMV pathotypes and different dominant resistance genes in the lines (Walsh and Jenner, 2002). RNA sequence comparison

has also been applied to group TuMV isolates, particularly using the coat protein sequences due to availability in a number of isolates (Walsh and Jenner, 2002).

Table 1.2 – Interactions of Turnip mosaic virus pathotypes with differential lines of *Brassica napus* (oilseed rape and swede).

Virus pathotype	Plant line			
	Rape S6	Rape R4	Swede 165	Swede S1
1	+[a]	0[b]	0	+
2	R[c]	R	0	R
3	+	$+_N$	0	+
4	+	+	+	+
5	+	+	0	+
6	+	$+_N$	0	R
7	+	0	0	R
8	+	$+_N$	R_N	R
9	+	R_N	0	R
10	+	+	0	0
11	R	+	0	R
12	+	+	$+_N$	+

[a] +, systemic infection, plants were susceptible. [b] 0, no infection, plants appeared to be immune. [c] R, local infection, no systemic spread. Local symptoms were chlorotic and systemic symptoms were mosaic unless indicated by N (necrotic). From Jenner and Walsh (1996).

The diversity of TuMV may result from point mutation (Jenner and Walsh, 1996) and recombination (Chen *et al*., 2002; Ohshima *et al*., 2002). RNA viruses have a relatively high ratio of misincorporation error, mostly 0.1-10 mutations per 10 kb molecule per replication cycle (Domingo and Holland, 1997). The reason is RNA polymerases lack 3' to 5' exonuclease proof-reading activity, and mismatch repair cannot take place on single-stranded progeny genomes. Moreover, replication slippage in the 5' terminus region of TuMV genome was detected by Hancock *et al*. (1995). The ability to evolve rapidly may be the reason why TuMV can infect a broad range of hosts. In other circumstances, however, this could introduce disadvantages for TuMV. For example, a mutant of TuMV isolate UK 1 that overcomes UK 1-resistance has less fitness compared to UK 1 (Jenner *et al.*, 2002).

1.3.5 Detection and management

TuMV can be differentiated from other potyviruses through serological techniques, especially with monoclonal antibodies (MAbs). One of the most variable regions of

potyvirus genome lies at N-terminus of coat protein (CP), which is surface located (Shukla *et al*., 1988). Thus CP is the typical section where MAbs have been developed against to be able to distinguish between TuMV isolates (Walsh and Jenner, 2002). EMA67, a MAb developed by Jenner *et al.* (1999) has so far been giving positive reactions with all TuMV isolates infecting brassicas. In addition to serological techniques, genetic methods have also been applied to identify TuMV isolates, such as analysis of RNA sequence, especially the CP region. CP sequences are available for a number of TuMV isolates (Walsh and Jenner, 2002).

As mentioned in 1.3.3, TuMV is non-persistently transmitted by aphids, which only need very short time to take up or transmit TuMV. The use of insecticides has proven insufficient in TuMV control (Walsh and Jenner, 2002), as aphids are capable of transmitting TuMV prior to being killed by many insecticides. On the contrary, use of insecticides may increase virus transmission by killing off natural enemies and by encouraging intensified aphid movement (Robert *et al*., 1993; Hooks *et al.*, 2007). Additionally, aphids that transmit virus non-persistently tend to not stay or reproduce on the plants they transmit virus to (Hooks *et al.*, 2007). Unlike bacterial and fungal pathogens where plants can be protected from infection directly by chemicals, there is no direct method to control viruses. Cultural approaches such as removing infected materials and scheduling planting dates may help to diminish the impact to some extent (Shattuck, 1992). In the long run, the most effective, economical and environmental friendly way to control TuMV is to develop immune or highly resistant cultivars. Therefore, the deployment of TuMV resistance genes and breeding of resistant crops are crucial.

1.4 Plant disease resistance

There are various rapidly evolving pathogens causing plant diseases, which heavily impact crop production and food security around the world. Moreover, plant pathogens can spread rapidly over large distances, with wind, water, insects and humans as vectors (Dangl *et al.*, 2013). Therefore, plant disease resistance is important for the reliability of food production. Unlike animals, plants cannot move from place to place to avoid unfavorable conditions. Also, plants do not have a circulation system and mobile immune cells, so they cannot detect intruders using

circulating immune receptors (Spoel and Dong, 2012). However, plants have sophisticated resistance mechanisms and they can start highly specific immune responses that have long-lasting effect.

1.4.1 Types of plant disease resistance

Overall, active plant disease resistance can be broadly categorized into two layers, preformed mechanisms and infection-induced responses of the immune system. The first layer includes preformed structures and compounds, such as the waxy cuticle layer, cell walls, cuticular lipids (Reina-Pinto and Yephremov, 2009), antimicrobial chemicals and proteins and enzyme inhibitors (Habib and Fazili, 2007). The plant cell wall can be reinforced to fend off pathogens through the deposition of callose following the induction of host defence pathways (Spoel and Dong, 2012). Once pathogens have overcome these defensive layers, they are systematically confronted by the plant immune system that contains two interconnected tiers of receptors, one outside and one inside the cell. These two tiers recognize different types of pathogen molecules (Dangl *et al.*, 2013). The first tier of the plant immune system is primarily governed by extracellular surface pattern-recognition receptors (PRRs) that particularly recognize conserved microbial elicitors named pathogen-associated molecular patterns (PAMPs). PAMPs are the essential and typical components associated with certain classes of pathogens, such as bacterial flagellin, fungal chitin, lipopolysaccharides and peptidoglycans. PRRs are stimulated after recognizing PAMPs, which leads to PAMP-triggered immunity (PTI), the first tier of the plant immune system (Dodds and Rathjen, 2010). PTI involves intracellular signaling, transcriptional reprogramming, and synthesis of a complex output response that restricts pathogen colonization (Monaghan and Zipfel, 2012).

PTI is sufficient to repel most pathogens that are nonadaptive and is therefore an important tier in disease resistance. However, some successfully adapted pathogens can circumvent PTI by delivering molecules called effectors into the plant cell, which results in effector-triggered susceptibility (ETS). For example, AvrPto1 is one of the effectors of *Pseudomonas syringae* pv. *tomato* that promotes pathogen virulence by interfering with PTI (Zipfel and Rathjen, 2008). Plants, in return, have developed the second tier of the immune system named effector-triggered immunity

(ETI). ETI is governed by intracellular immune receptors known as resistance (R) proteins that can detect the presence of specific pathogen effector molecules. R proteins and effectors (avirulence proteins) are extremely diverse both between and within species, and thus the recognition is quite specific. ETI restores and amplifies PTI antimicrobial defences, and is often involved with localised plant cell death, referred to as the hypersensitive response (HR). A local HR in a plant can induce long-lasting systemic immunity known as systemic acquired resistance (SAR), which is accompanied by a systemic increase in the levels of salicylic acid (SA) and pathogenesis-related (*PR*) gene expression (Cui *et al.*, 2015).

1.4.2 Classes of plant resistance (R) proteins

R proteins can be divided into 5 classes based on their location and combination of structural motifs (Dangl and Jones, 2001; Martin *et al.*, 2003). The first class is intracellular NB-LRR (nucleotide-binding site plus leucine-rich repeat) proteins. This is the largest class of R proteins and its members possess a C-terminal LRR domain, a central NB domain and a varying N-terminal effector domain. Between NB and LRR domains, there is a highly conserved region of homology that is also present in some eukaryotic cell death effectors such as apoptotic protease-activating factor 1 (APAF-1) and cell death protein 4 (CED-4). This region is called ARC domain because of its presence in APAF-1, R proteins and CED-4 (Dangl and Jones, 2001). Because the NB and ARC domains are contiguous, these two domains are often referred to as the NB-ARC domain. It was suggested that R proteins might control plant cell death by virtue of the NB-ARC domain (Martin *et al.*, 2003). NB is critical for ATP or GTP binding (Martin, 1999). A leucine-rich repeat (LRR) is a protein structural motif forming an α/β horseshoe fold. It consists of repeat 20-30 amino acid stretches that are rich in the hydrophobic amino acid leucine (Enkhbayar *et al.*, 2004). LRR domains are present in diverse proteins and function as sites of protein-protein interaction, peptide-ligand binding and protein-carbohydrate interaction (Kajava, 1998). Major R proteins rely on a limited number of structural and functional domains, of which LRR appears to play a central role. Based on N-terminus structural features, the NB-LRRs can be subdivided into two classes: Toll/interleukin 1 receptor domain NB-LRRs (TIR-NB-LRR) and coiled coil motif

NB-LRRs (CC-NB-LRR) (Dangl and Jones, 2001). NB-LRR proteins are intracellular and lack transmembrane (TM) domains (Fig. 1.4).

The second class of R proteins is LRR proteins encoding membrane-bound extracellular proteins. Cf proteins from tomato belong to this class. *Cf* confers resistance to the fungus *Cladosporium fulvum* (Dixon *et al.*, 1996). Cf proteins lack NB but instead have a TM, extracellular LRR and a small putatively cytoplasmic tail without obvious motifs (Fig. 1.4).

The third class of R proteins is intracellular protein Kinase containing just one member-"Pto", from tomato. *Pto* confers resistance to the bacterium *Pseudomonas syringae*, encoding a serine/threonine kinase (STK) that lacks any obvious receptor-like domain and TM (Tang *et al.*, 1996). A threonine at position 204 plays a key role in its interaction with AvrPto. This residue is conserved in a number of STKs but is absent from non-functional *Pto* alleles. It is considered that phosphorylation of this residue might lead to a conformational change of kinase which allows AvrPto binding.

The fourth class comprises receptor-like protein kinases with an extracellular LRR domain, represented by the Xa21 protein from rice against bacterium *Xanthomonas oryzae*. In addition to an extracellular LRR and a TM, it has a cytoplasmic serine/threonine kinase (STK) region (Song *et al.*, 1995) (Fig. 1.4).

The fifth class is R proteins without obvious protein interaction domains and that have novel structures. An example is the RPW8 protein, conferring resistance to powdery mildew in Arabidopsis, it encodes a small potential membrane protein with a possible coiled-coil domain and basically no other homology to known proteins (Xiao *et al.*, 2001) (Fig. 1.4).

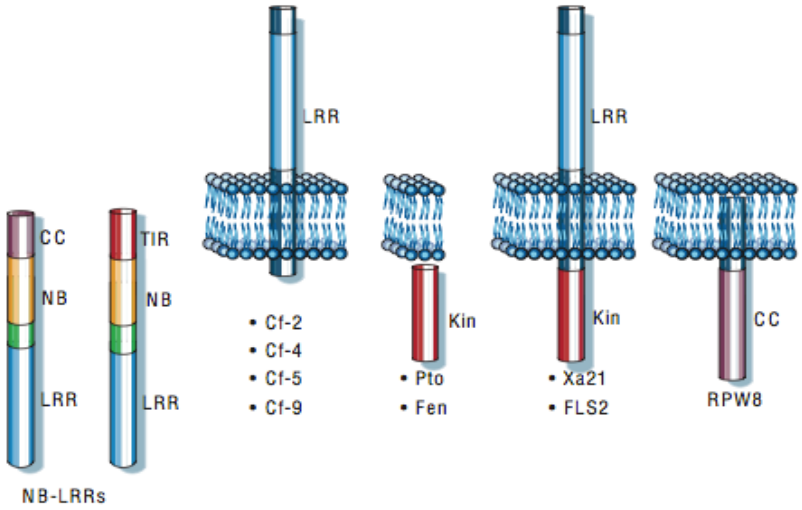


Figure 1.4 − Representation of the location and structure of the five classes of plant disease resistance proteins (Dangl and Jones, 2001).

1.4.3 Plant protein kinases and their relation to plant resistance

General introduction of kinases

In general, a kinase is a type of enzyme that catalyzes the transfer of phosphate groups from high-energy, phosphate-donating molecules to specific substrates. This process is known as phosphorylation. The substrate gains a phosphate group and the high-energy molecule of ATP (adenosine triphosphate) donates a phosphate group (Walsh and Ashby, 2013). Conversely, it is referred to as dephosphorylation when the phosphorylated substrate donates a phosphate group and ADP gains a phosphate group (producing a dephosphorylated substrate and the high energy molecule of ATP). The phosphorylation state of a molecule, whether it be a protein, lipid, or carbohydrate, can affect its activity, reactivity and its ability to bind other molecules. Therefore, kinases are critical in metabolism, cell signaling, protein regulation, cellular transport, secretory processes and countless other cellular pathways (Walsh and Ashby, 2013).

Kinases transmit signals and regulate complex processes in cells. Phosphorylation of molecules can enhance or inhibit their activity and modulate their ability to interact with other molecules. Kinases are broadly classified into groups by the substrate they act upon: protein kinases, lipid kinases, carbohydrate kinases (Walsh and Ashby, 2013).

Protein Kinases related to plant resistance

Recent studies revealed that higher plants possess genes coding for putative receptor kinases (Receptor-like Kinases, RLK). For instance, a completely sequenced Arabidopsis genome contains over 600 genes encoding RLKs (Shiu and Bleecker, 2001), suggesting that higher plants, like animals, use receptor kinase signaling commonly and broadly in responding to vast arrays of stimuli to modulate gene expressions.

A common feature of these putative receptor-like kinases (RLKs) is that each has an N-terminal signal sequence, an extracellular domain that varies in structure, a single transmembrane (TM) region, and a cytoplasmic protein kinase catalytic domain. Plant RLKs are classified into subfamilies based on the structural feature of the extracellular domain, which is thought to act as a ligand-binding site. One R protein possessing a kinase domain is Xa21 protein from rice against bacterium *Xanthomonas oryzae.* In addition to an extracellular LRR and a TM, it has a cytoplasmic serine/threonine kinase (STK) region (Song *et al.*, 1995). Xa21 belongs to the LRR-RLKs.

Pto, a cytoplasmic serine/threonine kinase, confers race-specific resistance to *Pseudomonas syringae* by recognition of AvrPto. Pto does not contain a known ligand-binding motif but is involved in both elicitor recognition and phosphorylation (Tang *et al.*, 1996). R protein receptors Xa21 and Pto fall into the same RLK (Receptor-like Kinase) subfamily (Afzal *et al*., 2008).

1.4.4 Plant recessive resistance

In research on plant disease resistance, Eckardt (2002) first introduced the term of susceptibility genes (S-genes), which are considered as the dominant genes whose modification gives rise to recessive resistance to plant pathogens. Plant recessive resistance occurs when there is mutation in specific host proteins (targets of pathogen effector) encoded by S-genes. According to the different types of S-gene products, plant recessive resistance can be divided into two categories (Pavan *et al*., 2010). The first category of recessive resistance results from the mutation of plant

negative defence regulators that counteract resistance responses. One well-characterised example in this category is the trans-membrane MLO protein, which negatively regulates the *PEN* gene conferring resistance to powdery mildew disease in both barley and Arabidopsis (Panstruga, 2005). The mutations of *Mlo* lead to efficient pre-invasion resistance to adapted powdery mildews (Humphry *et al.*, 2006). This type of recessive resistance has not been observed in plant virus resistances, although it is common in plant-fungus interactions (Truniger and Aranda, 2009). The second category of recessive resistance is induced by the loss-of-function mutation of susceptibility factors that are necessary for the growth and proliferation of plant pathogens. For example, plant eIF4E and eIF4G protein families are susceptibility factors. They function as translation initiation factors for potyvirus replication and translation through interaction with the potyvirus protein VPg (Viral Protein Genome-linked). The lack of interaction between VPg and the translation initiation complex will lead to recessive resistance in plants (Robaglia and Caranta, 2006). "eIF" stands for eukaryotic translation initiation factor. eIF4E binds with eIF4G to form the eIF4F complex, which provides scaffolding for other members of the translation initiation complex. Both eIF4E and eIF4G have isoforms in plants, named eIF(iso)4E and eIF(iso)4G, respectively. As with eIF4E, eIF(iso)4E binds with its eIF(iso)4G to form eIF(iso)4F complex (Browning, 1996). To date, potyvirus VPgs have been found to be able to interact with eIF4E, eIF4G and both of their isoforms (Robaglia and Caranta, 2006; Nicaise *et al*., 2007). Nicaise *et al*. (2007) suggested that potyviruses recruited eIF4E and eIF4G factors in a coordinated and selective manner. For example, CIYVV recruits eIF4E and eIF4G while LMV, PPV and TuMV recruit eIF(iso)4E and eIF(iso)4G.

Nearly half of more than 200 published virus resistance genes are recessively inherited, indicating that recessive resistance is more common for plant viruses than for other plant pathogens. Furthermore, recessive resistance is over-represented in plant resistance to potyviruses where more than half the resistance genes are recessively inherited, which is more frequent than any other plant virus families (Kang *et al*., 2005). Importantly, all characterised recessive resistances to viruses stem from the mutations in eIF4E or eIF4G protein families (Pavan *et al*., 2010).

1.5 Plant resistance to TuMV

There have been various studies on the resistances to potyviruses and recessive genes have been found to comprise a surprisingly high proportion (40%). Both recessive and dominant genes have proven effective against TuMV (Walsh and Jenner, 2002).

1.5.1 TuMV resistance in non-*Brassica* and *Brassica* species

There are studies that identified TuMV resistances in non-*Brassica* species. Provvidenti *et al*. (1979) conducted TuMV resistance tests on a collection of chicory (*Cichorium intybus*) and found most lines were resistant. Resistance to one strain of TuMV has been identified in garden balsam (*Impatiens balsamina*) (Provvidenti, 1982). In lettuce, the dominant gene *Tu* confers broad-spectrum resistance to all isolates of TuMV tested and it has been mapped (Robbins *et al*., 1994).

TuMV resistances have been found in a wide range of brassicas. In addition to the resistances identified in the differential lines in various differentiation systems, there have been resistance tests conducted on *B. rapa* (Chinese cabbage, turnip), *B. oleracea* (cabbage, cauliflower, broccoli, Brussels sprout and kohlrabi) and *B. napus* (swede and oilseed rape) species. Both dominant specific resistances and recessive broad-spectrum resistances have been found in brassicas.

1.5.2 TuMV resistance genes and their specificities

In the *Brassica* genus, several resistance genes against TuMV have been found, including *TuRB01* (Walsh *et al.*, 1999), *TuRB03* (Hughes *et al.*, 2003), *TuRB04* and *TuRB05* (Walsh and Jenner, 2002) in *B. napus*, and *TuRB01b* (Lydiate *et al.*, 2014; Rushholme, 2000), *retr01*, *ConTR01* (Rusholme *et al.*, 2007), *retr02* (Qian *et al.*, 2013), *TuRBCS01* (Li *et al*., 2014), *TuRB07* (Jin *et al.*, 2014) and *TuMV-R* (Chung *et al.*, 2014) in *B. rapa*. The genes mentioned above are all in the *Brassica* 'A' genome. *TuRB01*/*TuRB01b* is a dominant resistance gene conferring extreme resistance to TuMV pathotype 1, but is overcome by single nucleotide nutations in

the CI gene. *TuRB03* provides extreme resistance to TuMV isolate CDN 1 that belongs to pathotype 4 (Hughes *et al.*, 2003). *TuRB05* controls a necrotic response (hypersensitive response) to TuMV infection that restricts systemic spread of virus. *TuRB04* is epistatic to *TuRB05* and together confer extreme resistance to pathotypes 1 and 3 of TuMV. The recessive gene *retr01* is epistatic to the dominant gene, *ConTR01*, and in conjunction they confer broad-spectrum resistance to TuMV (Rusholme *et al.*, 2007). Recessive gene *retr02* itself carries broad-spectrum resistance to TuMV (Qian *et al.*, 2013). *TuRBCS01*, *TuRB07* and *TuMV-R* are all dominant genes and each provides resistance to TuMV strain C4.

There was one weak quantitative resistance gene identified in the 'C' genome of *B. napus*, named *TuRB02*. It confers quantitative resistance to TuMV pathotype 1 isolate CHN 1 (Walsh *et al.*, 1999). No TuMV resistance gene has been mapped in *Brassica* 'B' genome so far. In some circumstances, genes conferring systemic necrosis are considered to be proper resistance genes, as they are so called R genes mediating the HR form of defence reaction that can kill infected plants rapidly thus removing them as internal sources of virus infection. Nyalugwe *et al*. (2014) mapped a TuMV necrosis gene *TuRBJU 01* on 'A' genome of *B. juncea*. Liu *et al*. (2015) identified the TuMV necrosis gene *TuNI* in *Arabidopsis* ecotype Ler, which interacts with TuMV P3 gene in a gene-for-gene manner.

As mentioned previously, *B. juncea* is a very important species among brassica crops. However, most *B. juncea* cultivars tested are very susceptible to TuMV, resulting in severe losses (Fjellstrom and Williams, 1997; Kehoe *et al.*, 2010). Therefore, research on TuMV resistance and mapping and identification of natural resistance genes in *B. juncea* would be very useful in order to speed up breeding resistant crops through marker-assisted selection.

1.6 Development and application of molecular markers

In genetics, a molecular marker is a fragment of DNA or a single nucleotide that associated with a certain locus within the genome. Molecular markers are crucial tools for plant identification and improvement. The development of genetic markers has gone through several phases. Early markers based on phenotyping or isozymes

(non-DNA-based) were replaced by DNA-based methods due to the improvement of DNA analysis technologies. With the advent of polymerase chain reaction (PCR), early hybridisation-based molecular markers were overtaken rapidly by PCR-based markers, which increased the feasibility of high-throughput marker screening significantly. Because of very limited sequence information, early PCR-based methods relied on arbitrary primers. Then more robust markers such as simple sequence repeats (SSRs) were developed. Along with the development of sequencing technologies, sequence-based markers have been developed, such as single nucleotide polymorphisms (SNPs), with great versatility and utility (Henry, 2013).

1.6.1 Classes of molecular markers

First-generation markers relied on restriction enzymes and hybridisation

Restriction fragment length polymorphism (RFLP) is the representative marker of the first generation. An individual restriction enzyme recognises a specific nucleotide sequence and even a single nucleotide alteration can invalidate or create a restriction site. Thus there is polymorphism between individual homologous DNA molecules in terms of the locations of cutting sites and the lengths of DNA between them, resulting in restriction fragments of different sizes. The co-dominant nature and high reproducibility are the two major advantages of RFLP markers. The disadvantages are requirement for large amounts of pure DNA, low multiplex ratio, time-consuming and labor-intensive (Jones *et al.*, 2009). Variable number tandem repeat (VNTR) is another type of first generation marker, which is based on hybridisation such as DNA southern blot (Jeffreys *et al*., 1985).

Second-generation markers based on PCR

The utilisation of PCR has significantly accelerated the development and application of molecular markers in plants. PCR allows exponential copies of DNA sections to be produced, and thus PCR-based markers confer highly increased sensitivity and require much smaller quantities of DNA to start. These markers are also highly specific, easily automated and can be used in conjunction with other

techniques, *e.g.*, restriction enzymes and hybridisation (Henry, 2013). There are numerous types of markers in this category, to name a few, Random amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Cleavage amplification polymorphisms (CAPs), Simple sequence repeat (SSR), Inter-simple sequence repeat (ISSR), Sequence tagged sites (STS), sequence characterized amplification region (SCAR), etc. (Jones *et al.*, 2009).

Third-generation markers based on DNA sequencing

When conventional Sanger sequencing was predominating, development of DNA sequencing had been confined. Over the last few years, next-generation sequencing (NGS) technologies have overtaken and have dramatically increased sequencing efficiencies (Varshney and May, 2012). Further enhancements in sequencing will continue the trend of delivering larger volumes of sequence data at lower costs. There are a range of sequence-based approaches to marker discovery in plants, such as whole-genome sequencing, organellar sequencing, transcriptome sequencing, amplicon sequencing, enriched genome sequencing and genotyping by sequencing (GBS) (Henry, 2013). Single nucleotide polymorphism (SNP) and Insertion/deletion (InDel) markers were developed based on DNA sequencing.

1.6.2 SNPs genotyping

The ultimate minimum of genetic variation is the single nucleotide polymorphism (SNP), and thus they can provide the maximum number of markers. Generally, SNP frequencies in plants are in a range of one SNP every 100-300bp (Jiang, 2013). In terms of location in the genome, SNPs can be present in the coding regions, non-coding regions of genes or in the intergenic regions, with higher frequencies in non-coding regions than in coding regions. In the coding regions, there are synonymous and non-synonymous SNPs depending on whether the translated amino acid sequences change. SNPs are co-dominant markers and can have dense representation in genomic maps. Therefore, there is a high probability that many SNPs will have tight linkage with target genes near or adjacent to them, or within genes that they are literally located in (Jones *et al.*, 2009). Moreover, SNPs can be fairly easily automated and rapidly detected. Hence, SNP has become a

significantly important molecular marker in genetic study and breeding. The recent advances in DNA sequencing will also result in SNPs being increasingly applied for various purposes.

There are various ways of detecting and genotyping SNPs, depending on individual needs. Nowadays, SNPs are widely detected by sequencing. A convenient method for visualising SNPs is SNP-RFLP, CAPS or dCAPS marker technique. For example, if one allele confers a restriction site for a restriction enzyme whereas the other not, digestion of the two alleles will yield different fragments in length, which can be displayed by DNA staining and gel electrophoresis. SNPs genotyping characterises individuals for their SNP profile and the methods depend largely on the technology and sequence information available (Jones *et al.*, 2009). With the decreasing costs and growing accessibility of next-generation sequencing (NGS) and genotyping assays, high-throughput methods based on multiplexing are increasingly used on a large scale, such as the Affymetrix Genechips (Affymetrix Inc.), Amplifluor (Serological Corp.) and the TaqMan, SNPlex and SnaPshot assays from Applied Biosysterms. Lately, Illumina® has introduced the Goldengate and Infinium high-throughput genotyping assays (Hayward *et al.*, 2012).

1.6.3 Gene mapping and marker-assisted selection

Gene mapping is assigning a specific gene to a particular section of a chromosome and determining the distances between genes. Normally, a collection of molecular markers are assigned to their respective positions on the genome to construct genome maps, where genes can be viewed as one particular type of genetic marker and mapped in the same way. With the advances in the next-generation sequencing (NGS), high-quality SNPs established by genome sequencing are starting to take over from a wide range of other molecular markers for construction of genetic maps (Hamilton and Robin, 2012). In addition, SNPs identified within ESTs (Expressed sequence tags) or transcriptome NGS data also enable the evaluation of allele frequencies and allelic correlation with phenotypes of interest. Preliminary gene mapping can locate QTLs (Quantitative trait loci) to a region on the genome. Genotyping and identifying SNPs in these regions facilitates fine-mapping or extremely high density mapping of the QTLs. SNPs that have close linkage with

genes are identified as candidates for qualitative or quantitative trait nucleotides (QTNs), also named perfect markers, whereby different alleles are correlated to phenotypes. Perfect markers enable the rapid screening assays for genetic selection of germplasm, or marker-assisted breeding (Hayward *et al.*, 2012).

Marker-assisted selection (MAS) is an indirect selection measure where a trait of interest is selected not based on the trait itself, but on marker(s) tightly linked to the gene determining the trait. Markers can act as a proxy for the target phenotype, enabling efficient selection at an early stage for traits that are difficult or expensive to measure (Hayward *et al.*, 2012). Most of the markers used in MAS nowadays are DNA-based (molecular) markers, instead of morphological, biochemical or cytological markers previously used. To be practically effective, a molecular marker must be closely linked to the gene of interest (generally within 1 cM), and available with high throughput and highly consistent screening methods. In comparison with other molecular markers, the use of SNPs in MAS has two major advantages. Firstly, it is easier to obtain a tightly linked SNP to a gene of interest than any other type of molecular markers; and secondly, SNPs are much more likely to be the causative agent or perfect markers for the phenotype (Hayward *et al.*, 2012).

1.7 Plant interspecific crossing and embryo rescue

Plant interspecific hybridisation (or wide hybridisation) is a cross between individuals of different species or genera, which can combine separate genomes into one nucleus. Interspecific hybridisation breaks the barrier between plant species and enables interspecific transfer of genes. It induces variation in genotypes and phenotypes of the progenies. It is a very important and practical technique for crop genetic improvement and new germplasm development (Liu *et al.*, 2014).

1.7.1 Interspecific crossing barriers and the methods for overcoming these

Generally, the frequency of interspecific hybrids obtained by conventional crossing method is low due to the presence of incompatibility barriers. The incompatibility barriers may arise at any stage from pollination to seed formation or even at later

stages of developing into a fertile plant (Bhat and Sarla, 2004). Stebbins (1958) categorised interspecific crossing barriers into pre- and post-fertilisation barriers broadly. Pre-fertilisation barriers include failure of pollen germination, impaired pollen tube growth, poor penetration through stigma papillae and growth arrest of tubes in the style or ovules, all of which inhibiting fertilisation. Post-fertilization barriers include degeneration of the hybrid embryo, male and female sterility in the hybrid plants, hybrid inviability and segregation in progenies (Bhat and Sarla, 2004). According to the specific type of barrier, appropriate methods can be used to overcome both pre- and post-fertilization barriers (Pratap and Kumar, 2014).

The bud pollination (flower buds are emasculated and pollinated 2-3 days prior to anthesis) is the mostly used technique to overcome pre-fertilization barriers. In addition to emasculating bud prior to anthesis, sometimes even the stigma is removed and the cut end is pollinated, which is called stump pollination (Bhat and Sarla, 2004). The use of mixed pollen and irradiated mentor pollen was reported to be effective to overcome incompatibility (Brown and Adiwilaga, 1991; Pratap and Kumar, 2014). Application of plant growth hormones (such as auxins and cytokinins) to pedicel during or soon after pollination can facilitate pollen tube growth to achieve fertilisation (Pratap and Kumar, 2014). Furthermore, the direction of the crossing affects the outcomes of interspecific hybridisation. As a general rule, it is more productive to use plant species with higher chromosome number as the female parent than the reciprocals (Kumar and Pratap, 2014). Also, use of self-compatible species as the female parent tends to be more successful in interspecific hybridisation (Kaneko and Bang, 2014).

The most common post-fertilisation barrier is embryo degeneration resulted from abnormal development of the endosperm. This can be successfully overcome by using culture medium as the replacement of endosperm that supplies nutrients to the developing embryo. A technique called *in vitro* embryo rescue has been widely used in interspecific hybridisations to rescue hybrid embryo before natural abscission occurs (Pratap and Kumar, 2014).

1.7.2 Embryo rescue and polyploidy induction

The basis of embryo rescue technique is the aseptic isolation and transfer of the embryo to an appropriate medium for development under optimal conditions. Generally, in such cultures there are no problems with the disinfection of embryos. Pollinated flowers, siliques, ovaries, ovules and embryos can be used for embryo culture, as for which to use depending on specific case (Kaneko and Bang, 2014; Sahijram and Rao, 2015).

The essential aspect of embryo culture is the selection of the medium suitable for growth of embryo. In general, it is sufficient to have a standard basal plant growth medium with major salts and microelements. The mature embryos can be cultured in a basal salt medium with a source of carbon energy (such as sucrose). In comparison, immature embryos additionally require certain amino acids, vitamins and growth regulators. Even in some cases, natural endosperm extracts are needed (Sahijram and Rao, 2015).

In many cases of interspecific hybridisation and microspore culture, polyploidy induction is necessary. For example, in microspore culture, spontaneous or induced chromosome doubling is required to produce of DH (double haploid) plants that are fertile and homozygous. The rate of spontaneous doubling varies largely among plant species. Within a species, there are differences among genotypes as well (Sood and Dwivedi, 2015). The *in vitro* conditions can also influence the spontaneous doubling rate (Kasha *et al*., 2006). If no spontaneous doubling occurs, the artificial polyploidy induction is needed. The most frequently used method is the treatment with anti-microtubule chemicals (such as colchicine) that inhibit microtubule polymerization through binding to tubulin. For colchicine treatment, the optimal combination of concentration and treatment duration is critical for a successful chromosome doubling (Caperta *et al*., 2006).

1.8 Aims and Objectives

The main aim of the study was to exploit plant natural resistance to TuMV in *B. juncea*, by virtue of discovering novel resistance and utilising previously mapped

resistance in brassicas.

The specific objectives of the study were:

1) To seek resistance to TuMV in *B. juncea* and *B. nigra*.
2) To characterise and map any identified TuMV resistance in *B. juncea* and *B. nigra*.
3) To identify which candidate gene(s) is the TuMV resistance gene *TuRB01*/*TuRB01b* that is present in *B. napus* and *B. rapa*.
4) To introgress *TuRB01*/*TuRB01b* from *B. napus* and *B. rapa* into *B. juncea* through interspecific hybridisation.

Through mapping novel TuMV resistance genes and further research on a previously mapped resistance gene, more insight will be gained on the mechanisms of interactions between TuMV isolates and the TuMV-resistance genes in brassicas. Ultimately, in terms of the practical application, it is very important to develop durable TuMV-resistant brassica varieties using a well-established marker-assisted selection program.

Chapter 2 Materials and Methods

2.1 Plant lines

2.1.1 Plant materials

***Brassica juncea* and *Brassica nigra* lines**

For the TuMV resistance tests, 34 lines of *B. juncea* and 27 lines of *B. nigra* were collected and evaluated for their resistance to TuMV isolate UK 1. The seeds of these lines were obtained from Warwick Crop Centre Genetic Resources Unit (GRU), IPK Gatersleben Germany, Institute of Vegetable and Flowers (IVF) Beijing, Saskatoon Research and Development Centre Canada and commercial outlets, covering various genetic and geographic origins (Table 2.1).

Table 2.1 − Information of plant materials in TuMV resistance tests.

Plant line	Species	Geographical origin	Type	Source
TWBJ01	*B. juncea*	Malaysia	Leaf	Warwick GRU
TWBJ02	*B. juncea*	Bhutan	Oilseed	Warwick GRU
TWBJ03	*B. juncea*	Bhutan	Oilseed	Warwick GRU
TWBJ04	*B. juncea*	Bhutan	Oilseed	Warwick GRU
TWBJ05	*B. juncea*	Zimbabwe	N/A	Warwick GRU
TWBJ06	*B. juncea*	Zimbabwe	N/A	Warwick GRU
TWBJ07	*B. juncea*	Zimbabwe	N/A	Warwick GRU
TWBJ08	*B. juncea*	Zimbabwe	N/A	Warwick GRU
TWBJ09	*B. juncea*	Zimbabwe	N/A	Warwick GRU
TWBJ10	*B. juncea*	Zimbabwe	N/A	Warwick GRU
TWBJ11	*B. juncea*	Zimbabwe	N/A	Warwick GRU
TWBJ12	*B. juncea*	Zimbabwe	N/A	Warwick GRU
TWBJ13	*B. juncea*	Zimbabwe	N/A	Warwick GRU
TWBJ14	*B. juncea*	China	Root	IVF Beijing, China
TWBJ15	*B. juncea*	China	Leaf	IVF Beijing, China
TWBJ16	*B. juncea*	China	Leaf	IVF Beijing, China
TWBJ17	*B. juncea*	China	Stem	IVF Beijing, China
TWBJ18	*B. juncea*	China	Stem	IVF Beijing, China
TWBJ19	*B. juncea*	China	Leaf	Shandong, China
TWBJ20	*B. juncea*	China	Root	Shandong, China
TWBJ21	*B. juncea*	Bhutan	Oilseed	Warwick GRU
TWBJ22	*B. juncea*	Hong Kong	Leaf	Warwick GRU
TWBJ23	*B. juncea*	China	Root	Warwick GRU
TWBJ24	*B. juncea*	Uruguay	N/A	Warwick GRU
TWBJ25	*B. juncea*	Japan	Leaf	Warwick GRU
TWBJ26	*B. juncea*	Japan	Leaf	Warwick GRU
TWBJ28	*B. juncea*	SUN	N/A	Warwick GRU
TWBJ29	*B. juncea*	China	Leaf	Warwick GRU
TWBJ30	*B. juncea*	Japan	Oilseed	Warwick GRU
TWBJ31	*B. juncea*	India	Oilseed	Warwick GRU

TWBJ32	*B. juncea*	India	Oilseed	Warwick GRU
TWBJ33	*B. juncea*	U.K.	Leaf	Warwick GRU
TWBJ34	*B. juncea*	India	Oilseed	Warwick GRU
060DH17	*B. juncea*	N/A	N/A	Saskatoon,Canada
TWBN01	*B. nigra*	Germany	N/A	IPK
TWBN02	*B. nigra*	Poland	N/A	IPK
TWBN03	*B. nigra*	Greece	N/A	IPK
TWBN04	*B. nigra*	Italy	N/A	IPK
TWBN05	*B. nigra*	France	N/A	IPK
TWBN06	*B. nigra*	Germany	N/A	IPK
TWBN07	*B. nigra*	Hungary	N/A	IPK
TWBN08	*B. nigra*	Pakistan	N/A	IPK
TWBN09	*B. nigra*	Russia	N/A	IPK
TWBN10	*B. nigra*	Greece	N/A	IPK
TWBN11	*B. nigra*	Greece	N/A	IPK
TWBN12	*B. nigra*	Greece	N/A	IPK
TWBN13	*B. nigra*	Italy	N/A	IPK
TWBN14	*B. nigra*	Italy	N/A	IPK
TWBN15	*B. nigra*	France	N/A	IPK
TWBN16	*B. nigra*	Alaska	N/A	IPK
TWBN17	*B. nigra*	Turkey	N/A	IPK
TWBN18	*B. nigra*	India	N/A	IPK
TWBN19	*B. nigra*	India	N/A	IPK
TWBN20	*B. nigra*	Yugoslavia	N/A	IPK
TWBN21	*B. nigra*	India	N/A	IPK
TWBN22	*B. nigra*	Denmark	N/A	IPK
TWBN23	*B. nigra*	India	N/A	IPK
TWBN24	*B. nigra*	Czechoslovaki	N/A	IPK
TWBN25	*B. nigra*	N/A	N/A	KEW Gardens
Ni-100	*B. nigra*	N/A	N/A	Saskatoon, Canada
al-1-3	*B. nigra*	N/A	N/A	Saskatoon, Canada

B. rapa and *B. napus* lines

Resistant and susceptible lines of "Tropical Delight" were used for the experiments on *TuRB01b* identification and introgression**.** "Tropical Delight" (T. Sakata and Company) is an F_1 hybrid commercial Chinese cabbage (*B. rapa*) line, which has extreme resistance to TuMV isolate UK 1 (Lydiate *et al*., 2014). Tropical Delight was self-pollinated and progeny were inoculated and tested to confirm their homozygosity for resistance or susceptibility. *B. napus* lines R4, S6 (Jenner and Walsh, 1996), 22S (Hughes *et al*., 2003), DH12075, NO1D, NO2D and PSA12, together with *B. napus* cultivars Global DH, Yudal, Darmor, Karoo and Cabriolet were used for research aimed at the identification of *TuRB01*. TuMV-resistant *B. napus* line Westar (Walsh *et al*., 1999) was used for introgression of *TuRB01* into *B. juncea*.

2.1.2 Development of mapping populations for TuMV resistance gene(s) in *B. juncea*

Selfs and crosses of *B. juncea*

Because *B. juncea* is a self-compatible plant species, it is fairly straightforward to self. Successful self-pollination was accomplished by enclosing the racemes of a plant within a perforated transparent bread bag before flowering (Fig. 2.1). While making crosses and backcrosses, bud inoculation was conducted. Immature flower buds of appropriate size were emasculated by removal of anthers. The stigmas of these buds were then pollinated with pollen of the desired male parent. The racemes were labelled and enclosed within perforated transparent bread bags to prevent cross-contamination (Fig. 2.1). In some cases, pollinations were repeated.

Figure 2.1 – Self-pollinations and cross-pollinations of *Brassica juncea* in the glasshouse.

Resistant *B. juncea* lines and corresponding mapping populations

Reciprocal crosses were made between resistant individuals from four *B. juncea* lines (TWBJ03, TWBJ14, TWBJ20 and TWBJ23) and a well-characterised UK 1-susceptible *B. juncea* line (060DH17) for which there is genomic information. Four F_1 seed lines were produced and confirmed to be UK 1-susceptible. Four F_1 seed lines were selfed and backcrossed to S_1 seed lines of resistant parent to produce F_2 and BC_1 populations, respectively (Fig. 2.2).

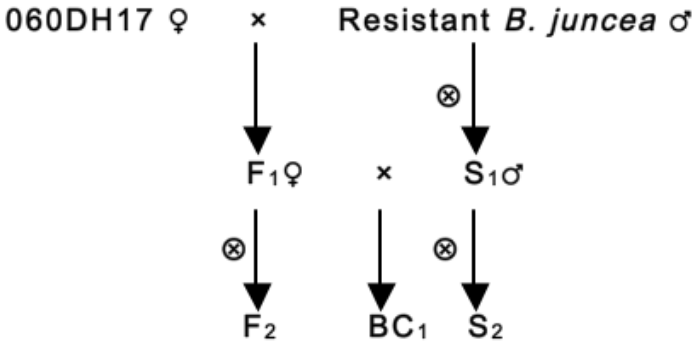


Figure 2.2 − Crossing strategy used to develop *Brassica juncea* BC_1 populations segregating for resistance to TuMV.

2.2 Plant growth

2.2.1 Glasshouse growth methods

The experimental plants were grown in an insect-proof glasshouse. Two compartments were used. One was for plant rearing prior to virus inoculation and the other was for virus inoculation (Fig. 2.3). The temperature is controlled at 18 ± 2°C.

Figure 2.3 − *Brassica juncea* plants following TuMV inoculation in the glasshouse.

2.2.2 Incubator growth methods

To produce resynthesised *B. juncea*, incubators were used. They were set at 25 ± 2°C with 12h photoperiod, 100 µmol $m^{-2}s^{-1}$ fluorescent light. Two types of incubators were used: Gallenkamp and Lab heat incubator (Fig. 2.4). After being transferred from medium into composts, seedlings of resynthesised *B. juncea* were grown in plant propagators for acclimation (Fig. 2.5).

Figure 2.4 − Embryo rescue of resynthesised *Brassica juncea* in incubators.

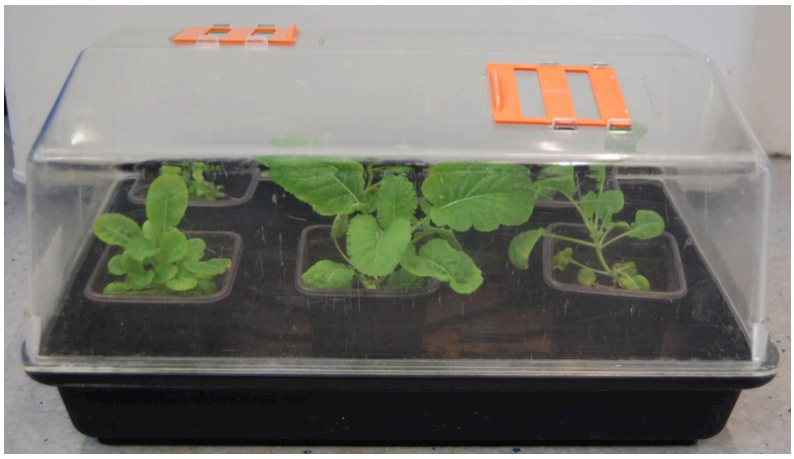


Figure 2.5 − Seedlings of resynthesised *Brassica juncea* in plant propagator.

2.3 Turnip mosaic virus (TuMV) transmission and detection

2.3.1 TuMV isolates

The TuMV isolates used were UK 1, vVIR24 and CDN 1, which belong to pathotypes 1, 3 and 4, respectively. These three pathotypes were found to be predominant in a study covering 124 isolates (Jenner and Walsh, 1996). TuMV isolate vVIR24 is an engineered mutant isolate of UK 1 with a single nucleotide substitution. It confers the ability to overcome the *TuRB01*-based resistance and has the same pathogenicity with pathotype 3 isolates of TuMV.

2.3.2 Mechanical transmission of TuMV and back inoculation

TuMV was mechanically transmitted as described by Jenner and Walsh (1996). Virus isolates were maintained in a susceptible host, mustard cv. Tendergreen (TGM). Systemically infected leaves of mustard were ground thoroughly in cold inoculation buffer (1% K_2HPO_4 + 0.1% Na_2SO_3) to produce inoculation sap. The leaves of test plant were dusted by 0.037mm carborundum. Then the inoculation sap was rubbed onto leaves using a small piece of muslin. Inoculated plants were kept in an insect-proof glasshouse at 18 ± 2°C.

When either the absence or presence of virus needed to be confirmed, back inoculations were conducted. Leaves from inoculated test plants were used for preparing sap to inoculate the indicator plants (e.g. TGM and *B. napus* line R4).

2.3.3 Visual assessment of infection

After inoculation, symptoms of test plants were scored at weekly intervals up to 4 weeks. Both inoculated and uninoculated leaves were assessed, in order to determine whether the infection was fully systemic or limited. Classification of phenotypes was as described by Jenner and Walsh (1996); 0, immune with no detectable symptom, R, resistance, infection limited to inoculated leaves but no systemic infection, +, susceptibility, systemic mosaic infection, $+_N$, susceptibility, systemic infection with necrosis. The visual phenotypes were checked by ELISA.

2.3.4 Enzyme-linked Immunosorbent Assay (ELISA)

At the end of visual assessment, uninoculated tip leaves were tested for the presence of TuMV in an indirect plate-trapped antigen (PTA) ELISA as described by Jenner and Walsh (1996). Leaf samples were ground between a pair of steel rollers (Meku-Pollahne, Wennigsen, Germany) and the sap was collected into separate Eppendorf tubes. Each well of ELISA plates (Nunc Immuno plate Maxi Sorp F96, Gibco Ltd., Uxbridge, UK) received 100µl of coating buffer (0.05 M sodium carbonate buffer), then 100µl of leaf sap of each plant was pipetted into duplicate wells of the plate which was kept at 4°C overnight. Antibodies were diluted in phosphate-buffered saline (PH 7.3) containing Tween 20 (PBS-T) and bovine serum albumin (0.5g/L) and incubated with samples for 2h at room temperature; the first antibody was a mouse monoclonal antibody EMA67 (Jenner *et al*., 1999), diluted in 1/500; the second antibody was goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma Chemical Co., Poole, UK), diluted in 1/3000. Finally the plates were incubated with the substrate p-nitrophenyl phosphate (1mg/mL in 0.1 M diethanolamine, PH 9.8) at room temperature. The subsequent reaction produced a colour change in the substrate. The colour strength indicated the quantity of virus. An Anthos Labtec HT2 microplate reader (Tech Gen International, London, UK) was used to measure the optical absorbance at 405 nm after 10-60 min (Fig. 2.6). Phenotypes were finalised by combining ELISA results and visual assessments as

follows: 0, immune, no symptoms, no virus detected by ELISA; R, local infection, no systemic infection, no virus detected by ELISA in uninoculated leaves; +, systemic infection without necrosis, virus detected by ELISA in uninoculated leaves, plant was susceptible; $+_N$, systemic infection with necrosis, virus detected by ELISA in uninoculated leaves, plant was susceptible.

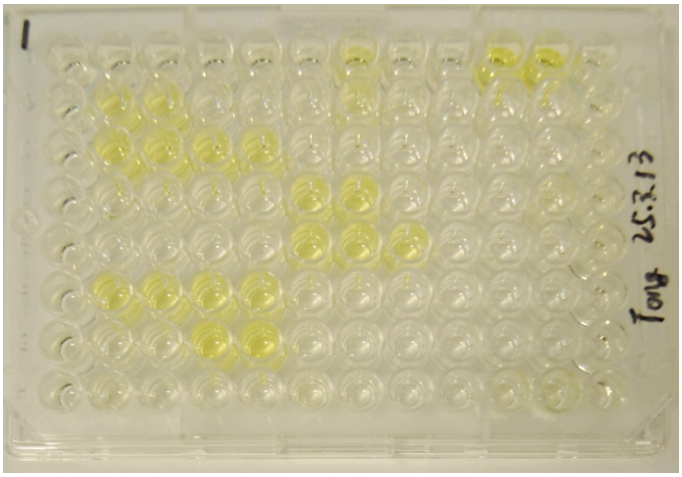


Figure 2.6 − ELISA plate test of TuMV-inoculated *Brassica juncea* plants.

2.3.5 Reverse transcription polymerase chain reaction (RT-PCR)

The TuMV genome comprises a single stranded RNA molecule. To detect the presence of TuMV in plant leaves, reverse transcription polymerase chain reaction (RT-PCR) is another option. TuMV sequences have been well studied in our research group and a selection of primers spread across the whole TuMV genome were available. After RNA extraction and RT-PCR, primer pair TuMV49 and TuMV9 was used for PCR amplification (Table 2.2). Then the presence of a band of the correct size on agarose gel confirms the presence of the TuMV. Sequencing of PCR products was performed to confirm virus identity and primer TuMV55 was used for sequencing (Table 2.2).

Table 2.2 − Primers used for TuMV gene amplification and sequencing.

Primer	Sequence (5'-3')	Direction	Target region
TuMV49	CAGGTTTTGGTCGGCTTTCA	Forward	TuMV CI gene
TuMV9	GGTGGGACGTCCTTTGGTAAC	Reverse	TuMV NIa gene
TuMV55	TCTTCAGGATCAAACCCATAC	Reverse	TuMV VPg gene

2.4 Nucleic acid techniques

2.4.1 Nucleic acid extraction

Collection of leaf samples

Fresh leaf samples were collected in 2ml Eppendorf microcentrifuge tubes (≤100 mg leaf for each tube) and immediately placed in liquid nitrogen for flash freezing. Leaf samples were stored in -80°C freezer prior to extraction.

Genomic DNA extraction

For manual DNA extraction, frozen leaf samples were disrupted thoroughly using a liquid-nitrogen-cooled electric drill. Extractions were performed using Qiagen DNeasy® Plant Mini Kit (Venlo, Netherlands), following the guidelines of DNeasy Plant Handbook which can be found at https://www.qiagen.com/gb/resources/.

Another approach was using the DNA extraction service at LGC Genomic Ltd. Leaf samples were freeze-dried and loaded on to 96 Deep Well plates. Then the plates were sent off and extracted frozen DNA samples were returned.

The concentration and quality of extracted DNA was validated using a NanoDrop® ND-100 spectrophotometer (Thermo Scientific). Some DNA samples prepared for Genotyping-by-Sequencing were quantified by Qubit™ Fluorometric Quantitation (San Diego, USA). Qubit™ fluorometric quantitation consists of the easy-to-use Qubit™ 3.0 Fluorometer and sensitive Qubit™ quantitation assays. This integrated system is more sensitive than UV absorbance–based quantification, because the detection is for target-specific fluorescence. Extracted DNA samples were kept at -20°C.

Total RNA extraction

Frozen leaf samples were disrupted into fine tissue powders using a liquid-nitrogen-cooled electric drill. Qiagen RNeasy® Plant Mini Kit (Venlo, Netherlands) was used for RNA extractions. The procedure follows the guidelines of the RNeasy

Mini Handbook, which can be found at https://www.qiagen.com/gb/resources/. The concentration and quality of extracted RNA was valuated using a NanoDrop® ND-100 spectrophotometer (Thermo Scientific). RNA samples were stored at -80°C.

2.4.2 Primers

PrimerSelect in DNASTAR's Lasergene® Core Suite version 10.1 was used to design primers for PCR amplification and sequencing. To design primers for the study of candidate genes of *TuRB01*, the sequence of a bacterial artificial chromosome (BAC) from a *B. napus* line was used as the template sequence. This BAC is 48302 bp long and covers all the candidate genes of *TuRB01* and their adjacent regions. The complete *Brassica* A genome sequence from *B. rapa* (Chiifu-401), provided on the web-based *Brassica* database (BRAD), was also used as a reference to design primers. The information of the primers was presented in Table 2.3 and Table 2.4.

Table 2.3 − Primers used for plant gene amplification.

Primer	Sequence (5'-3')	Direction	Target region
BR138	TTTGTTGGCTTATGTGTTGACG	Forward	5'UTR of *BORG1*
BR139	TGGTTCGACATACAGAATGTATTTC	Reverse	3'UTR of *BORG1*
BR164	CACCAAACCTGCAAAACAATCTCAACAAA	Forward	5'UTR of *BORG1*
BR165	GGAATATCGCACGCAGACAAGTAGTAAAC	Reverse	3'UTR of *BORG1*
BR183	AATAAAATTATTCTCAGTTGTCTAAAGC	Forward	5'UTR of *BORG2*
BR184	GCAATAGATCACAACAACAACAAAA	Reverse	3'UTR of *BORG2*
BR185	GTGGTTGGTGGCCTTCGTGGTC	Forward	5'UTR of *BORG2*
BR186	CGTTTTATTCTCCTTATTCGTCGCTGGC	Reverse	3'-end of *BORG2*
BR191	TCCAAAGAACGTCGCTGAAAG	Forward	5'-end of *BORG2*
BR192	TCCATCAGAATATCATCAACAGTGC	Reverse	3'-end of *BORG2*
BR200	CTAAGCTTGTTGACGGTGGAGACT	Forward	5'UTR of *BORG2*
BR202	ACGGCCTAGCGGGTGATGC	Reverse	3'UTR of *BORG2*
BR196	ATTCCTAGTGTTCAATTAAACTATCC	Forward	5'-end of Kinase
BR195	CTAACACGCCTAATACCGCTA	Reverse	3'-end of Kinase
BR211	ATAAGTTCCTCTGATTGATA	Forward	5'UTR of *BORG1*

Table 2.4 − Primers used for plant gene sequencing and analysis of gene expression.

Primer	Sequence (5'-3')	Direction	Target region
BORG1L	TTGATGATCTAAAACGCCGAATG	Forward	Exon1 of *BORG1*
BR160	GGGAGTAAGGATTGTTGGTGTAAA	Reverse	Exon3 of *BORG1*
BORG2L	ACTGATCTAGAACGTCACCTG	Forward	Exon1 of *BORG2*
BORG2R	CCCTCACATAGCTTCCAA	Reverse	Exon5 of *BORG2*
BR82	TGATGTATGGAAAAATGAAGACTGG	Forward	Exon1 of *BORG2*
BR148	TTCGTCGCTGGCACGCAACA	Reverse	Exon5 of *BORG2*
BR187	GCAGAGGGAGATCCGACAAAC	Forward	Exon2 of *BORG2*
BR188	GGACAAAACTCGGCAAACTG	Reverse	Exon4 of *BORG2*
BR189	TTCACCAAATGACCAACCAAGTC	Reverse	Exon3 of *BORG2*
BR190	CTCACGTGAATGCCACCTCATCC	Forward	Exon5 of *BORG2*
BR203	CTAAGCACACTGACACAGCCTG	Forward	Internal Kinase
BR204	CGAGCCCTAGAACCGTGAG	Reverse	Internal Kinase
BR205	TTCCAAAGGCTTCAGGTCTTGTTTATTCA	Forward	Internal Kinase
BR206	TGCCGCGAGCCCTAGAACCGT	Reverse	Internal Kinase
BR208	TTTCATATAAAAGGGTAGGA	Reverse	Exon3 of *BORG1*
BR210	TTTCATATAAAAGGGTAGCC	Reverse	Exon3 of *BORG1*

2.4.3 DNase treatment

There is no current RNA isolation procedure that removes 100% of the DNA. Because even a single molecule of DNA can be detected by PCR, RNA samples should be treated with DNase I before RT-PCR, otherwise it could result in false positive results. Thermo scientific DNase I (Carlsbad, USA) was used to digest single and double-stranded DNA into single bases or oligonucleotides. The enzyme is DNA-specific and does not affect the integrity of RNA (Vanecko and Laskowski, 1961). Application of the DNase treatment was according to the provided protocol. The prepared RNA was then used as a template for reverse transcription.

2.4.4 Reverse transcription reaction

The Invitrogen^{TM} SuperScript Reverse Transcriptase Kit (Carlsbad, USA) was used to perform reverse transcription reactions and produce complementary DNA (cDNA). Random hexamers (Invitrogen^{TM}) were used for RT-PCRs apart from the one on virus RNA using specific primers. The reactions were carried out in a themocycler (Bio-Rad, MyCycler^{TM}). Synthesised cDNA was kept at -20°C. The quality of the cDNA samples was tested by PCR using a primer pair

qRT2080F/qRT2080R. This primer pair was designed for detection of a plant housekeeping gene that encodes the 40S ribosomal protein S19 (Defilippi *et al*., 2005). The sequences of the forward and reverse primers are 5'-ATGGCAACTGGTAAAAC-3' and 5'-AGTGATTCTT-CTTCCTCTG-3', respectively.

2.4.5 Polymerase Chain Reaction (PCR)

Most of the PCRs were performed using Thermo Scientific Phusion High-Fidelity DNA Polymerase, which offers high-quality performance for all major PCR applications. From previous studies, the error rate of Phusion DNA polymerase in Phusion HF buffer is determined to be 4.4×10^{-7}, which is approximately 50-fold lower than Taq polymerase (Frey and Suppman, 1995). It's also adequate for amplification of long amplicons up to 7.5 kb in length. The denaturation and extension temperatures were 98°C and 72°C, respectively. The initial denaturation time was 2 minutes and 10s for each cycle. The extension time in each cycle was 30 seconds per 1 kb for genomic DNA, and 40 seconds per 1 kb for cDNA templates. The final extension duration was 7 minutes. Usually 0.5-1.0 U Phusion was used in 50µl reaction volume and 35 cycles were applied to most PCRs. A few PCRs were performed using normal Taq (Invitrogen™) and Elongase® enzyme (Invitrogen™), for which the denaturation temperatures were both 94°C. PCR reactions were performed in a thermocycler (Bio-Rad, MyCycler*™*).

2.4.6 Agarose Gel Electrophoresis

PCR products were separated using agarose gel electrophoresis. For most of my experiments, 1.5% agarose gels were prepared by dissolving certain amount of UltraPure™ agarose powder (Invirogen™) in 1× Tris-Borate-EDTA (TBE) buffer. GelRed™ (Biotium Inc.) was used as the fluorescent DNA stain, with a concentration of 2 µg/50 ml. The thickness of gels was usually 7-10 mm.

Before being loaded into gels, PCR products were mixed with 1/10 volume of 6× DNA loading buffer IV (0.25% Bromophenol blue, 40% (w/v) sucrose in dH_2O). The 1 Kb plus DNA ladder (Invirogen™) was used at a concentration of 0.5 µg/µl to estimate molecular mass of DNA bands and indicate concentration. Gels were

run under 70-120 V at room temperature. Following electrophoresis, gels were viewed on a Syngene G Box transilluminator, using software GeneSnap 7.07.

2.4.7 Purification of DNA fragments

Qiagen QIAquick® Gel Extraction Kit (Venlo, Netherlands) was used to purify targeted DNA fragments. The protocol for this kit indicates it is for the purification of up to 10µg DNA (70bp to 10kb). This was often used when a sequencing of PCR product was unsuccessful.

2.4.8 Sequencing

Two companies were used for sequencing. One was GATC Biotech, Konztanz Germany, and the other Source Bioscience Sequencing, Nottingham UK. The preparations of sequencing samples were the same for both companies. A total volume of 10µl was prepared comprising 2µl template DNA, 1µl 10mM primer and 7µl dH_2O. The obtained sequences were analysed and assembled in SeqMan (DNASTAR, Lasergene v10.1).

2.5 Genetic mapping

2.5.1 Illumina infinium chip for single nucleotide polymorphisms (SNPs) genotyping

To map the resistance genes in two BC_1 segregating populations of *B. juncea*, the *Brassica* 60K Illumina® Infinium SNP Array (Illumina, San Diego, CA) was used to obtain genotypic data. The Illumina® Infinium chip is a high throughput SNPs genotyping system. The *Brassica* 60K beadchips were imaged using an Illumina HiScan system, and the SNP alleles were clustered and called automatically using the Illumina BeadStudio software. This was operated by the Batley lab lead by Professor Jacqueline Batley in University of Western Australia.

DNA samples were dried down using a vacuum centrifuge for 2h 30minutes. Approximately 500ng of each DNA samples were prepared and sent off for SNPs genotyping. SNPs results were received in comma-delimited format (“csv”). A

series of criteria were used for filtering SNP markers in order to control the quality of data used for subsequent analysis. SNP markers that had many missing genotypes across individual plants were discarded. SNP markers that had the same genotype and appeared heterozygous within parental lines were removed from the analysis. The monomorphic SNPs were excluded. Adjacent SNPs that had the same genotypes for all plants in a mapping population were compressed to one SNP marker.

2.5.2 JoinMap 4.0 for constructing linkage maps

After quality control and filtering, a subset of validated SNP markers remained for constructing a linkage map. Linkage analysis and map construction were performed separately for each of the two populations using JoinMap® v4.0. Linked loci were grouped using a LOD threshold of 5-8 and a maximum recombination fraction of 0.4. Grouped marker loci, including the newly mapped SNP markers, were arranged into a scoring matrix using JoinMap® v4.0. The data set was inspected for the presence of spurious double crossovers (identified using JoinMap® v4.0 data tool kit) generated by missing data and taken into account in the final linkage group construction. After the original scores were rechecked, a final linkage map was constructed for each of the two populations. Kosambi mapping function were used to translate recombination frequency into map distances in centiMorgans (cM) (Kosambi, 1944; Lorieux, 2012).

2.5.3 R-QTL for QTL mapping

R/QTL implemented in the RStudio statistical package was used to perform QTL (quantitative trait loci) mapping (Broman *et al*., 2003). The core of R/qtl is a series of functions calculating QTL genotype probabilities to simulate from the joint genotype distribution and to calculate the most likely sequence of underlying genotypes.

The data from the genetic linkage map was compiled in comma-delimited format (“csv”) to be imported into R/qtl. The function “scanone” was run to perform a single-QTL genome scan with a normal model, using maximum likelihood via EM algorithm (Lander and Botstein, 1989). The function “summary.scanone” shows the

maximum LOD score on each chromosome for which the LOD exceeds a specified threshold. Permutation tests (n=1000) were performed to get a genome-wide LOD significance threshold. In addition, based on the permutation result, genome-scan-adjusted p-values for inferred QTL could be estimated. It was then possible to get a report of all chromosomes meeting a certain significance level, with the corresponding LOD threshold calculated automatically. The function "scantwo" was run to perform a two-dimensional genome scan with a two-QTL model. It calculated a LOD score for the full model (two QTL plus interaction) and a LOD score for the additive model (two QTL but no interaction).

2.6 Embryo rescue

2.6.1 Interspecific hybridisations

The TuMV-resistant *B. rapa* line derived from Tropical Delight (Lydiate *et al*., 2014) and the TuMV-susceptible *B. nigra* line "al-1-3" were used as parental lines for interspecific hybridisation. Bud pollinations were performed. Immature flower buds (*B. rapa* as female) of appropriate size were emasculated by removal of anthers. The stigmas of these buds were pollinated with pollen of *B. nigra* (male parent). Racemes were labelled and enclosed within perforated transparent bread bags to prevent cross-pollination. Each pollination was repeated.

2.6.2 Preparation of MS medium and rooting medium

Preparation of MS medium for primary cultivation

Basic MS medium with five macronutrients and seven micronutrients, two iron, five organics, and sucrose included was obtained from the media prep facility at the School of Life Sciences. Plant hormones 6-Benzylaminopurine (6-BA) and Naphthaleneacetic acid (NAA) were made up in 1M HCL and 1M NaOH solutions respectively, to a concentration of 1g/L. These plant hormone solutions were added to basic MS medium to a final concentration of 2 mg/L 6-BA and 0.1 mg/L NAA. Glutamine was added to the MS medium to a concentration of 400 mg/L. Activated carbon was added to prevent toxicity. The pH of the medium was adjusted to 5.7-5.8 using 1M HCL or 1M NaOH. Agar was added to the medium to a concentration

of 0.7%. Finally, all were mixed together and autoclaved. Then the medium was poured in to polystyrene sterile petri dishes (Greiner Bio-One, Stonehouse, UK). The petri dishes were 90 × 15mm in size with a single vent.

Ingredients of MS medium were as follows:
A. Major salts (macronutrients):
Ammonium nitrate (NH_4NO_3) 1,650 mg/l
Calcium chloride ($CaCl_2 \cdot 2H_2O$) 440 mg/l
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$) 370 mg/l
Potassium phosphate (KH_2PO_4) 170 mg/l
Potassium nitrate (KNO_3) 1,900 mg/l

B. Minor salts (micronutrients):
Boric acid (H_3BO_3) 6.2 mg/l
Cobalt chloride ($CoCl_2 \cdot 6H_2O$) 0.025 mg/l
Cupric sulphate ($CuSO_4 \cdot 5H_2O$) 0.025 mg/l
Manganese sulphate ($MnSO_4 \cdot 4H_2O$) 22.3 mg/l
Potassium iodide (KI) 0.83 mg/l
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) 0.25 mg/l
Zinc sulphate ($ZnSO_4 \cdot 7H_2O$) 8.6 mg/l

C. Iron stock:
$Na_2EDTA \cdot 2H_2O$ 37.2 mg/l
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$) 27.8 mg/l

D. Vitamins and organics:
Myo-Inositol 100 mg/l
Nicotinic acid 0.5 mg/l
Pyridoxine · HCl (VB6) 0.5 mg/l
Thiamine · HCl (VB1) 0.5 mg/l
Glycine 0.2 mg/l

Preparation of rooting medium for secondary cultivation

After the ovules had germinated and grown into plantlets, they were transferred to rooting medium in Magenta™ vessels (Sigma-Aldrich, Dorset, UK) of the size 77mm × 77mm × 97mm for further cultivation. The ingredients of rooting medium were ½ MS medium + 1mg/L IBA + 2% sucrose + 0.8% agar.

2.6.3 Culture of embryos and seedlings

The immature siliques were taken 12-15 days post pollination and surface-sterilized by soaking in 75% ethanol for 30s, followed by a 15-min treatment with 5% sodium hypochlorite solution, then rinsed three times with sterile deionized water.

The siliques were first cut in the end region where there were no ovules, and then carefully slit lengthwise with a surgical blade (preferably only at one junction between the valves to pull off their contents from the very first ovule to the cut end). For culture, ovules attached to part of the ovary tissue were excised with forceps on autoclaved filter paper soaked with sterile deionized water in petri dishes, and then inoculated onto media (MS medium supplemented with 3% sucrose, 0.2% active carbon, 400 mg/L glutamine, 2 mg/L BA and 0.1 mg/L NAA, 0.7% agar, at PH = 5.8) in petri dishes. After an initial dark culture of 24h, the dishes were moved to the incubator at 25±2°C with a 12h photoperiod, 100 μmol $m^{-2}s^{-1}$ fluorescent light.

2.6.4 Plant vegetative propagation

Vegetative propagation (vegetative reproduction, vegetative cloning) is a form of asexual reproduction of plants. After embryo rescue, surviving plants were propagated by taking cuttings. Some side shoots off the main stem were cut at their bases. The ends of cut shoots were dipped into 2-Strike powder (Bayer Garden). The extra powder was shaken off and the cut shoots were planted it into M2 compost. The pots with the cutting were bagged to stop them from wilting too much. The plastic bag was retained for around 5 days and then removed.

2.7 Colchicine treatment

2.7.1 Colchicine treatment of leaf axils

Once plants had established from the cuttings in the glasshouse (after 2-3 weeks), the apical meristem was removed and a 0.1% colchicine solution, with Nonidet^{TM} P-40 (Sigma-Aldrich, Dorset, UK) as a wetting agent, was applied to the leaf axils after scraping the lower part of the petiole with a clean scalpel.

2.7.2 Colchicine treatment of roots

The roots of established plants (cuttings) were rinsed carefully to remove the compost. Then the roots were immersed in 0.34% colchicine solution for 90 minutes for polyploidy induction. After that, roots were rinsed and planted back into M2 compost.

2.8 Plant cytogenetic analysis

2.8.1 Ploidy level testing

Plant Cytometry Services based in Netherlands was used for plant ploidy testing. It specialises in the determination of the amount of nuclear DNA in plant cells by flow cytometry. Fresh leaf samples were collected and packed in a sealed plastic bag with a piece of slightly moist filter paper. In addition to the test plants, leaf samples of standard *Brassica* species were also sent for ploidy testing. Results including relative DNA ratios and histogram of samples were obtained. The quantity of the fluorescence is representative of the amount of DNA in a cell nucleus. The intensity of the fluorescence of the cell nuclei of an unknown plant is compared with that of a control plant with known chromosome number, which provides an accurate indication of the chromosome number from the unknown plant.

2.8.2 Genome in situ hybridisation (GISH)

To obtain further information on the chromosome arrangements in the resynthesised plants, Genomic in situ Hybridisation (GISH) was performed by collaborators from Birmingham University researching meiosis differences between established and newly resynthesised *B. juncea*. Both *B. rapa* and *B. nigra* probes are available. Species-specific probes for both the *B. rapa* 'A' genome and the *B. nigra* 'B' genome were available.

Chapter 3

Evaluation of resistance to Turnip mosaic virus in *Brassica juncea* and *Brassica nigra*

3.1 Background

3.1.1 Studies on TuMV resistance in *B. juncea* and *B. nigra*

There have been limited studies on the TuMV resistances in *B. juncea* and *B. nigra*. Fjellstrom and Williams (1997) performed TuMV resistance tests on two *B. juncea* cultivars using TuMV strains C1, C2, C3 and C4 (Provvidenti, 1980). Resistance to TuMV C1 was found in one *B. juncea* cultivar and resistance to TuMV C2 in the other *B. juncea* cultivar, both resistances seemed not robust. No resistance was found against TuMV C3 or C4 (Fjellstrom and Williams, 1997). Kehoe *et al*. (2010) investigated TuMV resistances in a collection of 44 *B. juncea* lines and 5 *B. nigra* lines. TuMV isolates WA-Ap1, NSW-1 and NSW-2 were used, which belonged to pathotypes 8, 7 and 1 (Jenner and Walsh, 1996), respectively. All the lines were systemically infected (either mosaic or necrotic symptoms). The necrotic symptom was considered to be a resistance phenotype. Other studies have been done by the same research group; 69 *B. juncea* lines were tested for TuMV resistance using the same three TuMV isolates (Nyalugwe *et al*., 2014). However, all these lines were also systemically infected. Therefore, it is very important to search further for robust natural resistance in *B. juncea* and also in *B. nigra*.

3.2 Materials and Methods

As was mentioned in 2.1.1, 34 lines of *B. juncea* and 27 lines of *B. nigra* were collected and tested for resistance to TuMV UK 1. For each line, 12 plant individuals were grown, 10 plants inoculated and 2 plants left out as uninoculated controls. All plants grew in the same glasshouse compartment under the same conditions (section 2.2.1).

3.3 Results

3.3.1 Testing *B. nigra* lines for TuMV resistance

Following inoculation, all 27 accessions of *B. nigra* were found to be highly susceptible to TuMV UK 1. Only one line had systemic mosaic symptoms; the other 26 lines uniformly showed systemic necrotic symptoms (Table 3.1, Fig. 3.1-3.2). Most of the *B. nigra* plants died rapidly from the systemic necrosis, including two well-characterised lines obtained from Canada.

Table 3.1 − Reactions of *Brassica nigra* plant lines following mechanical inoculation with Turnip mosaic virus isolate UK 1.

Plant line	No. plants infected/no. tested	Phenotype	Systemic infection
TWBN01	10/10	$+_N$[1]	Yes
TWBN02	10/10	$+_N$	Yes
TWBN03	10/10	$+_N$	Yes
TWBN04	10/10	$+_N$	Yes
TWBN05	10/10	+[2]	Yes
TWBN06	10/10	$+_N$	Yes
TWBN07	10/10	$+_N$	Yes
TWBN08	10/10	$+_N$	Yes
TWBN09	10/10	$+_N$	Yes
TWBN10	10/10	$+_N$	Yes
TWBN11	10/10	$+_N$	Yes
TWBN12	10/10	$+_N$	Yes
TWBN13	10/10	$+_N$	Yes
TWBN14	10/10	$+_N$	Yes
TWBN15	9/9	$+_N$	Yes
TWBN16	8/8	$+_N$	Yes
TWBN17	9/9	$+_N$	Yes
TWBN18	10/10	$+_N$	Yes
TWBN19	10/10	$+_N$	Yes
TWBN20	7/7	$+_N$	Yes
TWBN21	10/10	$+_N$	Yes
TWBN22	10/10	$+_N$	Yes
TWBN23	10/10	$+_N$	Yes
TWBN24	10/10	$+_N$	Yes
TWBN25	10/10	$+_N$	Yes
Ni-100	4/4	$+_N$	Yes
al-1-3	4/4	$+_N$	Yes

[1] $+_N$, systemic infection with necrosis, virus detected by ELISA in unioculated leaves; [2]+, systemic infection without necrosis, virus detected by ELISA in unioculated leaves (plants were susceptible).

Figure 3.1 − Necrotic infection of *Brassica nigra* plants (phenotype $+_N$) from line TWBN17 following sap inoculation with TuMV isolate UK 1. The plant on the left is the uninoculated control.

Figure 3.2 − Mosaic infection of *Brassica nigra* plants (phenotype +) from line TWBN05 following sap inoculation with TuMV isolate UK 1. The plant on the left is the uninoculated control.

3.3.2 Testing *B. juncea* lines for TuMV resistance

Amongst 34 lines of *B. juncea*, resistance to TuMV UK 1 was identified in 8 lines (TWBJ03, TWBJ04, TWBJ06, TWBJ14, TWBJ15, TWBJ18, TWBJ20 and TWBJ23) (Table 3.2). The different reactions of the *B. juncea* plants are shown in Figures 3.3-3.5.

Table 3.2 − Reaction of *Brassica juncea* plant lines following mechanical inoculation with Turnip mosaic virus isolate UK 1.

Plant line	No. plants infected/no. tested	Phenotype	Systemic infection
TWBJ01	10/10	+	Yes
TWBJ02	10/10	+	Yes
TWBJ03	2/10	$+_N$ / 0	Yes/No
TWBJ04	7/10	$+_N$ / R	Yes/No
TWBJ05	10/10	$+_N$	Yes
TWBJ06	5/10	+ / 0	Yes/No
TWBJ07	10/10	+	Yes
TWBJ08	9/9	$+_N$	Yes
TWBJ09	10/10	+	Yes
TWBJ10	10/10	+	Yes
TWBJ11	10/10	+	Yes
TWBJ12	10/10	$+_N$	Yes
TWBJ13	10/10	+	Yes
TWBJ14	0/10	0	No
TWBJ15	0/3	R	No
TWBJ16	10/10	+	Yes
TWBJ17	10/10	$+_N$	Yes
TWBJ18	8/10	+ / R	Yes/No
TWBJ19	10/10	+	Yes
TWBJ20	1/10	+ / 0	Yes/No
TWBJ21	10/10	+	Yes
TWBJ22	10/10	+	Yes
TWBJ23	0/10	0	No
TWBJ24	7/7	$+_N$	Yes
TWBJ25	10/10	+	Yes
TWBJ26	10/10	+	Yes
TWBJ28	10/10	$+_N$	Yes
TWBJ29	10/10	+	Yes
TWBJ30	10/10	+	Yes
TWBJ31	10/10	$+_N$	Yes
TWBJ32	10/10	$+_N$	Yes
TWBJ33	10/10	+	Yes
TWBJ34	9/9	$+_N$	Yes
060DH17	1/1	$+_N$	Yes

0, no symptoms, no virus detected by ELISA; R, local infection, no systemic infection, no virus detected by ELISA in uninoculated leaves; +, systemic infection without necrosis, virus detected by ELISA in unioculated leaves (plants were susceptible); $+_N$, systemic infection with necrosis, virus detected by ELISA in unioculated leaves.

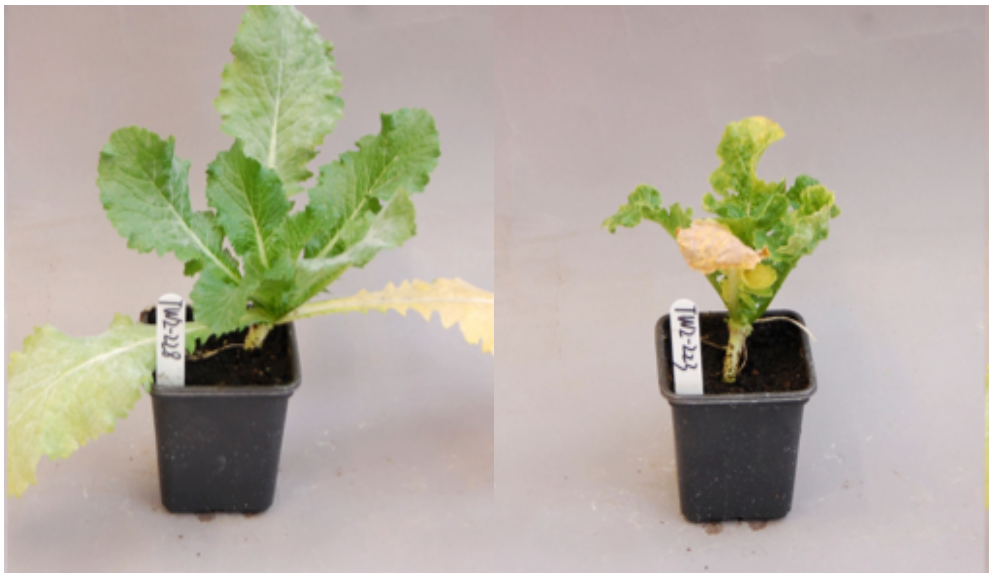


Figure 3.3 − Reaction of two *Brassica juncea* plants from line TWBJ18 that was segregating for resistance to TuMV isolate UK 1. The plant on the left is completely resistant (phenotype 0) and the one on the right is susceptible (phenotype +).

Figure 3.4 − Reaction of two *Brassica juncea* plants from line TWBJ03 that was segregating for resistance to TuMV isolate UK 1. The plant on the left is completely resistant (phenotype 0) and the one on the right is susceptible showing necrosis (phenotype $+_N$).

Figure 3.5 − Reaction of TuMV-resistant *Brassica juncea* plants from line TWBJ14 that was uniformly resistant to TuMV isolate UK 1 (phenotype 0). The plant on the left is the uninoculated control.

3.2.3 Investigation of TuMV resistance spectra in *B. juncea*

As resistance to TuMV isolate UK 1 was identified in *B. juncea*, whether the resistances are effective against other TuMV isolates was of interest. Using the method for self-pollinating in section 2.1.2, UK 1-resistant plants from *B. juncea* lines TWBJ14, TWBJ20 and TWBJ23 were selfed to obtain S_1 generations, from which resistant plants were further selfed to develop S_2 generations. The level of homozygosity of the resistance alleles should be reasonably high in S_2 generations. Plants in the three S_2 generations were planted and inoculated with TuMV isolates UK 1 (pathotype 1), vVIR24 (pathotype 3) and CDN 1 (pathotype 4). Results showed that the resistances in S_2 lines from TWBJ14 and TWBJ20 were effective against TuMV isolates UK 1, vVIR24 and CDN 1 (pathotypes 1, 3 and 4, respectively), whereas resistance in line TWBJ23 was not (Table 3.3). The phenotypes of these lines following inoculation with vVIR24 are shown in Figure 3.6. All virus isolates were inoculated to the *B. napus* differential plant line R4, in order to check their authenticity and stability. Results showed that all isolates maintained their authenticity (Table 3.3).

Table 3.3 − Phenotypes of *Brassica juncea* lines following challenge with different TuMV isolates.

Plant line	TuMV isolate (pathotype)					
	UK 1 (1)		vVIR24 (3)		CDN 1 (4)	
	No. resistant /no. tested	Phenoty -pe	No. resistant /no. tested	Phenot -ype	No. resistant /no. tested	Phenot -ype
TWBJ14 S_2	9/9	0[1]	9/9	0	9/9	0
TWBJ20 S_2	9/9	0	9/9	0	9/9	0
TWBJ23 S_2	9/9	0	0/9	+[2]	0/9	+
R4	2/2	0	0/2	$+_N$[3]	0/2	+
TGM[4]	0/2	+	0/2	+	0/2	+

[1]0, no symptoms, no virus detected by ELISA; [2]+, systemic infection without necrosis, virus detected by ELISA in unioculated leaves (plants were susceptible); [3]$+_N$, systemic infection with necrosis, virus detected by ELISA in unioculated leaves; [4]TGM, Tendergreen mustard (susceptible host of TuMV).

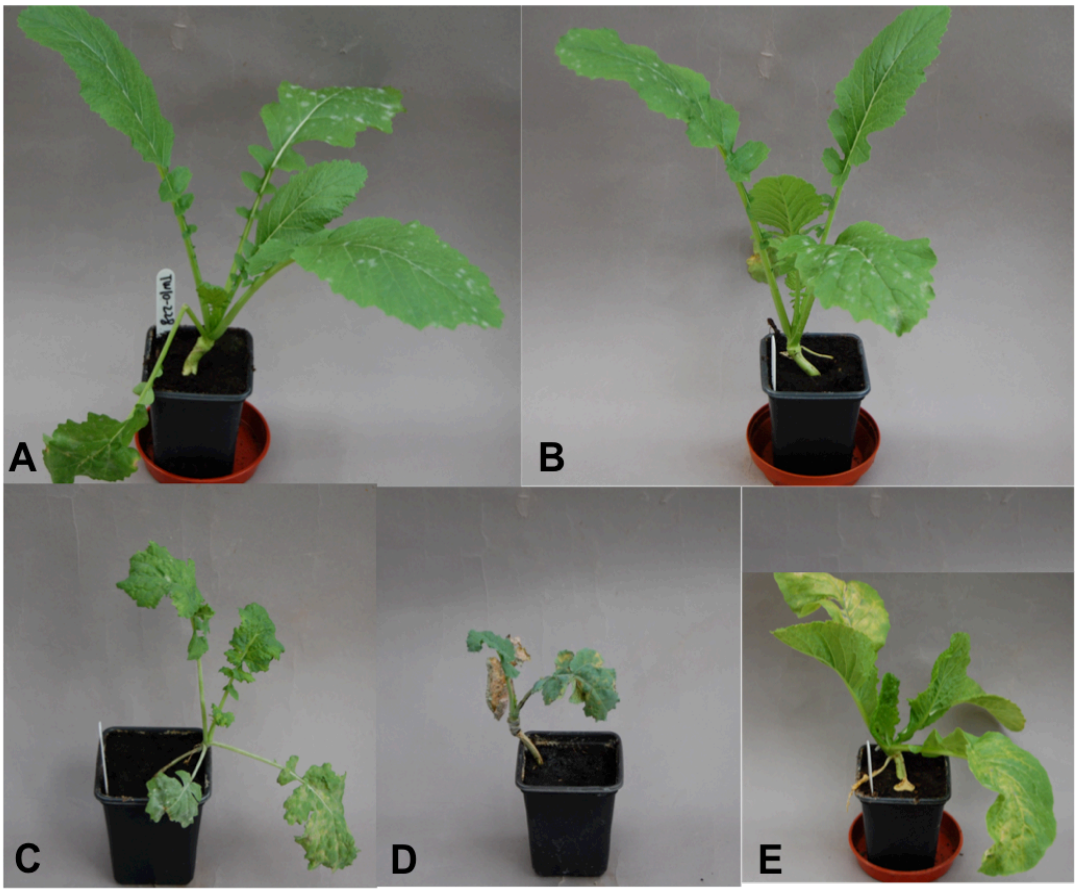


Figure 3.6 − Phenotypes of brassica plants following inoculation of TuMV isolate vVIR24. **A,** TWBJ14 (0); **B,** TWBJ20 (0); **C,** TWBJ23 (+); **D,** R4 ($+_N$); **E,** TGM (+).

The results suggested that the resistance in *B. juncea* lines TWBJ14 and TWBJ20 were effective against TuMV isolates UK 1, vVIR24 and CDN 1, representing the most prevalent pathotypes 1, 3 and 4. Thus, these two lines possess the broad-spectrum resistance to TuMV. Furthermore, a more stringent TuMV test has been performed on these two lines. Four weeks after inoculation with TuMV isolate UK 1, vVIR24 or CDN 1, one plant individual had both inoculated and uninoculated leaves assessed by back inoculation and ELISA (sections 2.3.2 and 2.3.4). The strategy for this TuMV test is shown in Figure 3.7. One inoculated leaf was cut in half, half was used for ELISA and the other half for back inoculation. The same procedure was performed on uninoculated leaves. This was to confirm the characterisation of TuMV resistance in TWBJ14 and TWBJ20. The results of these two proceedings of assessments indicated consistently that no virus was present, which confirmed that the resistances in *B. juncea* lines TWBJ14 and TWBJ20 were highly effective against TuMV isolates UK 1, vVIR24 and CDN 1, representing pathotypes 1, 3 and 4.

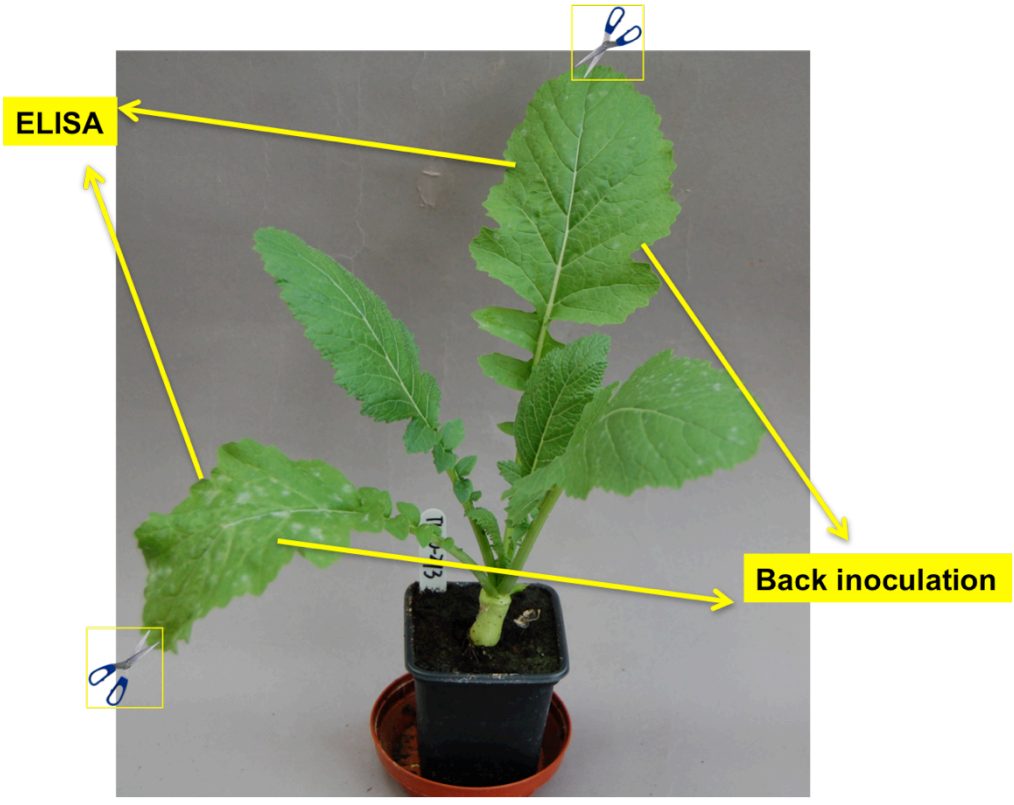


Figure 3.7− The strategy for virus detection that assesses the same leaf through both ELISA and back inoculation.

3.3 Discussion

3.3.1 TuMV resistances in *B. juncea* and *B. nigra*

The most effective, economical and environmentally friendly way to control plant disease is to use resistant plant cultivars. Therefore, identification and exploiting plant natural resistance is crucial (sections 1.3.5 and 3.1.1). The TuMV resistance test was designed to identify resistance to TuMV isolate UK 1 in *B. juncea* and *B. nigra* in the first instance, as there have been very limited studies and findings in this area previously (described in section 3.1.1). Although no resistance was found in any *B. nigra* lines tested, resistance to UK 1 was found in 8 lines of *B. juncea*. There were two resistance phenotypes, '0' in lines TWBJ03, TWBJ06, TWBJ14, TWBJ20 and TWBJ23, and 'R' in lines TWBJ04, TWBJ15, TWBJ18. Different mechanisms of resistance might be involved in these lines. Also, as *B. juncea* possess both 'A' and 'B' genomes, some of the resistances might be from the 'B' genome where no TuMV resistance has ever been identified previously. Additionally, the resistances were effective following severe TuMV mechanical inoculation, suggesting they could be robust under natural environmental conditions. These resistances have the potential for exploitation in commercial *B. juncea* plant lines.

3.3.2 Necrosis phenotype in *B. nigra*

Of 27 *B. nigra* lines tested, only one line showed '+' phenotype, whereas the others showed '$+_N$' uniformly. The necrosis phenotype has been considered as a hypersensitive response involved in programmed cell death, which is a typical resistance reaction called 'Effector-triggered immunity (ETI)' activated by R proteins (Spoel and Dong, 2012). The *B. nigra* lines used in this study could be good resources for mapping necrosis genes in the *Brassica* 'B' genome, which has never been studied before. To achieve this, either of the well-characterised *B. nigra* lines 'Ni-100' or 'al-1-3' can be used as one parent conferring '$+_N$' phenotype, and line 'TWBN05' can be used as the other parent conferring '+' phenotype. A segregated mapping population could be developed based on this.

3.3.3 The spectra of TuMV resistances in *B. juncea* lines TWBJ14 and TWBJ20

In the study of Jenner and Walsh (1996), TuMV pathotypes 1, 3 and 4 were the predominant pathoypes in the collection of 124 isolates gathered from around the world. TuMV isolates representing these three pathotypes were used for the test of TuMV resistance spectra. The resistances in *B. juncea* lines TWBJ14 and TWBJ20 were found to be effective against all three isolates. This has been confirmed in a stringent experiment (Fig. 3.7), in which both inoculated and uninoculated leaves were tested by ELISA and back inoculation, respectively. The resistance levels of these two lines were confirmed as immunity as no virus was detected even in inoculated leaves. Also, the use of both ELISA and back inoculation avoided any error in virus detection. The TuMV resistances in *B. juncea* lines TWBJ14 and TWBJ20 are very important in terms of both scientific study and practical application.

3.4 Conclusions

The main aim of the work in this chapter was to seek TuMV natural resistance(s) in *B. juncea* and *B. nigra*. No resistance was identified in *B. nigra*. Resistance to TuMV isolate UK 1 (pathotype 1) was found in 8 lines of *B. juncea*. The resistances in *B. juncea* lines TWBJ14 and TWBJ20 have been found to be also highly effective against TuMV isolates vVIR24 and CDN 1, representing pathotypes 3 and 4.

Chapter 4

Characterisation and mapping of Turnip mosaic virus resistance gene(s) in *Brassica juncea* lines TWBJ14 and TWBJ20

4.1 Background

4.1.1 Genetic mapping strategies

Broadly speaking, genetic mapping approaches can be split into two categories. The first approach is Quantitative Trait Loci (QTL) mapping based on a population segregating for the trait of interest. Derived from crossing of contrasting parental lines, there are several types of mapping populations, including F_2 (Second Filial population), BC_1 (First Backcross population), DH (Double Haploids) and RILs (Recombinant Inbred lines). The second approach is Genome Wide Association (GWA), which is a more recently developed approach using collections of hundreds of natural accessions as a mapping population. For both approaches, individual plants in a population are genotyped and phenotyped for the target trait. This allows the analysis of underlying associations between genetic and trait variation at markers across the genome (Vaughan, 2015). After an initial genetic mapping experiment, choosing what kind of approach to take in follow up work depends on the genetic architecture of traits. One or two major loci tend to be easier to identify through independent mapping populations or fine mapping, whereas multiple minor loci are likely to be difficult to identify in different genetic backgrounds (Alonso-Blanco and Koornneef, 2000).

QTL analysis is a statistical method that is widely used in the study of association between phenotypic and genotypic data. It can be utilised to target not only multiple genes, but also major Mendelian genes. Since QTL can be present throughout the genome, a large number of markers are required. A target of 10 to 50 markers per chromosome is needed to perform accurate QTL mapping (Collard *et al*., 2005). There are a number of approaches to identify QTL including single marker analysis, interval mapping and composite interval mapping (CIM), also termed multiple QTL

mapping (MQM).

With the advancement of modern genotyping techniques and the completion of the whole genome sequences of *B. rapa* (Wang *et al*. 2011), *B. oleracea* (Liu *et al*., 2014) and *B. napus* (Chalhoub *et al*. 2014), genetic mapping of target genes in *Brassica* 'A' and 'C' genomes has become easier.

4.2 Materials and Methods

4.2.1 Phenotypic assessment of TuMV resistance in *B. juncea* plants

TuMV inoculation and plant phenotypic assessments followed the method mentioned in section 2.3. Phenotypes of individual plants were finalised by combining ELISA results and visual assessments. Plants with phenotypic codes "0" were classified as resistant and "+" and "$+_N$" as susceptible. For each inoculation, uninoculated controls and susceptible TuMV indicator plants were included.

4.2.2 Development of mapping populations for TuMV resistance genes in *B. juncea*

Recombination for genetic mapping was generated experimentally by crossing contrasting plant lines and developing populations segregating for the trait of interest. The selfing and crossing followed the procedures and strategies described in section 2.1.2. Four BC_1 and F_2 populations in parallel have been developed by crossing a TuMV-susceptible *B. juncea* DH line (060DH17) with four TuMV-resistant *B. juncea* plants (TWBJ03, TWBJ14, TWBJ20 and TWBJ23) from different sources.

4.2.3 Complementation tests

In plant genetics, complementation testing is a method to check if two lines of a plant species, with the same mutant phenotype, possess the same or different homozygous recessive mutations in the genome. If the hybrid of two such lines produces the wild-type phenotype instead of the parental mutant phenotype, complementation occurs and it suggests the mutations are in different genes.

Complementation will not occur if the mutations are in the same gene.

As recessive resistances to TuMV were identified in four *B. juncea* lines (section 4.3.1), it is possible that the same resistance gene might be represented more than once within the four lines. Therefore, complementation tests were performed to investigate this. Crossings have been made between F_1 plants of these four lines (produced by crossing with 060DH17). Seeds generated from the cross between F_1 plants of TWBJ14 and TWBJ20 were planted and tested for resistance to UK 1.

4.2.4 Genotyping and QTL mapping of TuMV resistance genes

As mentioned in section 2.5.1, the *Brassica* 60K Illumina® Infinium SNP Array (Illumina, San Diego, CA) was used to obtain genotypic data. SNP genotyping was performed on three *B. juncea* parental lines TWBJ14, TWBJ20 and 060DH17, 65 TWBJ14 BC_1 plants (25 of "0", 20 of "+" and 20 of "$+_N$") and 40 TWBJ20 BC_1 plants (20 of "0" and 20 of "$+_N$"). Analysis of SNP markers and construction of genetic linkage maps was implemented according to sections 2.5.1 and 2.5.2. The software MapDraw (version 2.1) (Liu and Meng, 2003) was used to draw genetic linkage maps based on given genetic linkage data. Then the phenotypic data (in a binary version), locus genotype data and the linkage map data were compiled and imported into R/qtl for QTL analysis.

The QTL mapping followed the procedure showed in section 2.5.3. For the **scanone** interval mapping based on a single-QTL model, three methods were used. Firstly, standard interval mapping (SIM) via the EM algorithm was performed, followed by the multiple imputation method (Sen and Churchill, 2001). Permutation tests (1000 permutations) were performed to get a genome-wide LOD significance threshold to determine the significance of QTLs identified. The genome-wide significance level of 5% (alpha = 0.05) was employed. To define the QTL confidence interval, the 95% Bayes credible interval was calculated (Broman and Sen, 2009). Flanking markers were then nominated to define the QTL interval. The third single-QTL mapping method performed was composite interval mapping (CIM), which aimed to reduce residual variation and so clarify evidence for further significant QTL. It was a step towards the multiple-QTL model. After single-QTL analysis, a two

dimensional QTL analysis based on a two-QTL model (**scantwo**) was performed. The permutation test (100 permutations) for a two-QTL model was then carried out to determine a significance threshold at a 5% significance level. Pair(s) of QTL positions that fell above the threshold were reported. It was then decided whether it was a full model (two QTL plus interaction) or an additive model (two QTL with additive effect) according to the LOD scores calculated. The functions **makeqtl** and **fitqtl** were performed to fit a defined QTL model and to determine the contribution to phenotypic variation of this model (Broman and Sen, 2009).

4.3 Results

4.3.1 Phenotypic assessment of TuMV resistance in *B. juncea* S_1 and F_1 populations

A number of UK 1-resistant *B. juncea* plants were self pollinated to produce S_1 seeds. S_1 seed lines have been tested for their resistance to UK 1 to see if there was any segregation. As a result, S_1 seed lines of TWBJ14 and TWBJ20 were all resistant to UK 1 without segregation (Table 4.1), indicating the homozygosity at the resistance gene loci. Although the tested plants of the original line TWBJ23 were uniformly resistant to UK 1, the resistance in the S_1 seed line segregated (22 Resistant: 5 Susceptible). For the other S_1 seed lines tested, the proportion of resistant plants was notably lower, particularly for line TWBJ06 and TWBJ18 where no resistance was identified in S_1 lines (Table 4.1). Following the phenotyping of S_1 families, a number of S_1 resistant plants were self pollinated to produce S_2 seeds.

For *B. juncea* lines TWBJ03, TWBJ14, TWBJ20 and TWBJ23, F_1 seeds from reciprocal crosses (i.e. F_1 seeds generated both on resistant and susceptible parent plants) were produced (sections 2.1.2 and 4.2.2) and tested for resistance to UK 1. The results showed that all F_1 seed lines were susceptible, indicating the resistances in these lines are recessive.

Table 4.1− Resistance to Turnip mosaic virus (TuMV) isolate UK 1 in resistant *Brassica juncea* parental lines and progenies.

Resistant plant line	No. TuMV-resistant plants /no. tested	Phenoty-pe	Resistance to UK 1 in progenies		
			Generation of progenies	No. TuMV-resistant plants /no. tested	Phenotype
TWBJ03	8/10	0[1] / $+_N$[2]	S_1	2/28	0 / $+_N$
			F_1	0/4	$+_N$
			F_1(Re[3])	0/11	+[4]
TWBJ14	10/10	0	S_1	28/28	0
			F_1	0/5	$+_N$
			F_1(Re)	0/8	+
TWBJ20	9/10	0 / $+_N$	S_1	28/28	0
			F_1	0/15	$+_N$
			F_1(Re)	0/14	+
TWBJ23	10/10	0	S_1	22/27	0 / $+_N$
			F_1	0/8	$+_N$
			F_1(Re)	0/9	+ / $+_N$
TWBJ04	3/10	0 / $+_N$	S_1	3/28	0 / $+_N$
TWBJ06	5/10	0 / +	S_1	0/10	+
TWBJ15	3/3	0	S_1	3/18	0 / $+_N$
TWBJ18	2/10	0 / +	S_1	0/28	$+_N$ / +

[1] 0, no symptoms and no virus detected by ELISA; [2] $+_N$, systemic infection with necrosis, virus detected by ELISA in uninoculated leaves; [3] Re, reciprocal cross. F_1 crosses were susceptible (♀) × resistant (♂) and reciprocal crosses were resistant (♀) × susceptible (♂); [4] +, systemic infection without necrosis, virus detected by ELISA in uninoculated leaves;

4.3.2 Phenotyping and analysis of genetic inheritance of TuMV resistances in *B. juncea* BC_1 and F_2 populations

Although four BC_1 and F_2 populations were produced, it was impossible to phenotype all these populations with limited time and glasshouse space. Also the funding was not available to map four populations. Efforts were focused on two *B. juncea* lines, TWBJ14 and TWBJ20, due to their consistent resistance to UK 1 in S_1 generations and their broad-spectrum resistance to TuMV (section 3.2.3). BC_1 populations from both resistant lines were phenotyped. In addition, the F_2 population of line TWBJ14 was phenotyped.

Segregation of UK 1 resistance in the BC_1 and F_2 populations derived from a cross between TWBJ14 and 060DH17

A large number of BC_1 and F_2 seeds were produced from the cross between TWBJ14 and 060DH17. Randomly selected seeds from both populations were planted and phenotyped in terms of resistance to UK 1. In total, 222 BC_1 plants and 159 F_2 plants were planted and phenotyped in two experiments (BC_1 Table 4.2 and F_2 Table 4.3), together with plants from parent lines 060DH17 and S_2 seed lines of TWBJ14 (Table 4.2 and Table 4.3).

Table 4.2 − Responses of the *Brassica juncea* BC_1 population derived from a cross between TWBJ14 and 060DH17 to Turnip mosaic virus isolate UK 1.

Plant line	Number of plants			Expected ratio	Goodness of fit	
	Resistant (0)	Susceptible ($+/+_N$)	Total	(0: $+/+_N$)	χ^2	*P* value
TWBJ14 S_2	5	0	5			
060DH17	0	5	5			
BC_1	53	169	222	1:3[1]	0.0966[2]	>0.05[3]

[1] The expected ratio of resistant to susceptible plants for the model of two recessive genes in a BC_1 population is 1:3.
[2] With one degree of freedom, $\chi^2=0.0966<\chi^2_{0.05}=3.841$.
[3] The segregation ratio of resistant to susceptible plants was not significantly different from 1:3.

The segregation ratio of resistant to susceptible plants in TWBJ14 BC_1 population was not significantly different from 1:3, which is based on a two recessive gene model for a BC_1 population. Both visual assessment and ELISA were performed to determine the phenotypes of all plants. The 53 resistant plants in this BC_1 population developed one phenotype - "0", whilst 169 susceptible plants developed two phenotypes, "+" and "$+_N$", 109 and 60 plants respectively (Fig. 4.1).

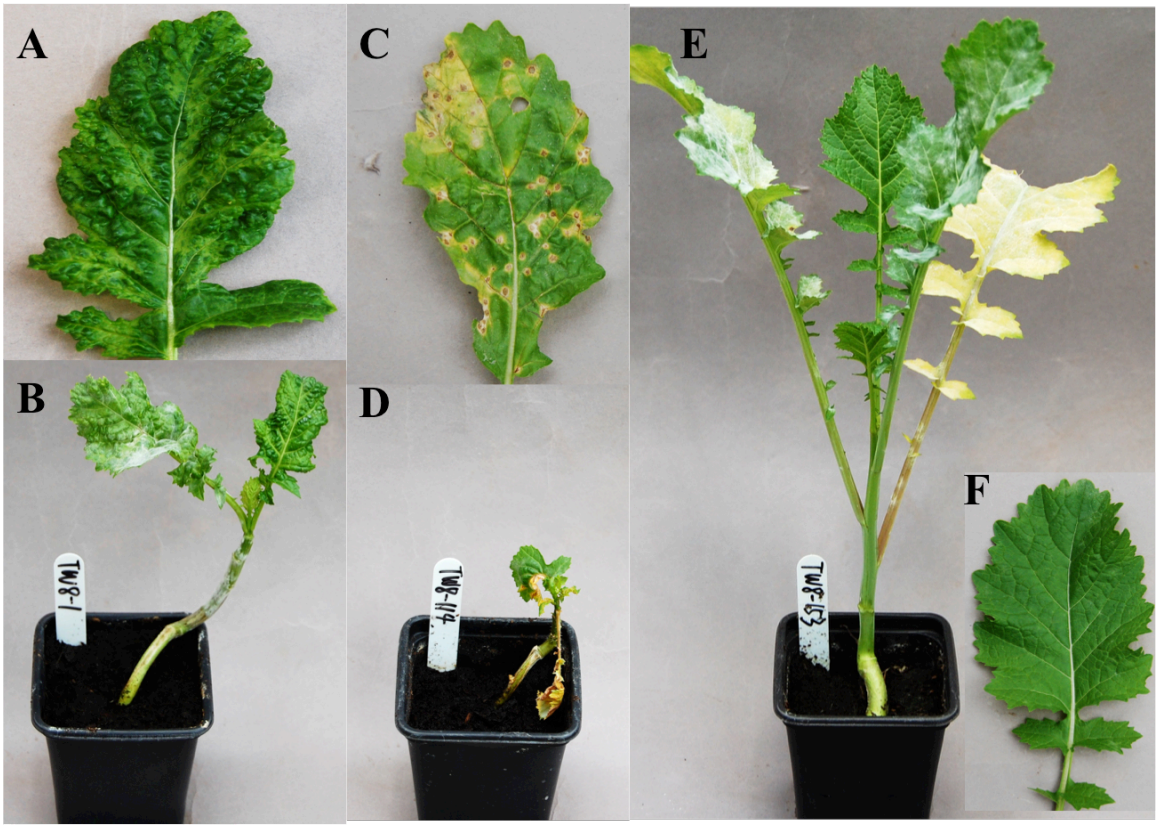


Figure 4.1 − Different phenotypes of plants in the TWBJ14 BC_1 population following challenge with Turnip mosaic virus isolate UK 1. **A,** infected leaf with mosaic symptoms; **B,** infected plant with systemic mosaic symptoms; **C,** infected leaf with necrotic symptoms; **D,** infected plant with systemic necrotic symptoms; **E,** resistant plant; **F,** uninfected leaf from a resistant plant.

Table 4.3 − Responses of the *Brassica juncea* F_2 population derived from a cross between TWBJ14 and 060DH17 to Turnip mosaic virus isolate UK 1.

Plant line	Number of plants			Expected ratio	Goodness of fit	
	Resistant (0)	Susceptible ($+/+_N$)	Total	(0: $+/+_N$)	χ^2	*P* value
TWBJ14 S_2	5	0	5			
060DH17	0	5	5			
F_2	9	150	159	1:15[1]	0.0944[2]	>0.05[3]

[1] The expected ratio of resistant to susceptible plants for the model of two recessive genes in an F_2 population is 1:15.
[2] With one degree of freedom, $\chi^2=0.0944<\chi^2_{0.05}=3.841$.
[3] The segregation ratio of resistant to susceptible plants was not significantly different from 1:15.

The segregation ratio of resistant to susceptible plants in the TWBJ14 F_2 population is not significantly different from 1:15, which is based on a two recessive genes model for an F_2 population. The 9 resistant plants in this F_2 population developed one phenotype "0", whilst 150 susceptible plants developed two phenotypes, "+" and "$+_N$", 81 and 69 plants respectively.

In conclusion, the phenotypic segregation of both BC_1 and F_2 populations of *B. juncea* line TWBJ14 fit a two recessive resistance gene model.

Segregation of UK 1 resistance in the BC_1 population derived from a cross between TWBJ20 and 060DH17

By random selection, 205 BC_1 plants of line TWBJ20 were phenotyped for resistance to TuMV isolate UK 1. This revealed that 41 plants were resistant, with phenotype "0" and 164 plants were susceptible, with phenotype "$+_N$" (Table 4.4, Fig. 4.2). In comparison to line TWBJ14, there was no "+" phenotype in the TWBJ20 BC_1 population, only "$+_N$" (Fig. 4.2).

Table 4.4 − Responses of the *Brassica juncea* BC_1 population derived from a cross between TWBJ20 and 060DH17 to Turnip mosaic virus isolate UK 1.

Plant line	Number of plants			Expected ratio (0: $+_N$)	Goodness of fit	
	Resistant (0)	Susceptible ($+_N$)	Total		χ^2	*P* value
TWBJ20 S_2	20	0	20			
060DH17	0	20	20			
BC_1	41	164	205	1:3[1]	2.71[2]	>0.05[3]

[1] The expected ratio of resistant to susceptible plants for the model of two recessive genes in a BC_1 population is 1:3.
[2] With one degree of freedom, $\chi^2=2.71<\chi^2_{0.05}=3.841$.
[3] The segregation ratio of resistant to susceptible plants was not significantly different from 1:3.

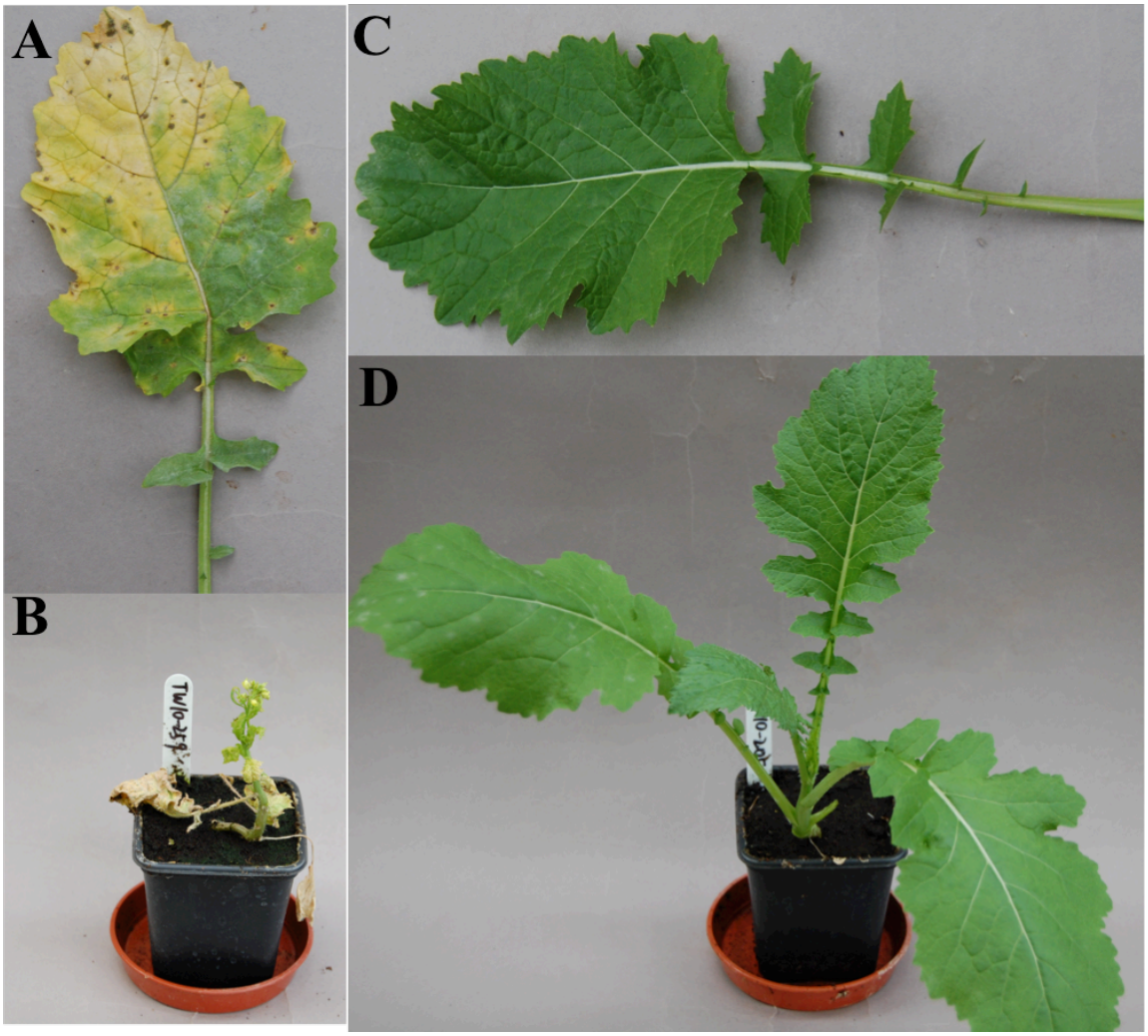


Figure 4.2 − Different phenotypes of plants in the *Brassica juncea* TWBJ20 BC_1 population following challenge with Turnip mosaic virus isolate UK 1. **A.** Infected leaf with necrotic symptoms; **B.** Infected plant with systemic necrotic symptoms; **C.** Uninfected leaf from a resistant plant; **D.** Resistant plant.

The Chi square test suggests that the segregation ratio of resistant (0) to susceptible ($+_N$) plants is not significantly different from a Mendelian model based on the action of two recessive genes (Table 4.4).

4.3.3 Complementation test

As mentioned in section 4.2.3, ten plants generated from a cross between F_1 plants of TWBJ14 and F_1 plants of TWBJ20 were inoculated with TuMV isolate UK 1. Phenotypic assessment showed that all of these ten plants were infected, with uniform "$+_N$" symptoms.

4.3.4 Genetic linkage analysis

In total, 65 plants from BC_1 population of TWBJ14 and 40 plants from BC_1 population of TWBJ20 were genotyped (section 4.2.4), generating a large set of SNP genotypic data. After filtering and quality control of SNP markers, 717 and 501 validated markers remained for BC_1 populations derived from TWBJ14 and TWBJ20, respectively. These markers were taken forward for genetic linkage analysis and construction of linkage maps. For both populations, 10 linkage groups have been constructed by MapDisto 2.0, using the physical position of makers as a reference. The total number of markers on each chromosome is listed in Table 4.5.

The linkage map of the TWBJ14 BC_1 population (Fig. 4.3) has a total length of 687.6 cM and comprises 239 non-overlapping SNP markers. The longest group is A06 (86.2 cM) and the shortest group is A04 (44.1 cM). The greatest inter-marker distance is 14.7 cM and the smallest is 1.6 cM. The average inter-marker distance of the whole map is 3.1 cM.

The linkage map of the TWBJ20 BC_1 (Fig. 4.4) population has a total length of 661.7 cM and comprises 193 non-overlapping SNP markers. The longest group is A09 (95.2 cM) and the shortest group is A04 (43.9 cM). The greatest inter-marker distance is 14.3 cM and the smallest is 1.6 cM. The average inter-marker distance of the whole map is 3.6 cM.

Table 4.5 − Distribution of markers and marker density of linkage groups in *Brassica juncea* TWBJ14 and TWBJ20 BC_1 populations.

Population	Linkage Group	No. Markers	No. Non-overlapping Markers	Length (cM)	Average Inter-marker distance (cM)
TWBJ14 BC_1	A01	74	21	69.0	3.5
	A02	76	26	71.8	2.9
	A03	125	34	85.7	2.6
	A04	39	14	44.1	3.4
	A05	77	25	74.6	3.1
	A06	60	24	86.2	3.7
	A07	52	22	65.4	3.1
	A08	65	18	49.3	2.9
	A09	84	33	82.4	2.6
	A10	65	22	59.1	2.8
TWBJ20 BC_1	A01	56	26	83.2	3.3
	A02	43	18	88.8	5.2
	A03	85	22	56.2	2.7
	A04	42	14	43.9	3.4
	A05	54	20	63.3	3.3
	A06	36	17	59.2	3.7
	A07	45	22	66.6	3.2
	A08	51	16	44.0	2.9
	A09	65	22	95.2	4.5
	A10	24	16	61.4	4.1

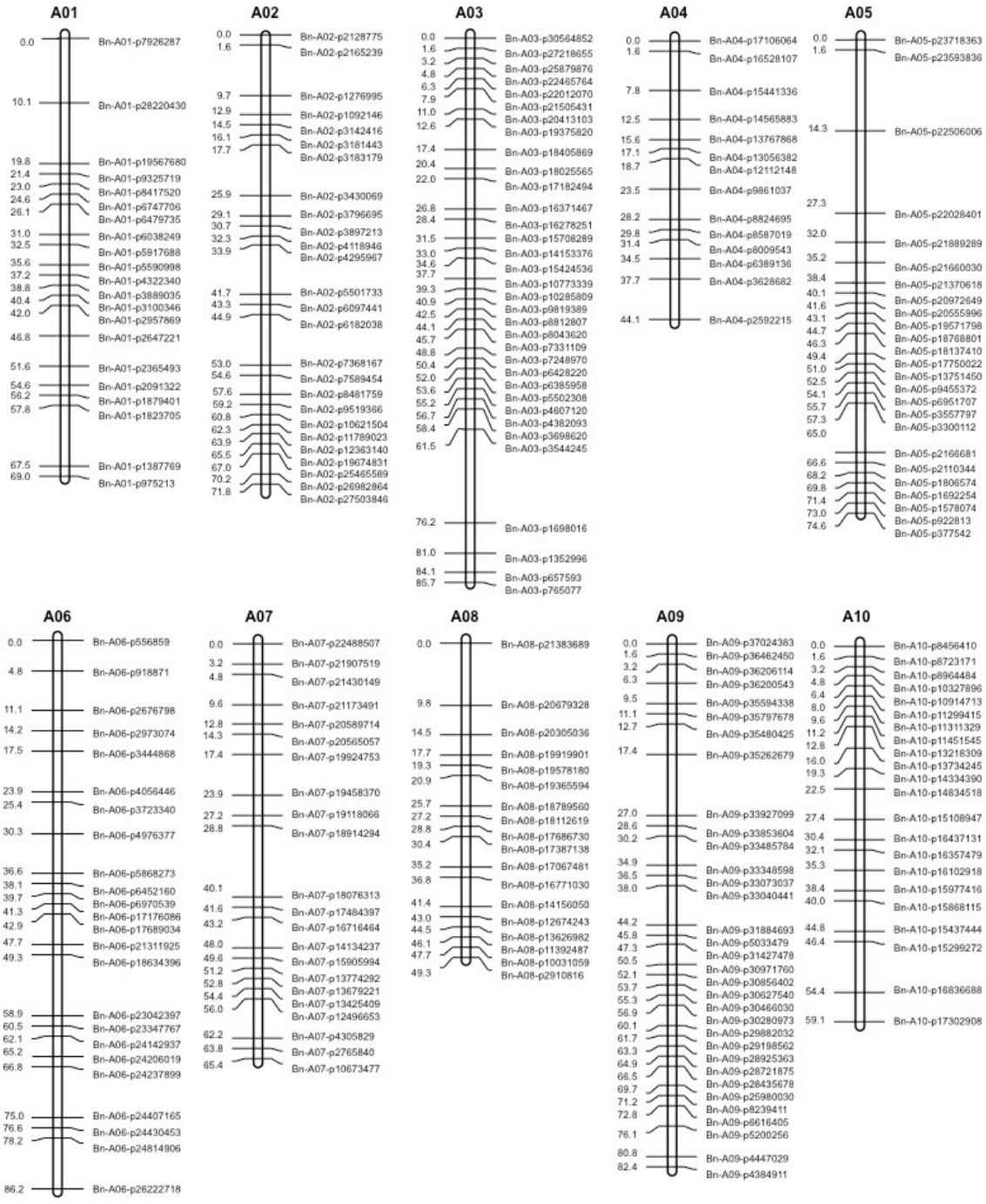


Figure 4.3 − Linkage map of *Brassica juncea* TWBJ14 BC_1 population.

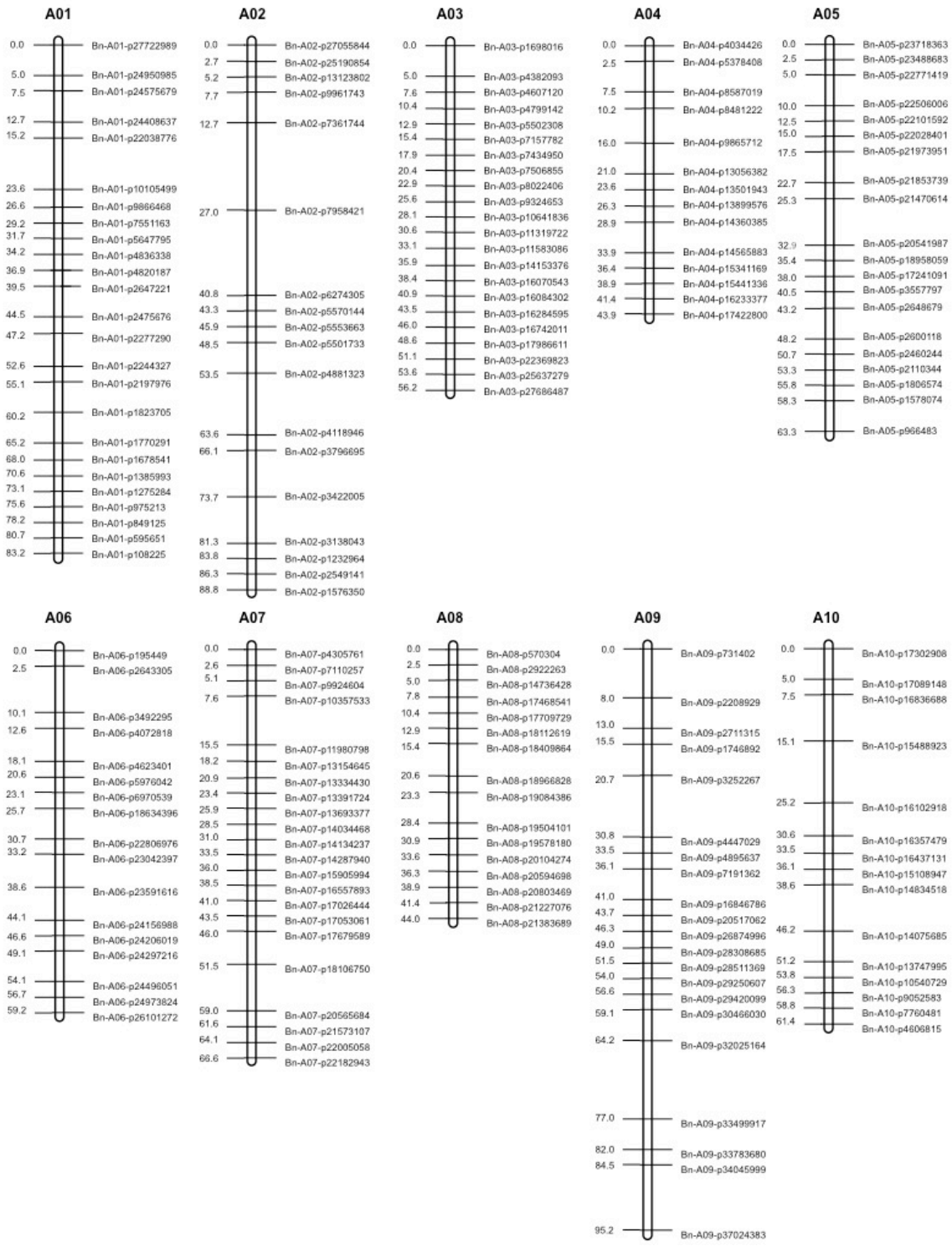


Figure 4.4 − Linkage map of *Brassica juncea* TWBJ20 BC_1 population.

4.3.5 QTL analysis

In addition to the QTL analysis of TuMV resistance in both BC_1 populations, a necrosis gene in TWBJ14 BC_1 population has also been mapped. Since the analysis was on binary traits (resistant or susceptible, necrotic or not-necrotic), the binary trait mapping model was adopted, using the argument **model = "binary"**.

TuMV resistance genes in *B. juncea* line TWBJ14

The result of **scanone** using the standard interval mapping via an EM algorithm is shown in Figure 4.5. The significance threshold was 2.63 at the 5% significance level. The LOD score of the QTL on A06 is 6.29 and it was the most significant QTL. The LOD score of the QTL on A02 was 2.75, which was also above the significance threshold though less significant than the one on A06. The QTL on A06 was detected at peak LOD of 6.29 at position 42.9 cM, flanked by markers Bn-A06-p17176086 at position 41.3 cM and Bn-A06-p21311925 at position 47.7 cM. The QTL on A02 was detected at peak LOD of 2.75 at position 43.3 cM, flanked by markers Bn-A02-p5501733 at position 41.7 cM and Bn-A02-p6182038 at position 44.9 cM. The confidence interval of the QTL on A06 was 27.8 cM, and 32 cM for that on A02, based on the calculation of 95% Bayes credible interval.

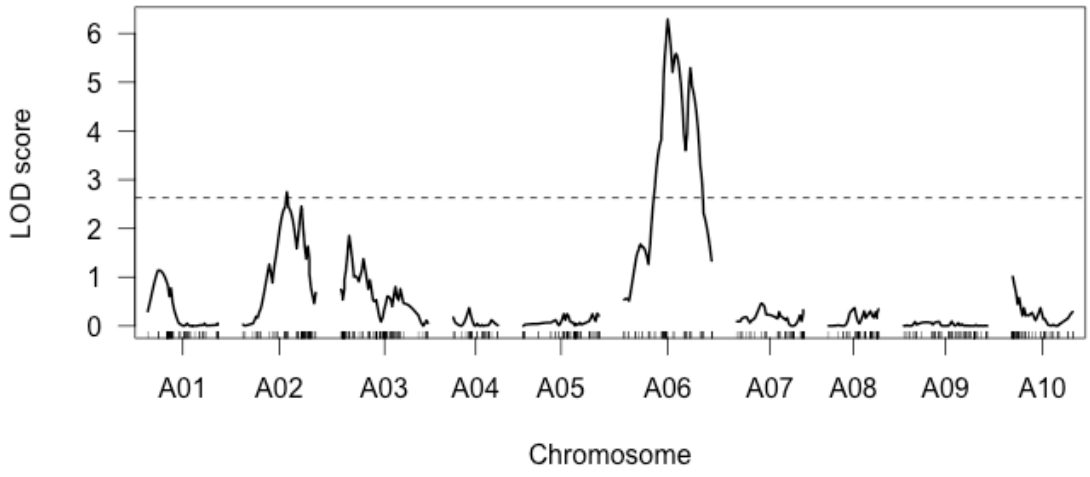


Figure 4.5 − Detection of QTL for Turnip mosaic virus resistance in *Brassica juncea* line TWBJ14 using standard interval mapping via EM algorithm in R/qtl. A significant LOD score (2.63) was determined by a permutation test (1000 times) at the significance level of 5% and is indicated by the dotted horizontal line.

The second interval mapping method performed was a multiple imputation method (Fig. 4.6). The significance threshold was 2.86 at the 5% significance level. The LOD scores of the QTL on A06 (LOD=7.09) and A02 (LOD=2.97) were both above the significance threshold. The positions of the peak LOD of these two QTLs are the same as the ones found by standard interval mapping. The confidence interval of the QTL on A06 was 12.3 cM, and 30.1 cM for that on A02, based on the calculation of 95% Bayes credible interval.

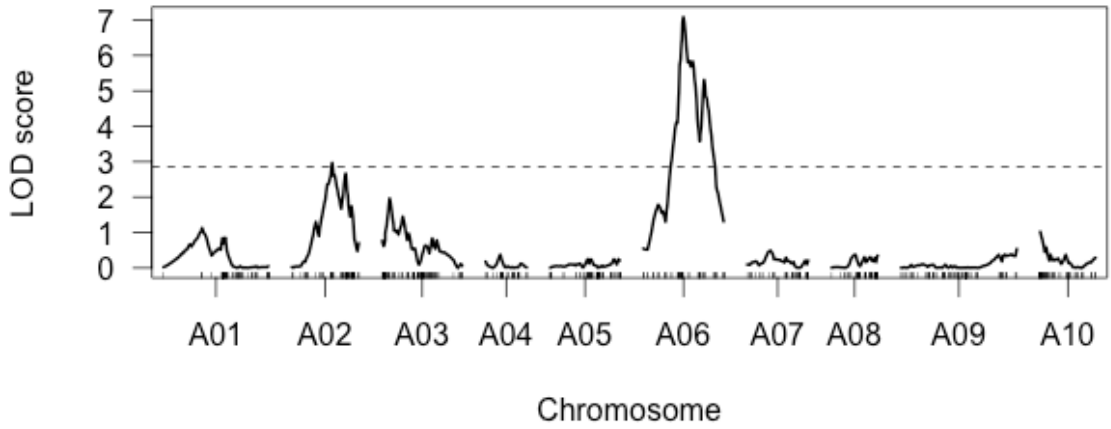


Figure 4.6 − Detection of QTL for Turnip mosaic virus resistance in *Brassica juncea* line TWBJ14 using the multiple imputation method in R/qtl. A significant LOD score (2.86) was determined by a permutation test (1000 times) at the significance level of 5% and is indicated by the dotted horizontal line.

A third single-QTL mapping method (composite interval mapping) was performed to clarify the evidence for any further QTL. The results showed that there were no more significant QTL other than the ones on A06 and A02.

After the single-QTL analysis, the function **scantwo** (a two-dimensional genome scan with a two-QTL model) using the multiple imputation method was performed (Fig. 4.7). For every pair of positions, the LOD scores for both the full model (two QTL plus interaction) and additive model (two QTL with additive effect) were calculated.

A permutation test in **scantwo** with the multiple imputation method was performed (n.perm=100). At the significance level of 5%, the thresholds for the full (T_f),

conditional-interactive (T_{fv1}), interaction (T_i), additive (T_a) and conditional-additive (T_{av1}) LOD scores are 5.85, 4.46, 4.20, 4.57 and 2.29, respectively. After the calculation of each LOD score, only the pair of A02 and A06 was reported as of interest and no other possible pairs were shown due to the lack of significance. These results suggest that it is an additive effect rather than an epistatic interaction between the two QTL on A02 (at position 43.3 cM) and A06 (at position 42.9 cM). Based on this defined additive model, the function **fitqtl** was performed. The result indicated the reliability of this model for TuMV resistance in line TWBJ14 and the two QTL on A02 and A06 together accounted for 48.9% of the total phenotypic variation.

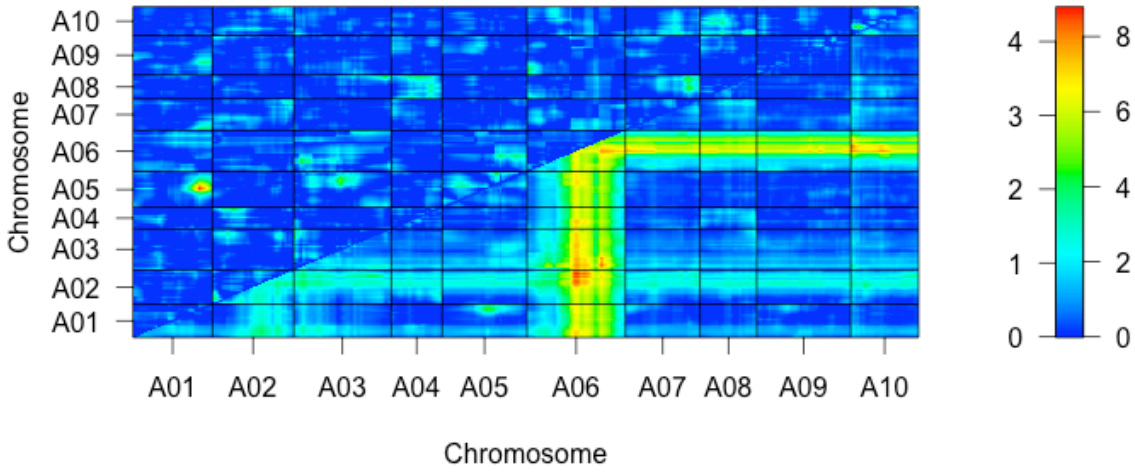


Figure 4.7 − Detection of QTL for Turnip mosaic virus resistance in *Brassica juncea* line TWBJ14 using a two-dimensional genome scan based on a two-QTL model.

TuMV resistance genes in *B. juncea* line TWBJ20

The result of **scanone** using the standard interval mapping via an EM algorithm is shown in Figure 4.8. The significance threshold was 2.79 at the 5% significance level. The LOD score of the QTL on A06 was 6.93, which suggested it was the most significant QTL. The LOD score of the QTL on A08 was 3.35 and it was the second significant QTL as it was also above the threshold. The QTL on A06 was detected at the peak LOD of 6.93 at position 28 cM, flanked by markers Bn-A06-p18634396 at position 25.7 cM and Bn-A06-p22806976 at position 30.7 cM. The

confidence interval of this QTL on A06 was 10.1 cM, calculated by the 95% Bayes credible interval method. The QTL on A08 was detected at peak LOD of 3.35 at position 5 cM, flanked by markers Bn-A08-p12599446 at position 2.5 cM and Bn-A08-p17468541 at position 7.8 cM. The confidence interval of this QTL was 10 cM, calculated by the 95% Bayes credible interval method.

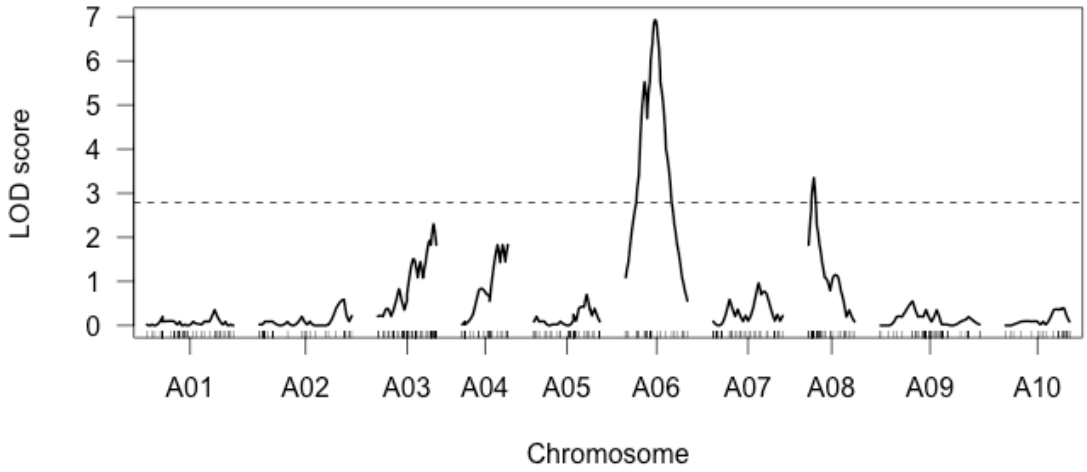


Figure 4.8 − Detection of QTL for Turnip mosaic virus resistance in *Brassica juncea* line TWBJ20 using standard interval mapping via EM algorithm in R/qtl. A significant LOD score (2.79) was determined by a permutation test (1000 times) at the significance level of 5% and is indicated by the dotted horizontal line.

The multiple imputation method was performed on the TWBJ20 BC_1 mapping population (Fig. 4.9). The same two significant QTLs were identified. The significance threshold was 3.14 at the 5% significance level. The LOD scores of the QTL on A06 (LOD=11) and A08 (LOD=3.88) were both above the significance threshold. The positions of the peak LOD of these two QTLs were the same as the one found by standard interval mapping. The confidence intervals of QTL on A06 and A08 were 4.7 cM and 5.8 cM, respectively and calculated by the 95% Bayes credible interval method.

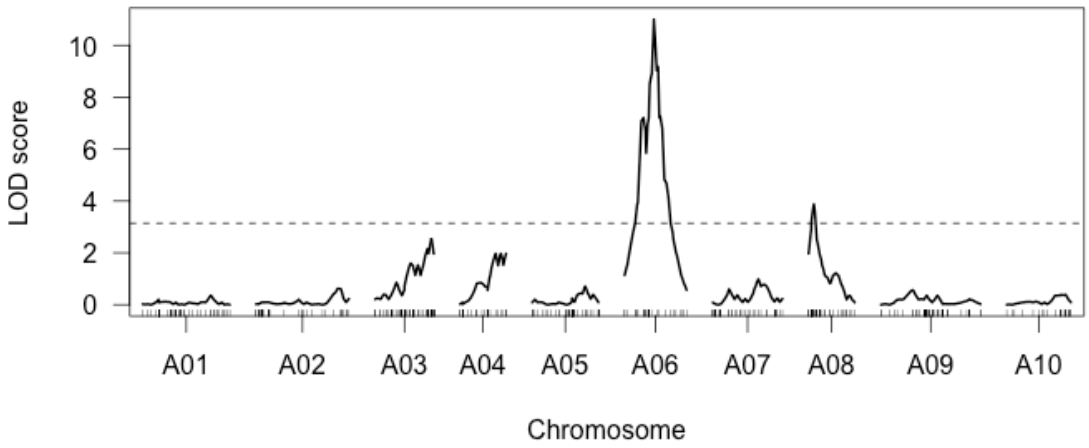


Figure 4.9 − Detection of QTL for Turnip mosaic virus resistance in *Brassica juncea* line TWBJ20 using a multiple imputation method in R/qtl. A significant LOD score (3.14) was determined by a permutation test (1000 times) at the significance level of 5% and is indicated by the dotted horizontal line.

Composite interval mapping was also performed to clarify the evidence for any further QTL. The results showed that there were no more significant QTL other than the ones on A06 and A08.

In the same way as the two QTL scan for line TWBJ14 (section 4.5.1), the QTL analysis using function **scantwo** via the multiple imputation method was performed for line TWBJ20 (Fig. 4.10). The permutation test (n.perm=100) decided the thresholds (at the 5% significance level) for the full (T_f), conditional-interactive (T_{fv1}), interaction (T_i), additive (T_a) and conditional-additive (T_{av1}) LOD scores. These were T_f=6.21, T_{fv1}=4.79, T_i=4.43, T_a=5.31 and T_{av1}=2.55. The only significant pair of positions reported was A06 and A08. No other possible pairs were shown due to the lack of significance. This result suggested that it was an additive effect rather than an epistatic interaction between the two QTL on A06 (at position 28 cM) and A08 (at position 5 cM). This model is supported by the result of **fitqtl** analysis, which indicated that the two QTL on A06 and A08 together accounted for 76.9% of the total phenotypic variation.

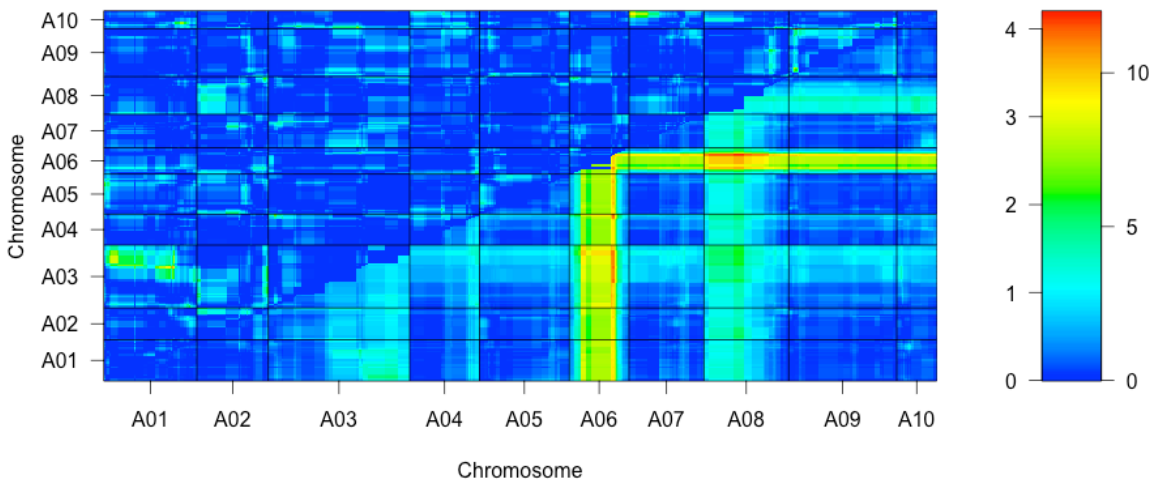


Figure 4.10 − Detection of QTL for Turnip mosaic virus resistance in *Brassica juncea* line TWBJ20 using a two-dimensional genome scan based on a two-QTL model.

The TuMV necrosis gene in *B. juncea* TWBJ14 BC_1 population

Attempts were made to map a necrosis gene in the TWBJ14 BC_1 population. Using the same genetic map as the one used in mapping the TuMV resistance in the TWBJ14 BC_1 (Fig. 4.3), a subset of 40 individual plants were used for the QTL mapping of the gene, comprising 20 plants with "$+_N$" phenotype and another 20 plants with "+" phenotype.

The result of the standard interval mapping via an EM algorithm is shown in Figure 4.11. The significance threshold was 2.84 at a significance level of 5%. One significant QTL on A06 was identified across all chromosomes. The LOD score of QTL on A06 was 4.29, peak at position of 57 cM, flanked by markers Bn-A06-p18634396 at position 49.3 cM and Bn-A06-p23042397 at position 58.9 cM. The confidence interval of this QTL was 16 cM according to a 95% Bayes credible interval method.

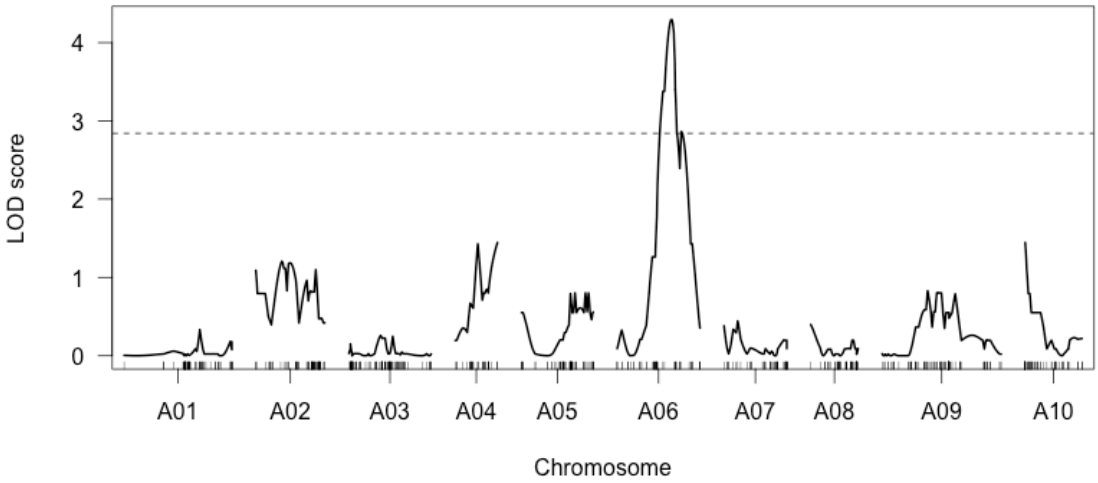


Figure 4.11 − Detection of putative QTL controlling the necrotic response to Turnip mosaic virus in *Brassica juncea* line TWBJ14 using standard interval mapping via an EM algorithm in R/qtl. A significant LOD score (2.84) was determined by a permutation test (1000 times) at the significance level of 5% and is indicated by the dotted horizontal line.

The multiple imputation method was applied in this QTL mapping as well (Fig. 4.12). The significance threshold was 3.22 at the significance level of 5%. One significant QTL was identified on A06. The peak LOD (4.94) was detected at position 56 cM. The confidence interval was 10.5 cM calculated by the 95% Bayes credible interval method. This major QTL explained 45.3% of the total phenotypic variation observed, strongly indicating a single gene effect.

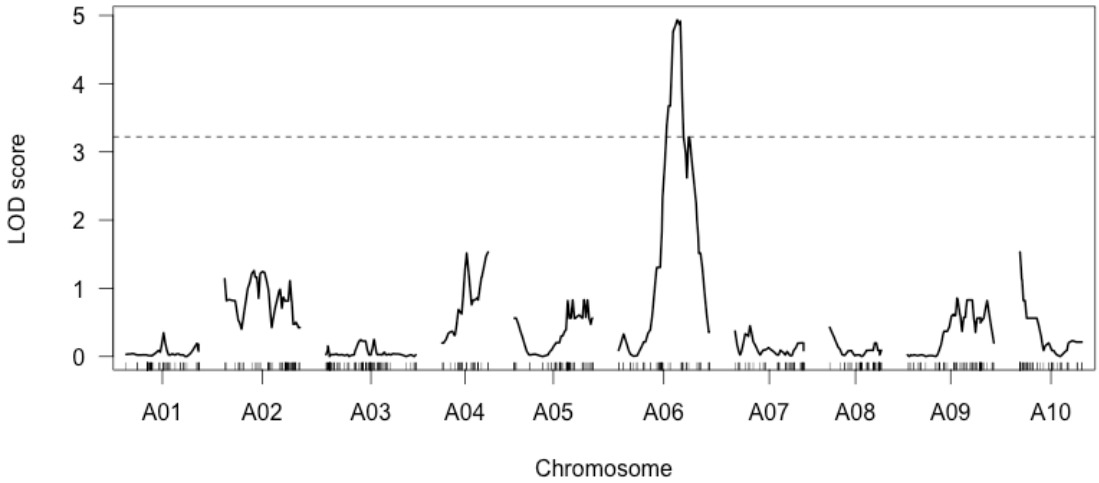


Figure 4.12 − Detection of putative QTL controlling the necrotic response to Turnip mosaic virus in *Brassica juncea* line TWBJ14 using a multiple imputation method in R/qtl. A significant LOD score (3.22) was determined by a permutation test (1000 times) at the significance level of 5% and is indicated by the dotted horizontal line.

Composite interval mapping was also performed on the TWBJ14 BC_1 mapping population. The results suggested that there were no more significant QTL other than the one found on A06.

4.4 Discussion

4.4.1 TuMV resistance genes in two resistant *B. juncea* lines

Both BC_1 and F_2 populations have been developed for the TuMV-resistant *B. juncea* lines TWBJ14 and TWBJ20. For line TWBJ14, a number of plants in both BC_1 and F_2 populations were phenotyped and the resistance/susceptibility segregation ratio closely fitted a Mendelian model based on the action of two recessive genes (1:3 and 1:15 respectively). For line TWBJ20, only the BC_1 population was phenotyped due to time limitations. However, the resistance/susceptibility segregation ratio of this population was not significantly different from a Mendelian model based on the action of two recessive genes (1:3).

The complementation test performed suggested that at least one resistance gene was not shared between these two lines. There were three phenotypes (0, + and $+_N$) in TWBJ14 BC_1 and F_2 populations, in comparison to the two phenotypes (0 and $+_N$) in TWBJ20 BC_1 population. This also suggested there were different resistance genes involved in these two resistant lines.

Two BC_1 segregating populations were used for genetic mapping because of their higher mapping efficiency than F_2 populations. Due to the limitation of research funding for a PhD project, it was not possible to genotype all the 427 individual plants in the two BC_1 populations. Therefore, subsets of selected plants have been genotyped using the Illumina® Infinium SNP Array. This might bring down the mapping efficiency to a certain degree. However, based on the genotypic data obtained, it appears both genetic linkage analysis and QTL mapping have been properly implemented. In both genetic maps, decent numbers of markers were spread across the chromosomes, with 14-34 non-overlapping markers on each chromosome.

The QTL analysis suggested that it was an additive two-QTL model involved in the TuMV resistances in both resistant *B. juncea* lines. One highly significant QTL and one less significant QTL contributed to the resistance independently with an additive effect. In both BC_1 populations, the QTL mapping results were consistent with the two recessive gene model suggested by the phenotypic distributions. Plant recessive resistance occurs when mutation arises in specific host proteins (targets of pathogen effector) encoded by susceptibility genes (S-genes) (Eckardt, 2002; Pavan *et al*. 2010). Recessive resistance is more prevalent for plant viruses than for other plant pathogens (Kang *et al.*, 2005). There have been several reports about pathogen resistances controlled by two recessive genes in plants. Ruffel *et al.* (2006) reported that complementation of two recessive resistance genes, *pvr2* and *pvr6*, were necessary for the resistance to Pepper veinal mottle virus (PVMV) in *Capsicum* (PVMV and TuMV both belong to the *Potyvirus* genus). Vallejos *et al*. (2010) identified two recessive resistance genes *bs5* and *bs6* in *Capsicum* with resistance to *Xanthomonas euvesicatoria* that causes bacterial spot disease. The combined effect of these two genes gave full resistance to all races of bacterial spot in peppers. Iyer and McCouch (2004; 2007) detected and cloned two recessive genes (*xa5* and *xa13*) controlling resistance to different strains of *Xanthomonas oryzae* pv. *oryzae* and suggested a new model for the function of recessive resistance in plant-bacterial interactions. As for resistances to insects, Hou *et al*. (2011) detected two recessive genes to the brown planthopper in rice using QTL analysis. Two QTL named *bph22*(t) and *bph23*(t) had LOD scores of 2.92 and 3.15 and explained 11.3% and 14.9% of phenotypic variation, respectively.

As mentioned in section 1.4.4, more than half of the reported *Potyvirus* resistance genes are recessive, which are believed to be based on a passive mechanism. There have been numerous reports on natural recessive virus resistance genes associated with translation initiation factors (eIF4E / eIF(iso)4E and eIF4G / eIF(iso)4G) in Arabidopsis, brassicas, lettuce, pepper, bean, rice, as well as many others (Le Gall *et al*., 2011). In *Brassica rapa*, several TuMV resistance genes related to the absence of susceptibility factors eIF4E / eIF(iso)4E have been reported, such as *retro01*, *ConTR01* (Rusholme *et al*., 2007) and *retro02* (Qian *et al*., 2013). Therefore, it is possible that an *eIF4* gene could be responsible for the observed resistances in *B. juncea* lines TWBJ14 and TWBJ20. According to the previous

studies, three copies of *eIF4E* and three copies of *eIF*(iso)*4E* have been identified and sequenced in a genomic library of the TuMV-susceptible *B. rapa* line R-o-18 (Jenner *et al*., 2010) and a genomic library of TuMV-resistant *B. rapa* line RLR22 (Nellist *et al*., 2014). Based on the conserved domains and motifs found in eIF4E and eIF4G using hmmpfam, Qian *et al*. (2013) identified all the *eIF4* genes in the *B. rapa* genome (eIF4E and eIF(iso)4E share certain similar protein domains and motifs, as do eIF4G and eIF(iso)4G). A total of 11 *eIF4E* / *eIF*(iso)*4E* and 14 *eIF4G* / *eIF*(iso)*4G* gene candidates were identified across *B. rapa* genome (Qian *et al*. 2013). Additionally, I have searched any other annotations of *eIF4* genes in different online databases. With this information, the positions of these candidate genes were compared to the regions of the QTL that I identified for the TuMV resistances in *B. juncea* lines TWBJ14 and TWBJ20 (Fig. 4.13 and Fig. 4.14).

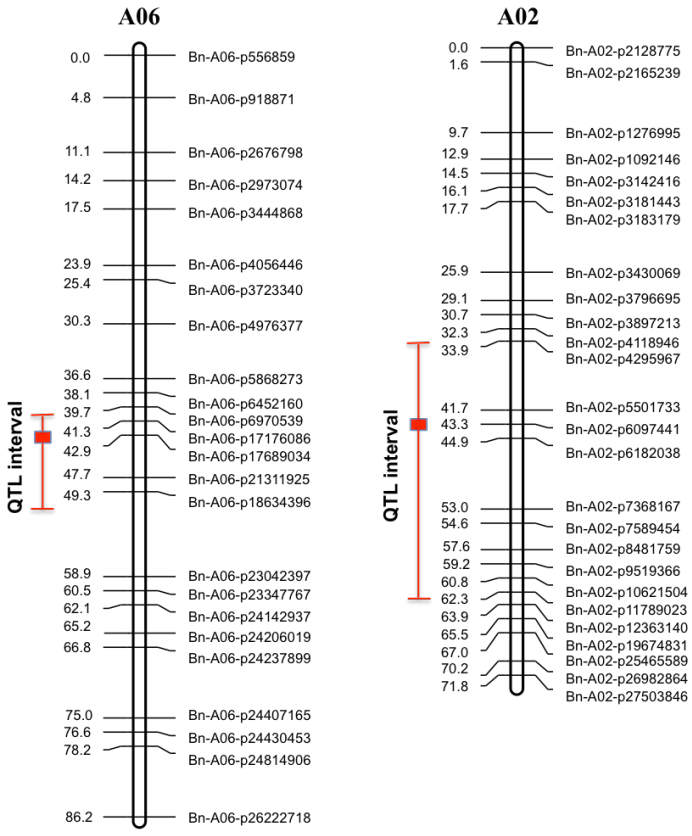


Figure 4.13 − Linkage groups A06 and A02 of *Brassica juncea* TWBJ14 BC_1 population showing QTL involved in Turnip mosaic virus resistance and linked markers.
QTL are indicated on left hand side. The red vertical bar and the rectangle represent confidence interval and peak LOD scores. The numbers on the left hand side of the linkage group represent genetic distance in cM at named markers shown to the right.

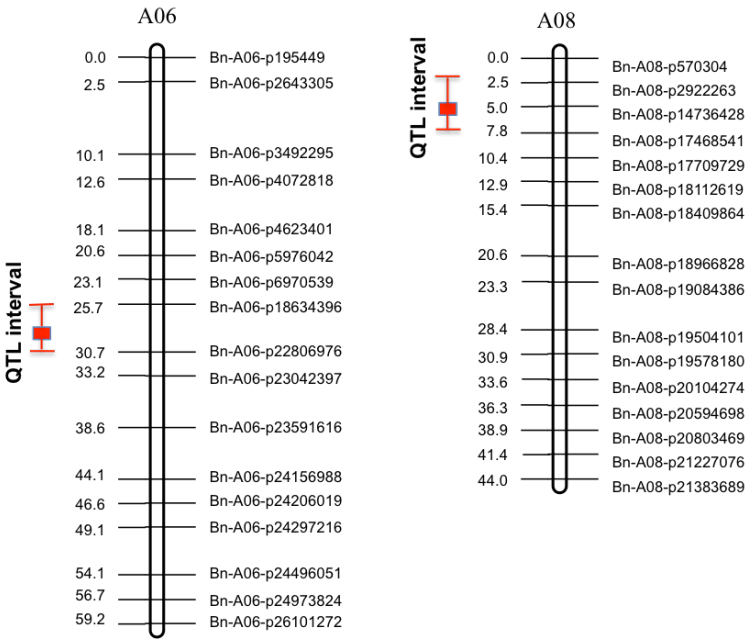


Figure 4.14 − Linkage groups A06 and A08 of *Brassica juncea* TWBJ20 BC_1 population showing QTL involved in Turnip mosaic virus resistance and linked markers.
QTL are indicated on left hand side. The red vertical bar and the rectangle represent confidence interval and peak LOD scores. The numbers on the left hand side of the linkage group represent genetic distance in cM at named markers shown to the right.

After comparisons of candidate locations with QTL confidence intervals, one predicted *eIF4G* candidate gene (*Bra038615*) on A06 lies in the confidence interval (12.3 cM) of the QTL on A06 in line TWBJ14, according to the physical position. What is more, *Bra038615* is not far from the TuMV resistance QTL on A06 in line TWBJ20, with the physical distance being approximately 3,829,000-7,802,000 bp. Additionally, the confidence interval of the QTL on A06 in line TWBJ14 includes the TuMV resistance QTL on A06 in TWBJ20, according to the physical position. This implies that *Bra038615* could be the candidate gene for the resistance QTL on A06 in both lines.

In addition, another two *eIF4G*-like genes *Bra008429* and *Bra020407* (Qian *et al.*, 2013) lie in the confidence interval (30.1 cM) of the TuMV resistance QTL on A02 in line TWBJ14. The candidate genes *BraA.eIF4E.c* (*Bra021026*), *BraA.eIF*(iso)*4E.c* (*Bra035531*) and one *eIF4G*-like gene *Bra010275* lie in the confidence interval (5.8 cM) of the QTL on A08 in line TWBJ20.

4.4.2 TuMV necrosis gene in *B. juncea* TWBJ14 BC_1 population

As described in section 1.5.2, in some circumstances, genes involved in the necrotic responses to pathogens have been deemed proper resistance genes, considering the fact that they induce the hypersensitive response, a defence reaction that restricts pathogen infection by killing infected plant cells rapidly. The segregation of systemic necrotic symptoms to TuMV infection in the TWBJ14 BC_1 population gave the opportunity to map the necrosis gene(s) involved. Forty individual plants with genotypic data and evenly segregated phenotypes (20 "+" and 20 "$+_N$") have been taken forward for QTL analysis based on the available linkage map. A single QTL responsible for the necrotic response was mapped on chromosome A06 (Fig. 4.15). Interestingly, dominant TuMV resistance genes *TuRB01*/*TuRB01b* (Walsh *et al*., 1999; Lydiate *et al*., 2014) and *TuBR03* (Hughes *et al.*, 2003) from the 'A' genome of *B. napus* and *B. rapa* are located in the confidence interval of this QTL on A06.

Another gene inducing necrosis to TuMV, *TuBR05* in *B. napus*, in conjunction with *TuBR04* confers resistance to pathotypes 1 and 3 of TuMV. *TuBR04* is epistatic to *TuBR05* (Walsh and Jenner, 2006). The positions of these two genes have not been reported yet. This model of resistance could be more prevalent than thought, and it might be applicable to the necrosis gene mapped on A06 in this study.

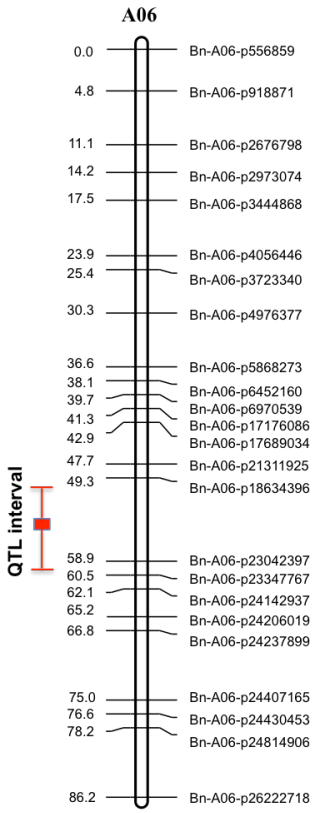


Figure 4.15 − Linkage group A06 of *Brassica juncea* TWBJ14 BC_1 population showing the QTL involved in the necrotic reaction to Turnip mosaic virus inoculation and linked markers.
QTL is indicated on left hand side. The red vertical bar and the rectangle represent confidence interval and peak LOD scores. The numbers on the left hand side of the linkage group represent genetic distance in cM at named markers shown to the right.

4.4.3 Standard interval mapping and Multiple imputation method in R/QTL

The most commonly used method for QTL analysis is interval mapping. It includes a subset of a mapping method such as Standard interval mapping, Haley-Knott regression and Multiple imputation method. There are two major problems in QTL mapping, the missing data problem and the model selection problem. The various interval mapping methods differ in their handling of missing genotype data. Standard interval mapping uses a maximum likelihood estimation under a mixture model, whereas the Haley-Knott regression method uses approximations to the mixture model. The multiple imputation approach uses the same mixture model but with multiple imputation in place of maximum likelihood. It dispenses with the missing data problem by filling in all missing genotype data, even at sites between

markers (on a grid along the chromosomes).

The multiple imputation method is more robust than standard interval mapping and has a little advantage over the extended Haley–Knott method for single-QTL models, particularly because of the large upfront effort to obtain the imputations. For the single-QTL analysis in this study, both standard interval mapping and multiple imputation methods have been performed. The results produced by the multiple imputation method appeared more accurate. Additionally, for the fit and exploration of multiple-QTL models, currently only multiple imputation and Haley-Knott regression have been implemented. However, Haley-Knott regression performs poorly in the case of selective genotyping. Therefore, the multiple imputation approach also has the greatest value for multiple-QTL analysis. What is more, the multiple imputation approach extends to the case of multiple-QTL models without modification. As with single-QTL and two-QTL analysis, the computation time for the imputations and for the fixed set of linear regressions to be performed at each putative QTL or QTL pair weakened the value of the approach, the imputations are performed just once. Therefore, in the exploration of multiple-QTL models, the multiple imputation approach is quite valuable (Broman and Sen, 2009).

Based on the comparisons above, the multiple imputation approach has been implemented for both single-QTL and two-QTL analysis in this study.

4.5 Conclusions

The main aim of this chapter was to characterise the genetic inheritance of the broad-spectrum TuMV resistance and map the genes conferring TuMV resistance in the *B. juncea* lines TWBJ14 and TWBJ20. Based on the phenotypes of the F_1, BC_1 and F_2 generations, the TuMV resistance in both lines fit a Mendelian model based on the action of two recessive genes. QTL mapping was performed based on SNP genotyping data of BC_1 plants. Two QTL with an additive effect were mapped for the TuMV resistance in both lines. One QTL was mapped for the necrotic response to TuMV in line TWBJ14. According to the positions of the mapped QTL, three candidate *eIF* genes were identified for the TuMV resistance in line TWBJ14. Four

candidate *eIF* genes were identified for the TuMV resistance in line TWBJ20. The involvement of two genes in the TuMV resistances might explain the fact these resistances have a broader spectrum of efficacy than single *R* genes.

Chapter 5

Identification of *TuRB01*/*TuRB01b* in the *Brassica* 'A' genome

5.1 Background

5.1.1 Introduction to *TuRB01*/*TuRB01b*

TuMV RESISTANCE IN BRASSICA 01 (*TuRB01*) is a single dominant gene that confers extreme resistance to pathotype 1 isolates of TuMV (Fig. 5.1). It was originally mapped on the chromosome N6 of *B. napus* (Jenner and Walsh, 1996; Walsh *et al.*, 1999). Subsequently dominant resistance was found in a *B. rapa* line, which had an identical specificity and mechanism to *TuRB01* resistance (Walsh *et al.*, 2002). This dominant resistance in *B. rapa* was controlled by a single gene *TuRB01b* (named after *TuRB01*) that was mapped on chromosome A6 of *B. rapa* (Rusholme, 2000; Lydiate *et al.*, 2014). Comparative mapping has confirmed A6 of *B. rapa* was equivalent to chromosome N6 of *B. napus* and that the genomic location of *TuRB01b* could be identical to that of *TuRB01* in *B. napus*. Therefore, it is most likely that *TuRB01b* and *TuRB01* represent the identical alleles at the same resistance locus on chromosome A6 of *Brassica* 'A' genome. Presumably, *B. napus* acquired *TuRB01* from the *B. rapa* gene pool following the natural synthesis of *B. napus* from *B. rapa* and *B. oleracea*.

Figure 5.1 − Plants of *Brassica napus* lines possessing (right) and lacking (left) *TuRB01* following inoculation with Turnip mosaic virus isolate UK 1 (Walsh and Jenner, 2002).

Although *TuRB01*/*TuRB01b* confers extreme resistance to TuMV isolate UK 1 and other TuMV pathotype 1 isolates, the resistance is overcome by TuMV isolate vVIR24, a mutant of UK 1, which possesses a single nucleotide substitution in the cytoplasmic inclusion (CI) gene of TuMV (Jenner *et al.*, 2000). This single mutation has resulted in the shift of UK 1 from avirulence to virulence against *TuRB01*/*TuRB01b*-based resistance (Walsh *et al.*, 2002). *TuRB01*/*TuRB01b*-based resistance is pathotype-specific and can be overcome by a number of different TuMV pathotypes, particularly pathotypes 3 and 4.

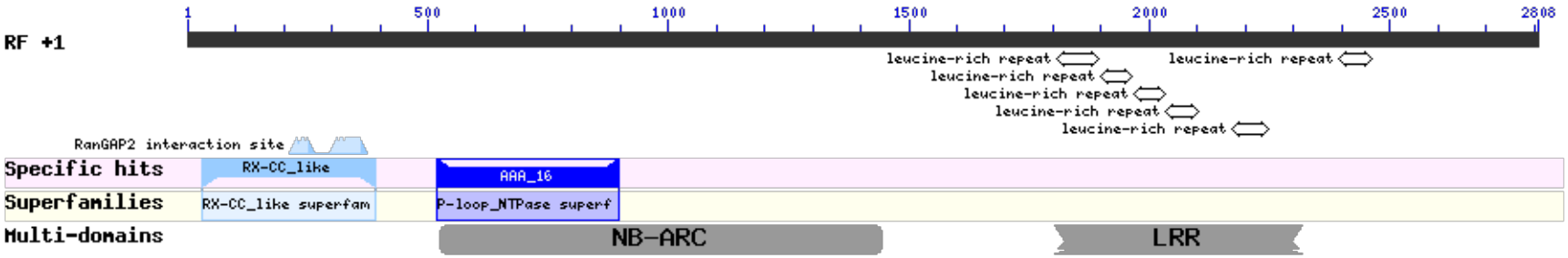
5.1.2 Previous unpublished work on the identification of *TuRB01*/*TuRB01b*

From previous work that was done in the plant-virus interactions group at University of Warwick, a large number of *B. napus* and *B. rapa* lines/cultivars have been tested for their resistance to TuMV isolates UK 1 and vVIR24. The results told us whether *TuRB01*/*TuRB01b* was present in each line. *TuRB01*/*TuRB01b* has been mapped to an interval containing three conventional *R* genes (NB-LRR genes, section 1.4.2), which were named *BORG1*, *BORG2* and *BORG3*. The genes were identified from a BAC (bacterial artificial chromosome) of 48302 bp long from a *B. napus* line possessing *TuRB01*/*TuRB01b*-based TuMV resistance. This BAC covers the whole interval and has been used as the reference sequence. Both *BORG1* and *BORG3* encode a CC-NB-LRR protein and they are highly homologous to each

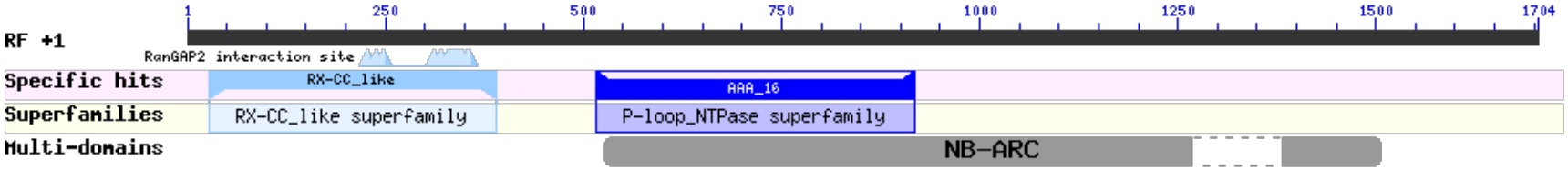
other, with the similarity of the CDS (coding DNA sequence) being 98% (A. R. Baker, University of Warwick, Personal communication). Additionally, both genes have an adjacent DNA segment that is homologous to a gene in *Arabidopsis* called *RCH1* (Root Clavata-Homolog 1), which is involved in the perception of root meristem growth factor in *Arabidopsis*. *RCH1* is an LRR receptor-like kinase gene (LRR-RKs, section 1.4.3) (Casamitjana-Martínez *et al*. 2003; Shinohara *et al*. 2016). *BORG2* was found to encode a truncated protein (Fig. 5.2). Compared to *BORG1* and *BORG3*, *BORG2* is shorter and lacks an LRR domain.

From previous work, DNA and RNA samples were extracted from a number of brassica lines/cultivars possessing and lacking *TuRB01*/*TuRB01b*. *BORG1* and *BORG3* were amplified by PCR, cloned and sequenced from a number of these brassica plant lines. The whole *BORG1* sequences have been obtained from 15 *B. napus* lines, four *B. rapa* lines and one *B. oleracea* line, whilst whole *BORG3* sequences have been obtained in 13 *B. napus* lines and two *B. rapa* lines (A. R. Baker, University of Warwick, Personal communication). Sequences were compiled and comparisons made to investigate the associations between genetic variation and functionality. Also, the expressions of *BORG1* and *BORG3* were studied in a number of brassica plant lines, the results showed that there was an association between *BORG1* expression and the presence or absence of *TuRB01*/*TuRB01b* in individual plant lines; there was no association for *BORG3* expression (A. R. Baker, University of Warwick, Personal communication).

BORG1



BORG2



BORG3

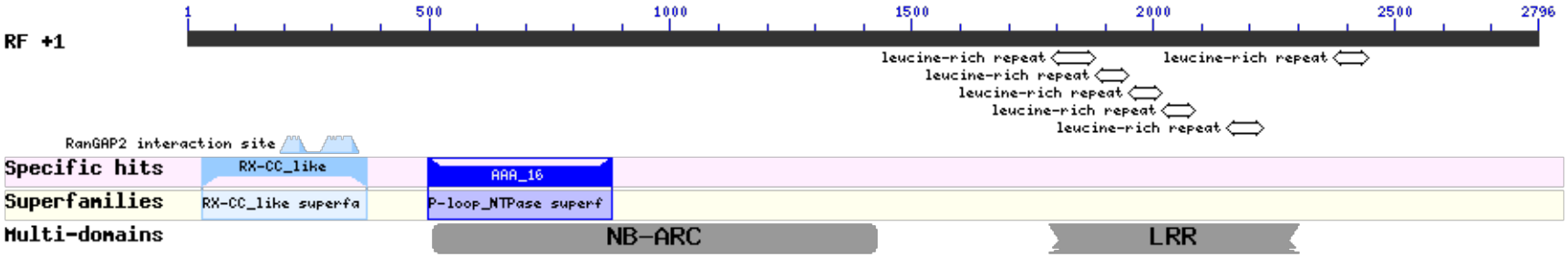


Figure 5.2 – Analysis of conserved domains on CDS (coding DNA sequence) of *TuRB01*/*TuRB01b* candidates *BORG1*, *BORG2* and *BORG3*, using the NCBI Conserved Domain Search Service.

Although *BORG2* was found to encode a truncated protein, whether it was involved in the *TuRB01*/*TuRB01b*-based resistance required experimental confirmation. Therefore, I PCR-amplified *BORG2* from a number of brassica lines and sequenced the products. In addition, the expression of *BORG2* in these lines was investigated. Comparisons were made between plant lines possessing and lacking *TuRB01*/*TuRB01b*. Furthermore, the potential *RCH1*-like gene adjacent to *BORG1* was investigated. It was sequenced and checked for expression in a number of plant lines.

5.2 Materials and Methods

The brassica plant lines used in this chapter are listed in section 2.1.1. Five primer pairs were used in attempts to PCR amplify *BORG2*, including BR183/BR184, BR185/BR186, BR191/BR192, BR185/BR202 and BR200/BR184 (Table 2.3 in section 2.4.2). *BORG2* internal primers BORG2L, BORG2R, BR82, BR148, BR187, BR188, BR189 and BR190 (Table 2.4 in section 2.4.2) were used for sequencing *BORG2*. The internal primer pair BR190/BR186 was used for the experiment on *BORG2* expression. For the experiments on the *RCH1*-like gene, primer pair BR196/BR195 (Table 2.3) was used for PCR amplification and sequencing and primer pair BR205/BR206 (Table 2.4) was used for expression studies.

5.3 Results

5.3.1 PCR amplification and sequencing of *BORG2* from different brassica plant lines

In total, PCR amplification of *BORG2* was attempted on 11 *B. napus* lines (listed in Table 5.1) and two *B. rapa* lines (TD-R and TD-S), using five different primer pairs. Successful amplification was achieved in nine *B. napus* lines, three of which possessed *TuRB01*, five lacked *TuRB01* and for one line (NO2D) it is not certain whether *TuRB01* is present or not. Whole *BORG2* sequence was obtained for eight lines. For line PSA12, as no other primer pairs but the internal primer pair BR191/BR192 worked for PCR amplification, partial sequence of *BORG2* was

obtained with no sequence of the 5' and 3' ends. For *B. napus* lines R4 and Karroo and *B. rapa* lines TD-R and TD-S, no PCR products were obtained using all the five primer pairs. The results for the *BORG2* amplification in four *B. napus* lines using primer pair BR185/BR186 are shown in Figure 5.3.

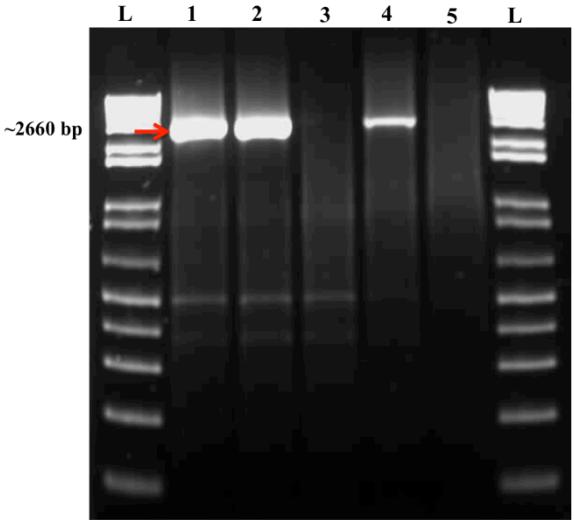


Figure 5.3 – PCR amplification of *TuRB01*/*TuRB01b* candidate *BORG2* from different *Brassica napus* lines using primer pair BR185/BR186.
L, 1 Kb Plus DNA ladder; **1,** DH12075; **2,** S6; **3,** R4; **4,** Global DH; **5,** ddH_2O (negative control).

BORG2 sequences in seven *B.napus lines* (DH12075, Global DH, Yudal, Darmor, 22S, S6 and NO1D) were all identical to the *BORG2* allele in the BAC and they were all of 2571 bp long (Table 5.1). Of these seven plant lines, three lines possess *TuRB01* and four lack *TuRB01*. Nucleotide sequence variation in the *BORG2* allele was found in plant line NO2D, with a homology of 98%. It is not certain whether NO2D possesses *TuRB01*/*TuRB01b*-based resistance as the specificity of TuMV resistance is not clear for this line. Partial *BORG2* sequence with a length of 2291 bp was obtained for line PSA12 with no sequence difference seen.

Table 5.1 – Results of sequencing and expression of *TuRB01*/*TuRB01b* candidate *BORG2* in different *Brassica napus* lines.

Plant line	Species	*TuRB01/TuRB01b*-based resistance	*BORG2* sequence and its homology to the allele in BAC	*BORG2* expression
DH12075	*B. napus*	✔[1]	1~2571 nt 100% identical	+[2]
Global DH	*B. napus*	✔	1~2571 nt 100% identical	+
Yudal	*B. napus*	✔	1~2571 nt 100% identical	+
R4	*B. napus*	✔	Not obtained	+
Karroo	*B. napus*	✔	Not obtained	+
Darmor	*B. napus*	✖[3]	1~2571 nt 100% identical	–[4]
22S	*B. napus*	✖	1~2571 nt 100% identical	+
S6	*B. napus*	✖	1~2571 nt 100% identical	+
NO1D	*B. napus*	✖	1~2571 nt 100% identical	+
PSA12	*B. napus*	✖	228~2518 nt 100% identical	+
NO2D	*B. napus*	?[5]	1~2571 nt 98% match	+

[1]**✔,** plant line possessing *TuRB01* resistance; [2]**+,** presence of *BORG2* expression; [3]**✖,** plant line lacking *TuRB01* resistance; [4]**–,** absence of *BORG2* expression; [5]**?,** not certain whether plant line possesses *TuRB01* resistance.

5.3.2 Expression of *BORG2* in different *B. napus* lines

The expression of *BORG2* was investigated in the 11 *B. napus* lines. PCR was carried out on cDNA samples of these lines using *BORG2* specific internal primer pair BR190/BR186 (Table 5.1; Fig. 5.4). A specific PCR band of approximately 560 bp was produced in 10 lines but not in line Darmor (Lane 4, Fig. 5.4). This indicated that of these 11 *B. napus* lines, *BORG2* was expressed in 10 lines but not in Darmor.

As there was no significant difference either in sequence or expression patterns of *BORG2* between *TuRB01*-possessing and *TuRB01*-lacking *B. napus* lines, *BORG2* is unlikely to be involved in *TuRB01*/*TuRB01b*-based resistance.

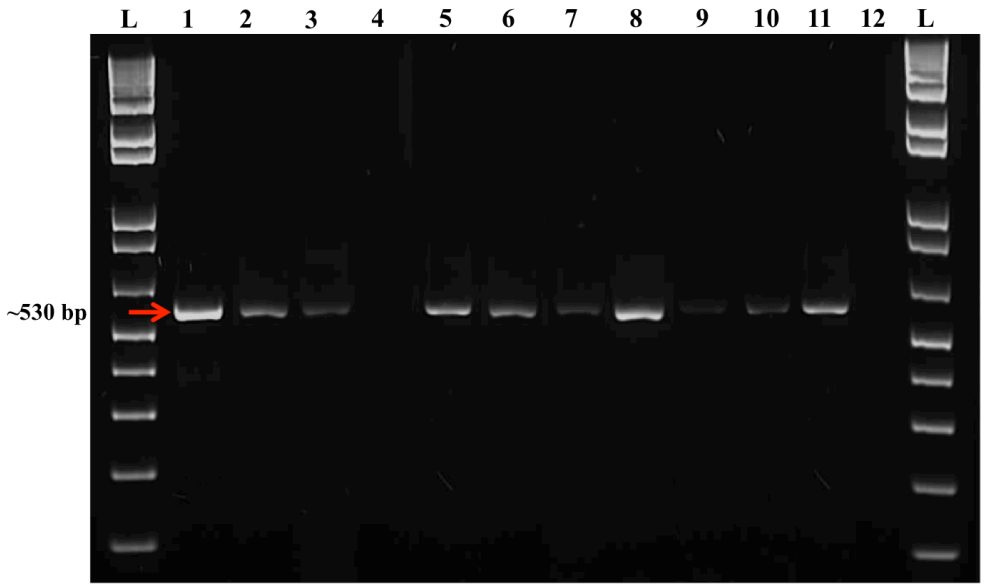


Figure 5.4 – Expression of *TuRB01* candidate *BORG2* in different *Brassica napus* lines.
L, 1 Kb Plus DNA ladder; **1,** DH12075; **2,** Global DH; **3,** Yudal; **4,** Darmor; **5,** 22S; **6,** S6; **7,** NO1D; **8,** NO2D; **9,** PSA12; **10,** R4; **11,** Karroo; **12,** ddH_2O (negative control).

5.3.3 Sequence and expression of *RCH1*-like gene in different brassica lines

PCR amplifications were done on 12 *B. napus* lines and two *B. rapa* lines (Table 5.2) using *RCH1*-like gene specific primer pair BR196/BR195. A specific PCR band of approximately 1190 bp was produced in 11 *B. napus* lines and both *B. rapa* lines (Fig. 5.5). No PCR products were obtained for *B. napus* line Cabriolet, which was identified as lacking *TuRB01*/*TuRB01b*-based resistance. As the reverse primer BR195 is an internal primer of *RCH1*-like gene, the whole gene sequence (636 bp) was not obtained following sequencing of the PCR product. Partial sequences (the first 416 nucleotides) of *RCH1*-like gene were obtained for these 13 brassica lines. These 13 partial sequences of *RCH1*-like gene were all identical to the allele in the BAC. For the *B. juncea* line 060DH17, the genome of which is being sequenced by our collaborators in Saskatoon Canada, the whole sequence of *RCH1*-like gene was obtained by analysing the sequence of a scaffold from this line (Isobel Parkin, Personal communication). The alignment shows the sequence of *RCH1*-like gene in 060DH17 is identical to the allele in the BAC (Table 5.2).

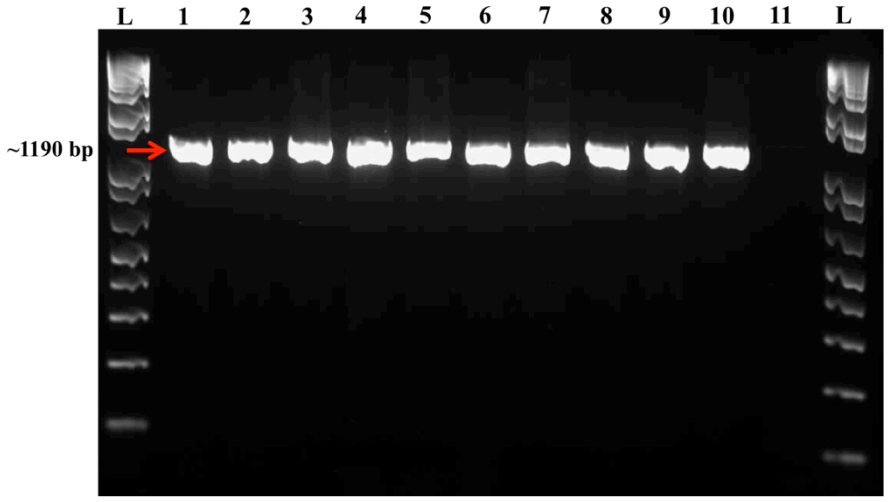


Figure 5.5 – PCR amplification of *TuRB01*/*TuRB01b* candidate *RCH1*-like gene from different brassica lines using primer pair BR196/BR195.
L, 1 Kb Plus DNA ladder; **1,** DH12075; **2,** TD-R; **3,** TD-S; **4,** R4; **5,** Yudal; **6,** Darmor; **7,** PSA12; **8,** NO1D; **9,** NO2D; **10,** Karroo; **11,** ddH_2O (negative control).

Table 5.2 – Results of sequencing and expression of *TuRB01*/*TuRB01b* candidate *RCH1*-like gene in different brassica lines.

Plant line	Species	*TuRB01/TuRB01b*-based resistance	*RCH1*-like gene sequence and its homology to the allele in BAC	*RCH1*-like gene expression	*BORG1* expression
DH12075	*B. napus*	✔[1]	1~416 nt 100% identical	+[2]	+
Global DH	*B. napus*	✔	1~416 nt 100% identical	+	+
Yudal	*B. napus*	✔	1~416 nt 100% identical	+	+
R4	*B. napus*	✔	1~416 nt 100% identical	+	+
Karroo	*B. napus*	✔	1~416 nt 100% identical	–	+
Darmor	*B. napus*	✖[3]	1~416 nt 100% identical	–[4]	–
22S	*B. napus*	✖	1~416 nt 100% identical	–	?[5]
S6	*B. napus*	✖	1~416 nt 100% identical	–	–
NO1D	*B. napus*	✖	1~416 nt 100% identical	+	+
PSA12	*B. napus*	✖	1~416 nt 100% identical	–	–
Cabriolet	*B. napus*	✖	Not obtained	+	–
NO2D	*B. napus*	?	1~416 nt 100% identical	+	+
TD-R	*B. rapa*	✔	1~416 nt 100% identical	+	+
TD-S	*B. rapa*	✖	1~416 nt 100% identical	+	–
060DH17	*B. juncea*	✖	1~636 nt 100% identical	–	–

[1]**✔,** plant line possessing *TuRB01/TuRB01b* resistance; [2]**+,** presence of gene expression; [3]**✖,** plant line lacking *TuRB01/TuRB01b* resistance; [4]**–,** absence of gene expression; [5]**?,** result was inconclusive.

As for the expression of *RCH1*-like gene, a target internal segment of 259 bp was amplified by PCR of cDNA samples of 15 brassica lines, using *RCH1*-like gene internal primer pair BR205/BR206. The specific PCR product was obtained in 10 brassica lines and absent in the other 5 lines, which reflected the expression patterns of *RCH1*-like gene in these lines (Table 5.2; Fig. 5.6).

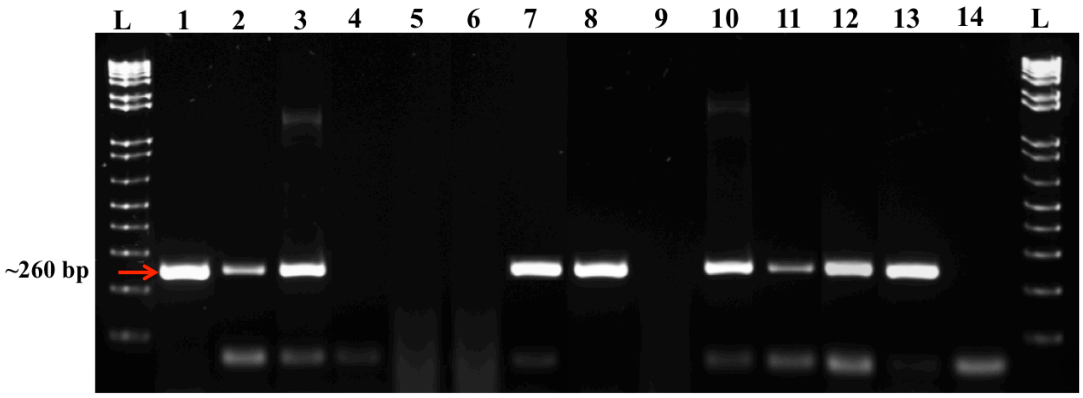


Figure 5.6 – Expression of *TuRB01/TuRB01b* candidate *RCH1*-like gene in different brassica lines using primer pair BR205/BR206.
L, 1 Kb Plus DNA ladder; **1,** DH12075; **2,** Global DH; **3,** Yudal; **4,** Darmor; **5,** 22S; **6,** S6; **7,** NO1D; **8,** NO2D; **9,** PSA12; **10,** R4; **11,** Karroo; **12,** TD-R; **13,** TD-S; **14,** ddH_2O (negative control).

Comparisons were made between *TuRB01*/*TuRB01b*-possessing and *TuRB01*/*TuRB01b*-lacking plant lines based on the expression of *RCH1*-like gene and *BORG1* (Table 5.2). Across these 15 brassica lines, an association between expression of *RCH1*-like gene and *TuRB01*/*TuRB01b* resistance was found in 10 lines. There was a lack of association in four lines (NO1D, Karroo, Cabriolet and TD(S)). As the specificity of TuMV resistance is not clear in line NO2D, it is not certain whether there is an association. In addition, the expression of *BORG1* is associated to *TuRB01*/*TuRB01b* resistance in 13 of these brassica lines and not associated in line NO1D. The *BORG1* expression in *B. napus* line 22S is ambiguous, as a previous result from the research group showed expression (unpublished), whereas I found no expression while repeating this experiment using fresh cDNA samples of 22S. For *TuRB01*-lacking *B. napus* line NO1D, both *BORG1* and *RCH1*-like gene expressions were detected, showing no association.

5.4 Discussion

To resist numerous pathogen infections, plants have evolved a variety of defence mechanisms. Effector-triggered immunity (ETI) is one of the sophisticated resistance mechanisms, which is initiated when a plant resistance (R) protein recognises a corresponding pathogen effector protein directly or more often indirectly. ETI often involves with localised plant cell death, referred to as the hypersensitive response (HR). The HR can be divided into two types depending on the scale, which is macroscopic HR and microscopic HR. Microscopic HR is invisible to the naked eye and infection is confined to very few plant cells. In contrast, macroscopic HR is visible. The form of HR can vary significantly in phenotype and timing during diverse plant-pathogen interactions (Mur *et al.* 2008).

The *TuRB01*/*TuRB01b*-based TuMV resistance is a pathotype-specific and appears to be ETI resistance. It is an extreme resistance that can be classified as immunity (Jenner and Walsh, 1996). A microscopic HR might be involved where single infected cells are killed and infection is prevented from spreading to adjacent cells. However, attempts to detect such microscopic HR have failed (J. A. Walsh, University of Warwick, Personal communication). It is also possible that *TuRB01*/*TuRB01b* induces operational immunity where cell-to-cell movement is impaired. *TuRB01*/*TuRB01b* is specifically effective against TuMV pathotype 1 isolates which are the most abundant TuMV isolates in Europe. However, pathotypes 3 and 4 are also common and both of them overcome *TuRB01*/*TuRB01b* (Jenner and Walsh, 1996). It would be interesting to combine *TuRB01*/*TuRB01b* with some other TuMV resistance genes to investigate durable resistance to TuMV in brassica crops. The mapping and identification of TuMV resistance genes will accelerate the introgression of such genes into desirable genetic backgrounds through marker-assisted selection.

The *TuRB01*/*TuRB01b* candidate genes *BORG1*, *BORG2* and *BORG3* are all homologous to an *Arabidopsis* gene *RPP8*. *RPP8* confers resistance to downy mildew *Peronospora parasitica* (McDowell *et al.*, 1998). It is a conventional *R* gene that encodes a CC-NB-LRR protein. Interestingly, *RPP8* has two allelic genes, *HRT* and *RCY1*, which confer resistance to Turnip crinkle virus and Cucumber

mosaic virus, respectively. Although these three alleles have strong homology, they confer ETI resistance to different pathogens. Meanwhile, the signal transduction requirements downstream of the *RPP8*/*HRT*/*RCY1* alleles also differ from one another (Takahashi *et al*., 2002).

In addition to *BORG3*, the previously eliminated *TuRB01*/*TuRB01b* candidate gene, another candidate gene *BORG2* has been studied and ruled out in this chapter. However, *BORG2* has a highly conserved sequence in 9 *B. napus* lines and it generally expresses in most lines (Table 5.1), which suggests that it could be functionally important.

The potential candidate *RCH1*-like gene adjacent to *BORG1* was also investigated. The sequence of this gene is significantly conserved amongst the brassica lines that were studied. However, the expression of this gene has a certain extent of correlation to the presence or absence of *TuRB01*/*TuRB01b*-based resistance. Although *BORG1* is the best candidate gene, potentially there could be another gene operative in addition to it. It is conceivable that *BORG1* is the predominant gene controlling the TuMV resistance and *RCH1*-like gene is a subordinate gene. Particularly for *B. napus* line 22S, assuming the previous result of positive *BORG1* expression is correct, it seems the expression of *BORG1* alone (without expression of *RCH1*-like gene) is not sufficient for the resistance to TuMV isolate UK 1 (Table 5.2). However, this is not the case for *B. napus* line Karroo where positive *BORG1* expression alone (without the expression of *RCH1*-like gene) is sufficient for the *TuRB01*/*TuRB01b*-based resistance. Additionally, for *TuRB01*/*TuRB01b*-lacking *B*. napus line Cabriolet and *B. rapa* line TD(S), without the expression of *BORG1*, they do not possess the resistance even though the *RCH1*-like gene is expressed (Table 5.2). The involvement of the *RCH1*-like gene in the *TuRB01*/*TuRB01b*-based resistance is inconclusive.

Further research on *BORG1* and *RCH1*-like gene is needed using site-directed mutagenesis or transformation into susceptible brassica lines. Additionally, it would be of interest to have an investigation of the signal transduction requirements downstream of *BORG1*.

5.5 Conclusions

The main aim of this chapter was to identify TuMV resistance gene *TuRB01*/*TuRB01b* on chromosome A6 of *Brassica* 'A' genome. Previously, one candidate gene *BORG3* was found not to be involved in the resistance. Another candidate gene *BORG2*, was ruled out in my study in this chapter, making *BORG1* (the only remaining candidate *R* gene) the best candidate. There is an ambiguity of the involvement of a second gene (*RCH1*-like gene) in this resistance, which is adjacent to *BORG1* and encodes an LRR receptor-like kinase (LRR-RK).

Chapter 6

Introgression of *TuRB01*/*TuRB01b*-based resistance into *Brassica juncea*

6.1 Background

6.1.1 Introgression of desirable gene(s) through interspecific crossing

Alien gene introgression in plants has lead to significant improvement of traits in various crop species. The improvements include resistance to biotic and abiotic stresses, high productivity and quality, improved nutrition and other enhanced economic characteristics. There are two major categories of methods for alien gene introgression. The first category involves sexual hybridisation, such as interspecific (wide) hybridisation. The second category involves asexual methods, such as genetic transformation and somatic hybridisation. These techniques, recently aided by modern tools such as molecular marker technology, molecular cytogenetics and bioinformatics, have introduced hundreds of desirable genes into crops (Pratap and Kumar, 2014).

Most of the successful introgressions to date have been achieved by sexual hybridisation. In plant breeding, interspecific hybridisation is a very important technique for the improvement of crops and development of new varieties. It is usually accomplished by several rounds of backcrossing to the recipient parent and stringent selection for the desirable traits. One typical example of gene introgression is the transfer of leaf-rust resistance from *Aegilops umbellulata* into cultivated wheat (*Triticum aestivum*) (Sears, 1956). Following on from this, Riley *et al.* (1968) transferred yellow rust resistance from *Aegilops comosa* into cultivated wheat. In tomato breeding, Iltis (1988) developed new tomato cultivars with increased dry matter content. This trait was introgressed from a wild-type of Peruvian tomato (*Lycopersicon chmielewskii*) that has a high concentration of soluble solids. In brassica crops, Chiang *et al*. (1977) introgressed resistance to race 2 of *Plasmodiophora brassicae* (clubroot pathogen) from *B. napus* into *B. oleracea* through interspecific hybridisation. Banga *et al.* (2004) successfully introgressed

white rust resistance (caused by *Albugo candida*) from resistant *B. napus* and *B. carinata* into susceptible *B. juncea*. This was achieved by simple interspecific hybridisation and backcrossing, due to the close genomic affinity within these *Brassica* species. Sheng *et al*. (2012) attempted to introgress an extreme black rot (caused by *Xanthomonas campestris* pv. *campestris*) resistance from *B. nigra* into *B. rapa* through interspecific hybridisation, embryo rescue and backcrossing. BC_2 plants expressing resistance to black rot disease were obtained. Additionally, many useful genes have been incorporated into cultivated oilseed crops, food legumes and other crops (Kumar *et al*., 2011).

6.2 Materials and Methods

6.2.1 Plant materials and interspecific crossing procedures

Plant materials mentioned in this chapter are described in section 2.1.1. More specifically, for interspecific crossing between TuMV-susceptible *B. juncea* and TuMV-resistant *B. rapa* and *B. napus*, *B. juncea* DH line 060DH17 was used as the susceptible recipient parent, and *B. rapa* line TD-R (homozygous for the *TuRB01b* allele) (Lydiate *et al.* 2014) and *B. napus* line Westar (homozygous for the *TuRB01* allele) were used as the resistance donor parents. The *B. juncea* line 060DH17 was used as the recurrent parent for backcrossing. Two rounds of backcrosses were made (Fig. 6.1). The artificial bud pollination followed the method described in section 2.1.2.

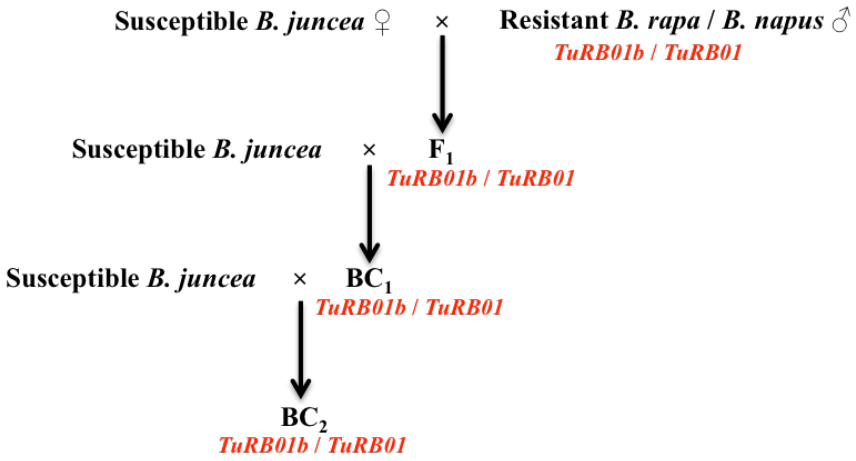


Figure 6.1 − Crossing strategy used to introgress *TuRB01*/*TuRB01b*-based resistance into the *Brassica juncea* line 060DH17 that is susceptible to Turnip mosaic virus. In each cross, the plant on the left is the female parent and the plant on the right is the male parent.

As for the interspecific crossing between TuMV-susceptible *B. nigra* and TuMV-resistant *B. rapa*, *B. nigra* line "al-1-3" (section 2.1.1, Table 2.1) and *B. rapa* line TD-R were used as the male and female parent, respectively. Two attempts at this cross were made. The first attempt did not use an embryo rescue technique. As this did not result in the production of any seed, the second attempt included an embryo rescue technique (Fig. 6.2).

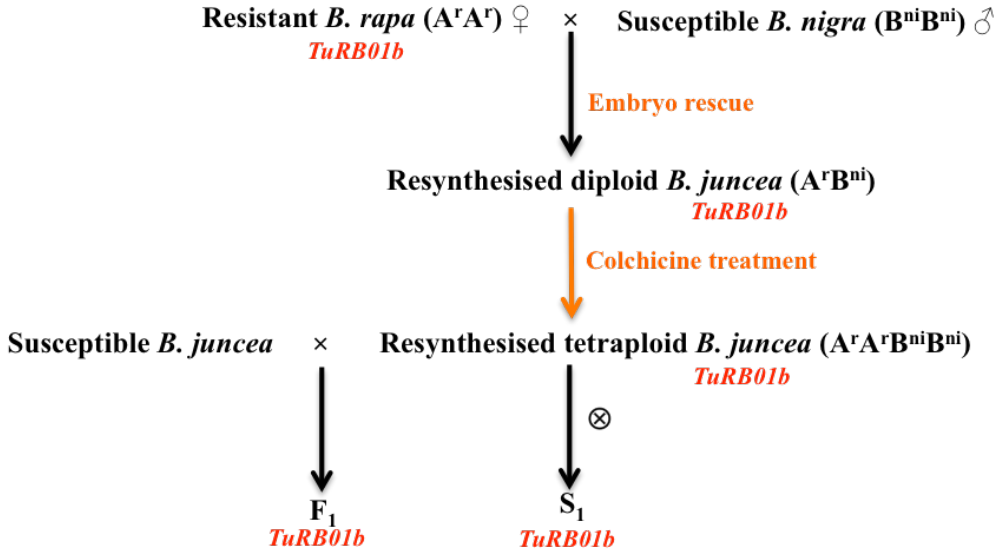


Figure 6.2 − Experimental procedure for the development of resynthesised *Brassica juncea* and progeny carrying *TuRB01b*. In each crossing, the plant on the left is the female parent and the plant on the right is the male parent. ⊗, self-pollination.

The experimental procedure included artificial bud pollination and transferring fertilised ovules onto a solidified MS medium (Fig. 6.3). The details of the techniques and methods used, including pollination, embryo rescue, colchicine treatment and plant cytogenetic analysis, were described in sections 2.6, 2.7 and 2.8.

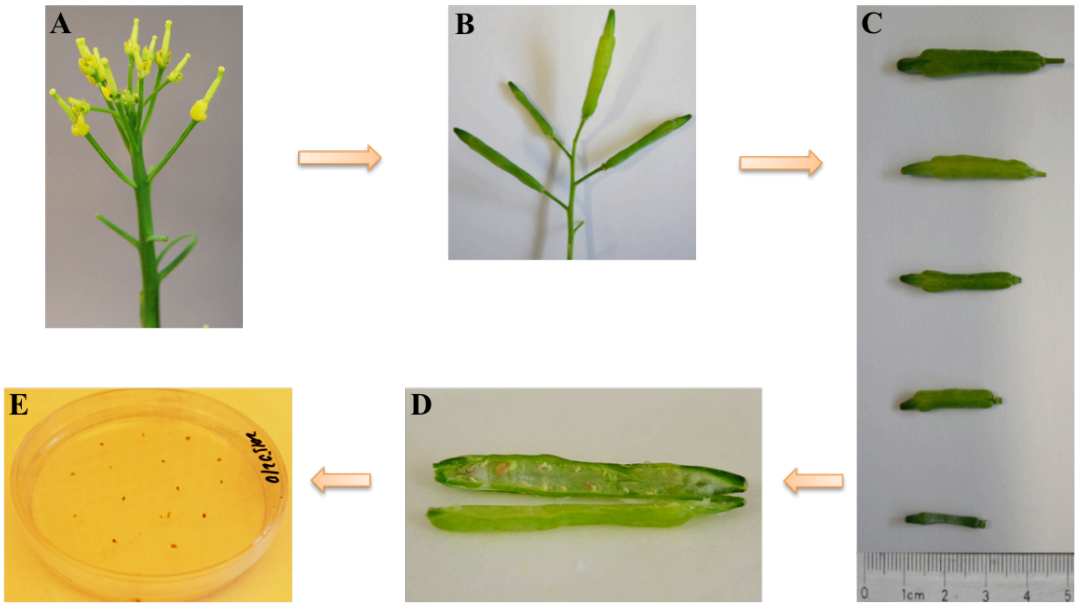


Figure 6.3 − Interspecific crossing between *Brassica rapa* and *Brassica nigra* from artificial bud pollination to fertilised ovule culture on a solidified MS medium. **A,** artificial bud pollination; **B,** fertilised siliques before embryo breakdown; **C,** different size of the fertilised siliques; **D,** fertilised ovules inside of siliques; **E,** culture of fertilised ovules on a solidified MS medium.

6.2.2 Cloning and sequencing of *BORG1* in *B. juncea* line 060DH17

Primer pairs BR138/BR158 and BR164/BR165 (section 2.4.2) were used to amplify *BORG1* by PCR. As the direct sequencing of PCR products did not give clean sequencing traces, cloning was carried out to sequence *BORG1* and bordering regions in the *B. juncea* line 060DH17. The TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, USA) was used to perform the cloning. The manufacturer's instructions were followed, including transforming chemically competent One Shot® *E. coli* cells (Invitrogen, Carlsbad, USA). The obtained sequences were analysed and assembled in the SeqMan software (DNASTAR Lasergene v10.1).

6.2.3 Sequence alignment and design of *BORG1*-specific primers

Both the nucleotide and amino acid sequences of *BORG1* alleles in *B. rapa*, *B. napus* and *B. juncea* were aligned using the software MegAlign (DNASTAR Lasergene v10.1). A primer pair specific for each allele was designed according to the differences between the alleles. Primer pairs for amplifying *BORG1* from both genomic DNA (gDNA) and complementary DNA (cDNA) (i.e. primer pairs detecting the presence and expression of specific *BORG1*) were designed. The details of primer design are described in section 2.4.2.

6.2.4 Selection of plants in different generations of the interspecific crossing

Stringent selections for *TuRB01*/*TuRB01b*-based resistance were made on the plants in different generations. Phenotypic analysis of plants was done by inoculating them with TuMV isolate UK 1 in order to select resistant plants; genetically, marker-based genetic testing using *BORG1*-specific primers was performed on both the gDNA and cDNA samples of plants. The primer pair BR205/BR206 was also used to detect the expression of the *RCH1*-like gene (section 5.2) in some plants in these generations. The sequences of these primers are shown in Table 2.3 and Table2.4 in section 2.4.2. The leaf samples for marker analysis were collected when the plants were at an early stage, prior to being challenged with TuMV. The genomic DNA and RNA extractions, RT-PCR and PCR were performed as described in sections 2.4.1, 2.4.4 and 2.4.5.

6.3 Results

6.3.1 Introgression of *TuRB01b* from *B. rapa* into *B. juncea*

For the interspecific crossing between *B. juncea* line 060DH17 and *B. rapa* line TD-R, approximately 180 F_1 hybrid seeds were obtained. Randomly selected seeds were planted and the plants tested for resistance to TuMV isolate UK 1. As a result, out of 19 F_1 plants that were inoculated, 18 plants were resistant (with phenotype "0", Fig. 6.4) and 1 plant was susceptible (with phenotype "$+_N$"). Both visual assessment and ELISA were carried out (Table 6.1). The infected leaves of the susceptible necrotic plant were ground up and back-inoculated to the *B. napus*

differential plant lines R4 and S6 (Jenner and Walsh, 1996). The result of the inoculation confirmed that the virus in the infected plant had not mutated to overcome *TuRB01b*.

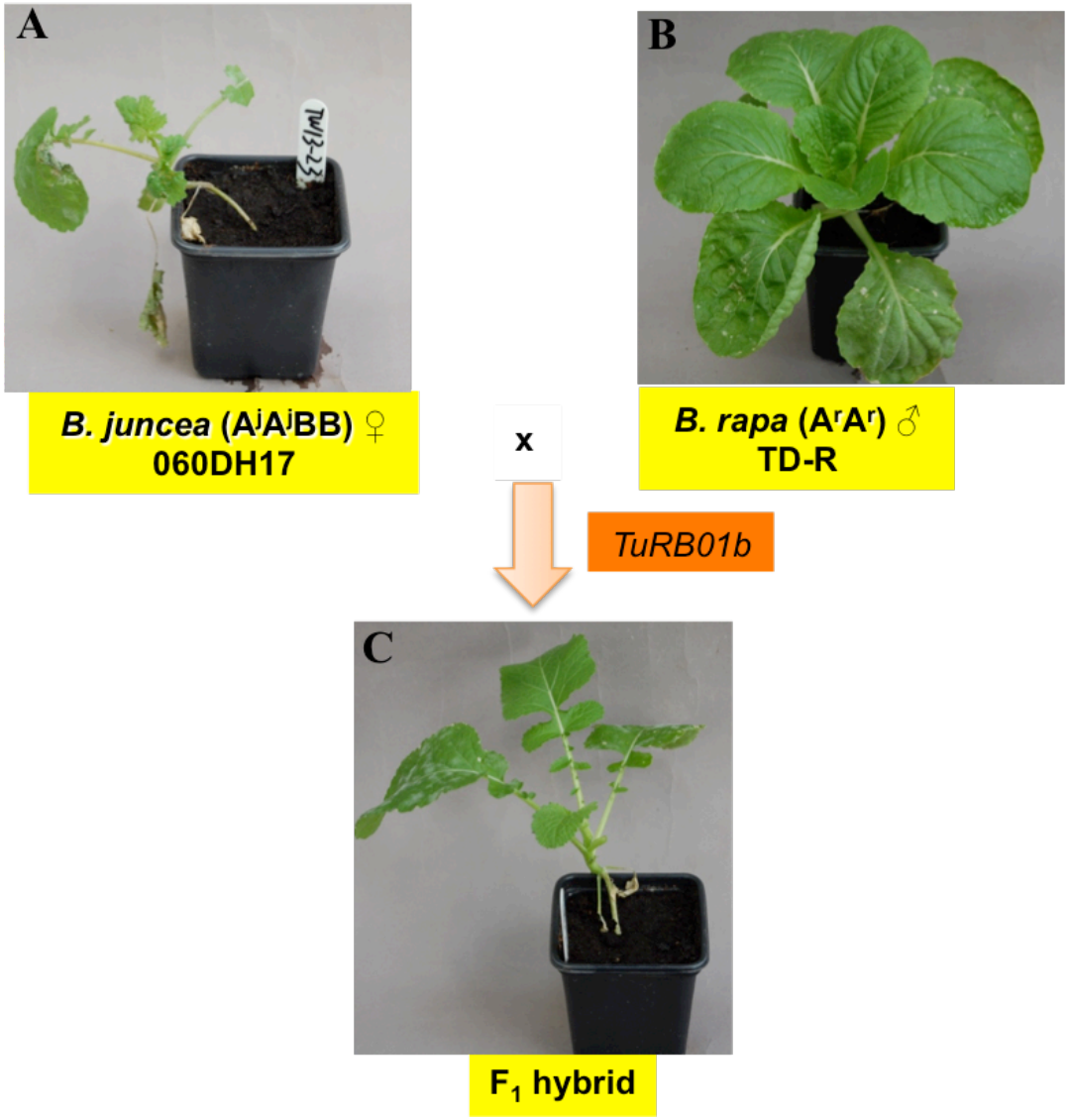


Figure 6.4 − Successful introgression of *TuRB01b* from *Brassica rapa* into F_1 hybrids through interspecific crossing. **A,** susceptible female parent *B. juncea* line 060DH17 (phenotype +); **B,** resistant male parent *B. rapa* line TD-R (phenotype 0); **C,** resistant F_1 hybrid (phenotype 0).

Table 6.1 − Phenotypes of F_1 and BC_1 plants generated from interspecific crossing, following challenge with Turnip mosaic virus isolate UK 1.

Original cross	Generation	No. TuMV-resistant plants / no. tested	Phenotype of resistant plants	Phenotype of susceptible plants
060DH17 × TD-R	F_1	18/19	0	$+_N$ [1]
	BC_1-1	3/11	0	+ [2]
	BC_1-2	2/7	0	+ / $+_N$
	BC_1-3	2/20	0	+
	BC_1-4	2/8	0	+
060DH17 × Westar	F_1	10/11	0	$+_N$
	BC_1	1/5	0	+

[1] $+_N$, systemic infection with necrosis, virus detected by ELISA in uninoculated leaves; [2] +, systemic infection without necrosis, virus detected by ELISA in uninoculated leaves.

Morphologically, the F_1 plants were intermediate to the parents (*B. juncea* and *B. rapa*) (Fig. 6.4). Ploidy level testing using flow cytometry was performed on 13 F_1 plants and their relative DNA contents were approximately half the sum of the relative DNA contents of the two parents (Table 6.2). This suggested that these plants were true F_1 hybrids generated from the interspecific crossing.

Table 6.2 − Result of ploidy level testing (using flow cytometry) of plants generated from interspecific crossing.

Plant line	**Relative DNA content**
B. rapa line TD-R	0.60
B. juncea line 060DH17	1.25
B. napus line Westar	1.37
F_1 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.90-0.96
F_1 from *B. juncea* (060DH17) × *B. napus* (Westar)	1.27-1.31

Several resistant F_1 plants were backcrossed to the recipient parent *B. juncea* line 060DH17 and BC_1 generations were developed. There were not many BC_1 seeds obtained due to the partial fertility of F_1 hybrids. Inoculation of TuMV isolate UK 1 revealed segregation of resistance in the BC_1 generation (Table 6.1). Resistant BC_1 plants were taken forward for further backcrossing to *B. juncea* line 060DH17, in order to produce BC_2 populations. In total, six BC_2 populations were developed from the interspecific crosses between *B. juncea* and *B. rapa*.

6.3.2 Introgression of *TuRB01* from *B. napus* into *B. juncea*

When the *B. juncea* line 060DH17 was crossed with the *B. napus* line Westar, approximately 80 F_1 seeds were obtained, a lower number of seeds compared to the interspecific cross with *B. rapa* line TD-R. The same experimental procedure was carried out as in section 6.3.1. Randomly selected F_1 seeds were planted and the plants tested for resistance to TuMV isolate UK 1. Out of 11 F_1 plants that were inoculated, 10 plants were resistant (with phenotype "0", Fig. 6.5) and 1 plant was susceptible (with phenotype "$+_N$"). Both visual assessment and ELISA were performed (Table 6.1).

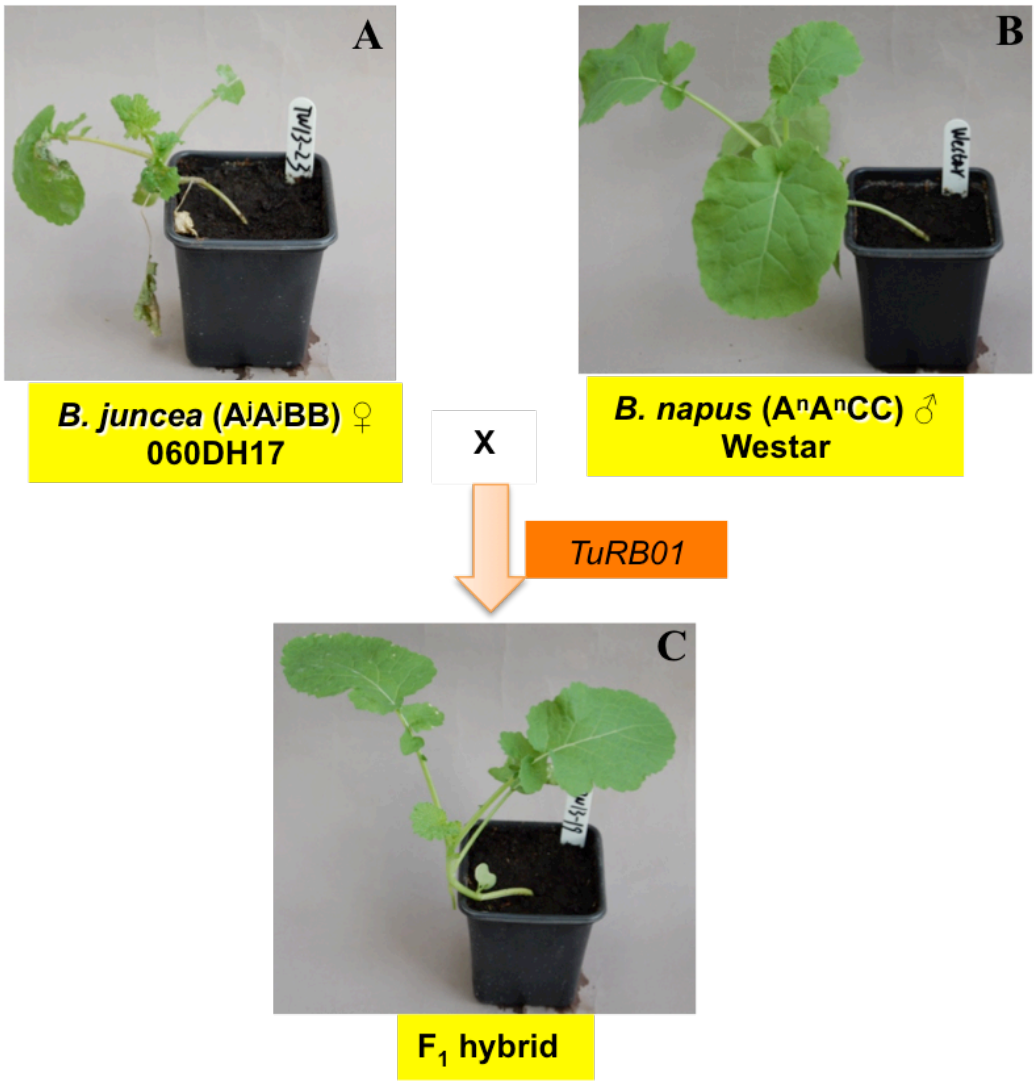


Figure 6.5 − Successful introgression of *TuRB01* from *Brassica napus* into F_1 hybrids through interspecific crossing. **A,** susceptible female parent *B. juncea* line 060DH17 (phenotype +); **B,** resistant male parent *B. napus* line Westar (phenotype 0); **C,** resistant F_1 hybrid (phenotype 0).

The F_1 hybrid plants were intermediate to the parents (*B. juncea* and *B. napus*) morphologically. Flow cytometry was performed on 9 F_1 plants and their relative DNA contents were intermediate to that of two parents (Table 6.2). This indicated their true hybrid nature generated through interspecific crossing.

Three resistant F_1 plants were backcrossed to *B. juncea* line 060DH17 and BC_1 generations were developed. The number of BC_1 seeds was poor. Five plants in this BC_1 population were challenged with TuMV isolate UK 1 and one plant was resistant (Table 6.1). The resistant BC_1 plant was backcrossed to *B. juncea* line 060DH17 and one BC_2 population was produced.

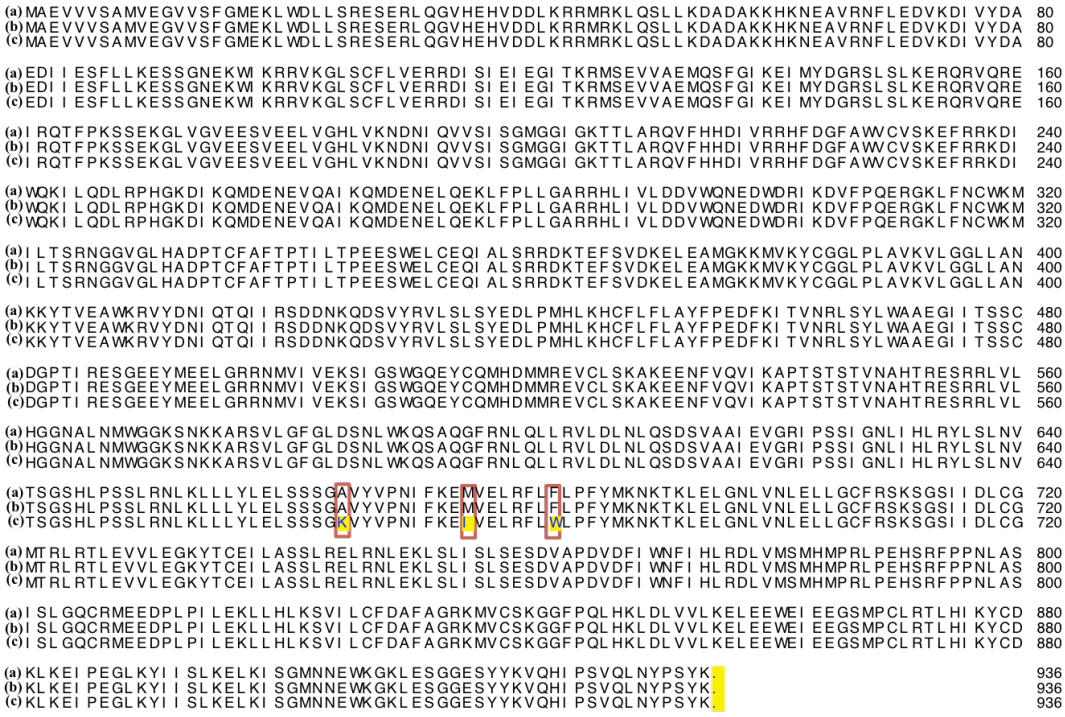
6.3.3 Differences between alleles at the *BORG1* locus in the TuMV-resistant *B. rapa*/*B. napus* and the TuMV-susceptible *B. juncea* lines and design of *BORG1*-specific primers

As described in chapter 5, the conventional *R* gene *BORG1* is the best candidate for the TuMV resistance gene *TuRB01*/*TuRB01b*. *BORG1* had been cloned previously and sequenced from the TuMV-resistant *B. rapa* line TD-R and *B. napus* line Westar in the plant-virus interactions group, University of Warwick (A. R. Baker, personal communication). The nucleotide sequence of *BORG1* is identical in these two lines. In this project, I cloned and sequenced the allele at the *BORG1* locus in the TuMV-susceptible *B. juncea* line 060DH17. The alignment of nucleotide sequences suggested that there were six nucleotide differences between alleles at the *BORG1* locus in *B. rapa*/*B. napus* and *B. juncea*. These differences were located in positions in the Exon 3 of *BORG1* (Fig. 6.6). The alignment of amino acid sequences revealed three non-synonymous mutations, A666K, M676I and F683W (Fig. 6.6A). The amino acid differences between *BORG1* in TuMV-resistant *B. rapa*/*B. napus* and allele at the *BORG1* locus in TuMV-susceptible *B. juncea* were located in the LRR (Leucine-rich repeat) domain of the gene, which is considered responsible for pathogen effector recognition in plants.

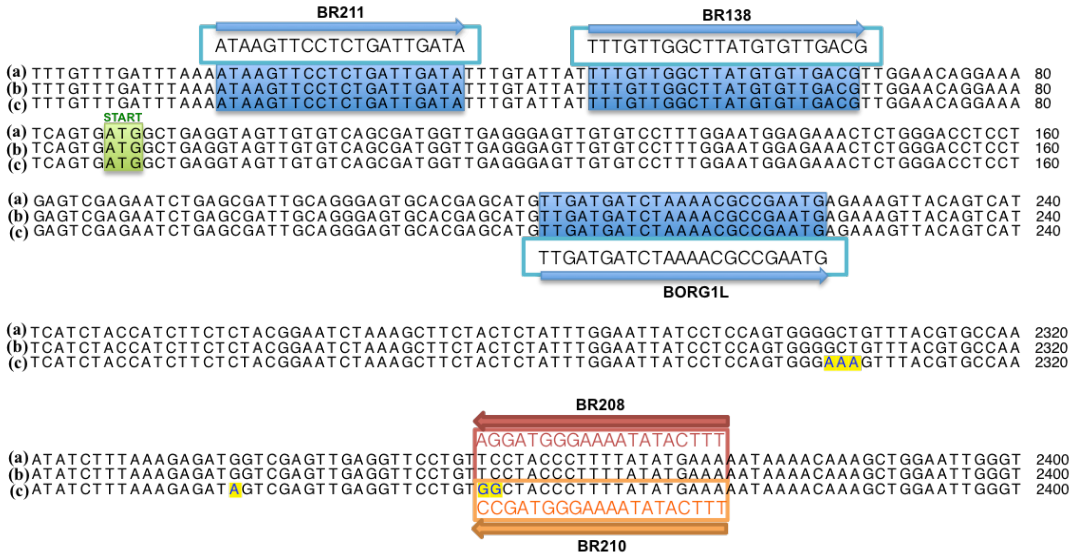
Specific primer pairs were designed for both versions of the alleles at the *BORG1* locus. The locations of primers are shown in Figure 6.6-B. The forward primers BR211 and BR138 were both located at the 5' UTR (untranslated region) of

BORG1. Due to the high homology between the *BORG1* and *BORG3* sequences (section 5.1.2), BR211 and BR138 were located in the *BORG1*-specific region to discriminate from *BORG3*. Another forward primer BORG1L was located in the Exon 1 of *BORG1* and was designed to specifically amplify *BORG1* from cDNA samples in order to detect the expression. The reverse primers BR208 and BR210 were located in the same position of Exon 3 of *BORG1* and differed from each other in the last two nucleotides ("GA" and "CC", the 5'-3' direction). BR208 was designed to be specific for *BORG1* in *B. rapa* and *B. napus*, whilst BR210 was specific for the allele at the *BORG1* locus in *B. juncea* (Table 6.3).

A



B



(a) *Brassica rapa* line TD-R; **(b)** *Brassica napus* line Westar; **(c)** *Brassica juncea* line 060DH17.

Figure 6.6 − A. Alignment of amino acid sequences of alleles at the *BORG1* locus in *Brassica rapa* line TD-R, *Brassica napus* line Westar and *Brassica juncea* line 060DH17. **B.** Locations of *BORG1*-specific primers in the nucleotide sequence. The positions containing sequence differences are highlighted in yellow.

(a) *Brassica rapa* line TD-R; **(b)** *Brassica napus* line Westar; **(c)** *Brassica juncea* line 060DH17.

Table 6.3 − Primer pairs specific for *BORG1* alleles in *Brassica rapa*/*Brassica. napus* and *Brassica juncea.*

Primer pair	Primer use
BR211/BR208	Amplification of *BORG1* in *B. rapa*/*B. napus* from gDNA
BR138/BR210	Amplification of *BORG1* in *B. juncea* from gDNA
BORG1L/BR208	Amplification of *BORG1* in *B. rapa*/*B. napus* from cDNA
BORG1L/BR210	Amplification of *BORG1* in *B. juncea* from cDNA

6.3.4 Genotyping of F_1 and BC_1 plants of interspecific crossing

The PCR conditions for *BORG1*-specific primer pairs (described in Table 6.3) were optimised using DNA samples of the three parental lines (*B. rapa* TD-R, *B. napus* Westar and *B. juncea* 060DH17) (Fig. 6.7). PCR amplifications using these primers were performed on selected F_1 and BC_1 plants from two interspecific crosses (060DH17 × TD-R and 060DH17 × Westar) (Table 6.4).

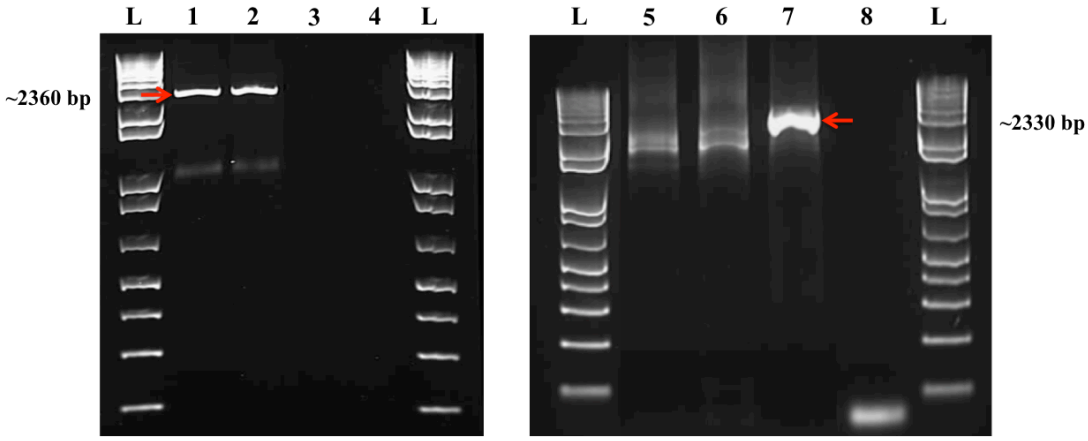


Figure 6.7 – PCR amplification of DNA samples from parental lines of interspecific crosses using *BORG1*-specific primers.
L, 1 Kb Plus DNA ladder; **1 and 5,** *Brassica rapa* line TD-R (R); **2 and 6,** *Brassica napus* line Westar (R); **3 and 7,** *Brassica juncea* line 060DH17 (S); **4 and 8,** ddH_2O (negative control). **1-4,** PCR using primer pair BR211/BR208; **5-8,** PCR using primer pair BR138/BR210. (R), resistant to Turnip mosaic virus isolate UK 1; (S), susceptible to Turnip mosaic virus isolate UK 1.

Table 6.4 – Genotyping of selected F_1 and BC_1 plants from interspecific crosses using within-gene specific molecular markers.

Original cross	Plant individual No.	Generation of plant individual	Pheno-type vs. UK 1	*B. rapa* (*B. napus*) *BORG1* presence	*B. rapa* (*B. napus*) *BORG1* expression	*B. juncea BORG1* presence	*B. juncea BORG1* expression	*RCH1*-like gene expression
060DH 17 × TD-R	1	F_1	0[1]	✔[2]	✔	✔	✔	✔
	2	F_1	0	✔	✔	✔	✔	✖[3]
	3	F_1	0	✔	✔	✔	✔	✖
	4	F_1	$+_N$[4]	✔	✔	✔	✔	✔
	5	BC_1	0	✔	✔	✔	✔	✔
	6	BC_1	0	✔	✔	✔	✔	✔
	7	BC_1	0	✔	✖	✔	✖	✔
	8	BC_1	+[5]	✖	✖	✔	✔	✔
	9	BC_1	+	✖	✖	✔	✖	✔
	10	BC_1	+	✖	✖	✔	✔	✔
060DH 17 × Westar	11	F_1	0	✔	✔	✔	✔	✔
	12	F_1	0	✔	✔	✔	✔	✔
	13	F_1	0	✔	✔	✔	✔	✔
	14	F_1	$+_N$	✔	✔	✔	✔	✔
	15	BC_1	0	✔	✔	✔	✔	✔
	16	BC_1	+	✖	✖	✔	✔	✔
	17	BC_1	+	✖	✖	✔	✔	✔
	18	BC_1	+	✖	✖	✔	✔	✔

[1] 0, no symptoms and no virus detected by ELISA; [2] ✔, presence of a specific band of PCR product; [3] ✖, absence of a specific band of PCR product; [4] $+_N$, systemic infection with necrosis, virus detected by ELISA in uninoculated leaves; [5] +, systemic infection without necrosis, virus detected by ELISA in uninoculated leaves;

PCR amplification using primer pair BR138/BR210 produced a specific PCR product in all the F_1 and BC_1 plants tested, indicating that the *B. juncea* allele at the *BORG1* locus was present in the genomes of these plants. The results of PCR amplification using primer pair BR211/BR208 suggested that the resistant plants tested all possessed the *B. rapa*/*B. napus* allele of *BORG1*, which was absent in susceptible plants with the "+" phenotype. This genotyping correlated with the phenotyping results. As for the PCR amplification detecting the expression of the specific version of *BORG1*, the correlation to individual phenotypes was present in the tested plants apart from plants No. 7 and No. 9, where neither primer pair (BORG1L/BR208 or BORG1L/BR210) produced a PCR product.

The results showed that plants No.4 and No.14 (two F_1 hybrids with the "$+_N$" phenotype) possessed and expressed *BORG1* and the *B. juncea* allele at the *BORG1* locus, as was seen in a typical F_1 hybrid that was resistant (phenotype "0") to TuMV isolate UK 1. The authenticity of UK 1 in plants No.4 and No.7 and lack of any mutation to overcome *TuRB01* were proven by the result of back-inoculation to the *B. napus* line R4 possessing *TuRB01* (section 6.3.1).

The expression of the *RCH1*-like gene that is adjacent to *BORG1* was also investigated. This gene was expressed in all the plants tested apart from plants No. 2 and No. 3.

6.3.5 Development of resynthesised *B. juncea* for introgression of *TuRB01b* by crossing *B. rapa* with *B. nigra*

For interspecific crossing between the *B. rapa* line TD-R and the *B. nigra* line al-1-3, in the first attempt, six groups of inflorescences on one TD-R plant were bud-pollinated by pollen from *B. nigra* line al-1-3. Although fertilised ovules started to develop, they degenerated and all the siliques were empty when harvested. This was due to the abortion of hybrid embryos and abnormal development of endosperms (section 1.7.1). Therefore, it was necessary to use the embryo rescue technique, so a second attempt was carried out using embryo rescue. The germination rate of the fertilised ovules in this experiment was over 10% and 48 germinated ovules were obtained (Table 6.5; Fig. 6.8).

Table 6.5 – Fertilisation and ovule germination of the interspecific crossing between *Brassica rapa* line TD-R and *Brassica nigra* line al-1-3.

Number of pollinated flower buds	Number of siliques	Percentage of siliques from which fertilised ovules obtained	Number of ovules transferred to culture medium	Number of ovules geminated	Germination rate
518	140	27%	424	48	11.3%

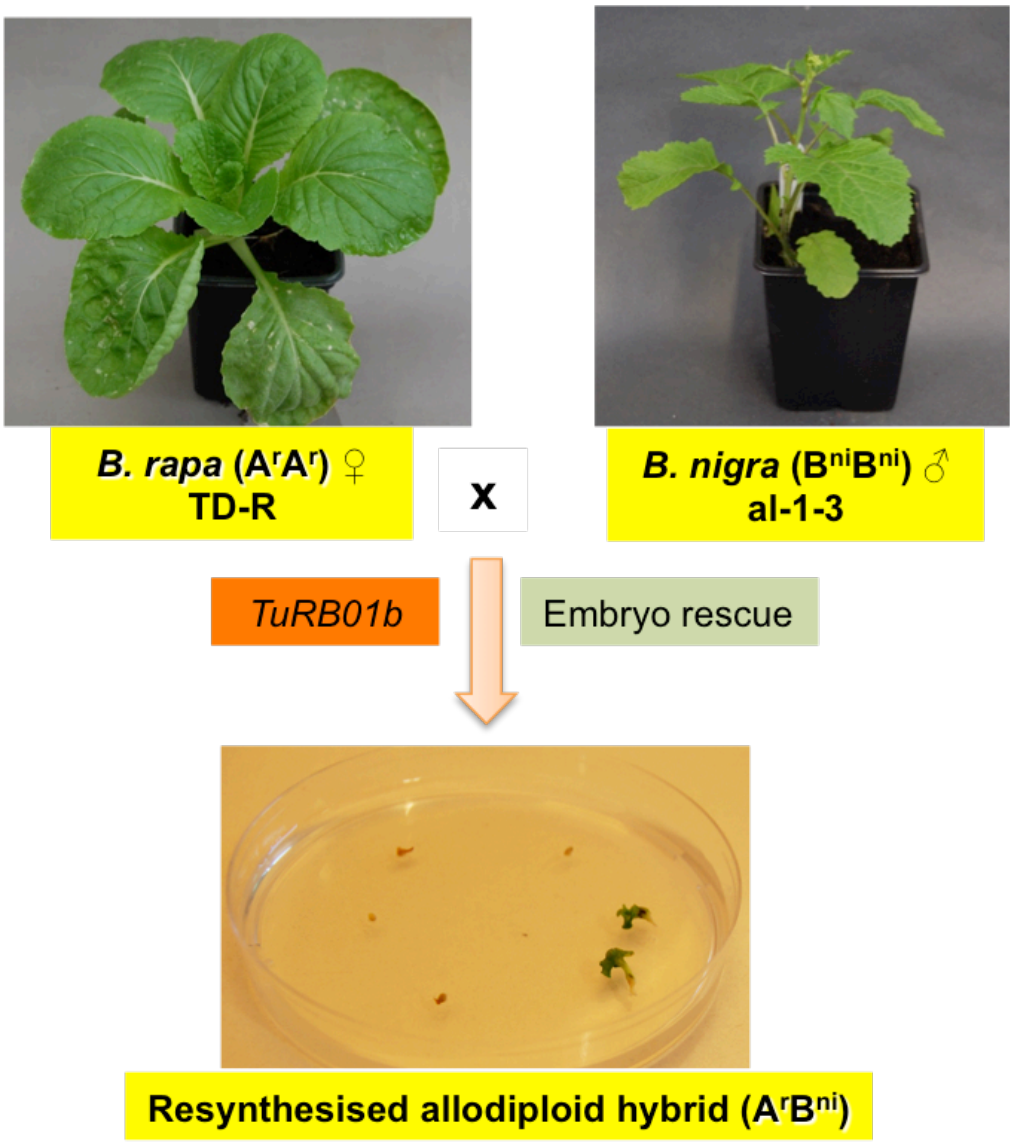


Figure 6.8 – Germination of fertilised ovules (generated from the interspecific crossing between the *Brassica rapa* line TD-R and *Brassica nigra* line al-1-3) on a solidified MS medium.

The germinated seedlings were separated on the same MS medium and then transplanted onto a root-induction medium (Fig. 6.9B; section 2.6.2). The rooted plants were then transplanted into M2 compost and kept in plant propagators for two weeks for acclimation (Fig. 6.9C). After this, the plants were moved to the glasshouse to grow under normal conditions (Fig. 6.9D).

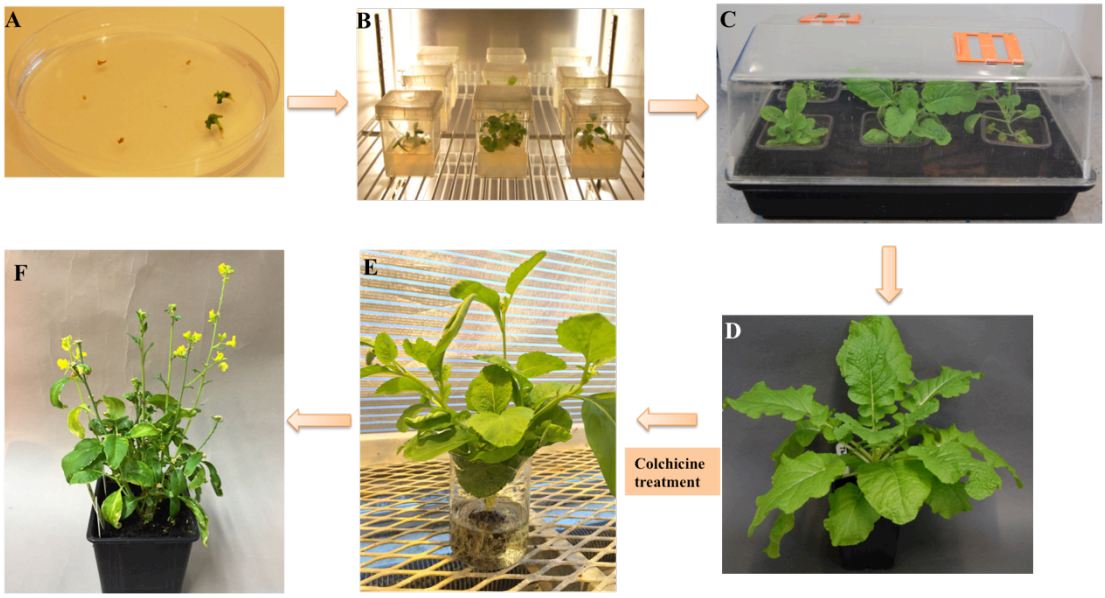


Figure 6.9 – Different stages of plants in the process of resynthesis and *Brassica juncea* development. **A,** germination of fertilised ovules; **B,** root-induction of the germinated seedlings; **C,** acclimation of resynthesised plants in plant propagator; **D,** resynthesised allodiploid hybrid growing in the glasshouse; **E,** colchicine treatment of the allodiploid hybrid; **F,** resynthesised *Brassica juncea* plant with the allotetraploid genome AABB.

The F_1 hybrid plants resembled normal *B. juncea* and had a morphology intermediate between that of the two parents. The flow cytometry result suggested that the relative DNA content of F_1 plants was intermediate between that of the parent species (Table 6.6), indicating that they were true hybrids, namely resynthesised allodiploid hybrid with the expected 'AB' genomic constitution. In addition, this was confirmed by the GISH result which showed that the F_1 hybrid possessed 18 chromosomes, including 10 chromosomes from *B. rapa* 'A" genome and 8 chromosomes from *B. nigra* 'B' genome (Fig. 6.10). The resynthesised allodiploid hybrids were male-sterile and produced no pollen (Fig. 6.11A, B). No spontaneous chromosomal doubling was seen in these plants, therefore, colchicine treatment was performed for polyploidisation.

Colchicine treatment of leaf axils (section 2.7.1) was performed on 6 resynthsised allodiploid hybrid plants. No polyploidisation was induced and no pollen was produced. However, colchicine treatment of roots (section 2.7.2; Fig. 6.9E) induced chromosomal doubling. Three out of four treated plants were male-fertile and produced pollen (Fig. 6.11C, D). One plant was confirmed to be tetraploid *B.*

juncea (with genome 'AABB') by the results of both flow cytometry (Table 6.6) and GISH (Fig. 6.10). Both resynthesised allodiploid hybrids and resynthesised tetraploid *B. juncea* plants were propagated vegetatively through the method outlined in section 2.6.4.

Table 6.6 − Ploidy level testing (using flow cytometry) of the plants generated from the interspecific cross between *Brassica rapa* and *Brassica nigra*.

Plant line	Relative DNA content
B. rapa line TD-R	0.60
B. nigra line al-1-3	0.69
Resynthsised allodiploid hybrid	0.65-0.66
Resynthsised tetraploid *B. juncea*	1.30

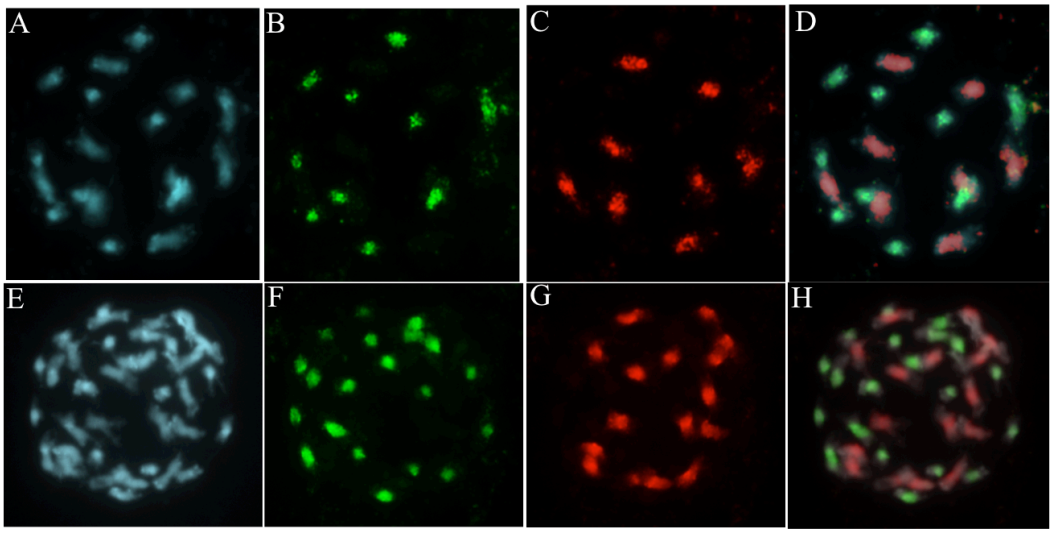


Figure 6.10 − Bicolor GISH with two genomic probes of *Brassica rapa* 'A' (green) and *Brassica nigra* 'B' (red) at mitotic metaphase of the resynthesised allodiploid hybrid (**A-D**) and resynthesised tetraploid *Brassica juncea* (**E-H**). Counterstaining was applied with DAPI (blue). Courtesy of Zeeshan Shamim and Dr. Susan Armstrong, Birmingham University.

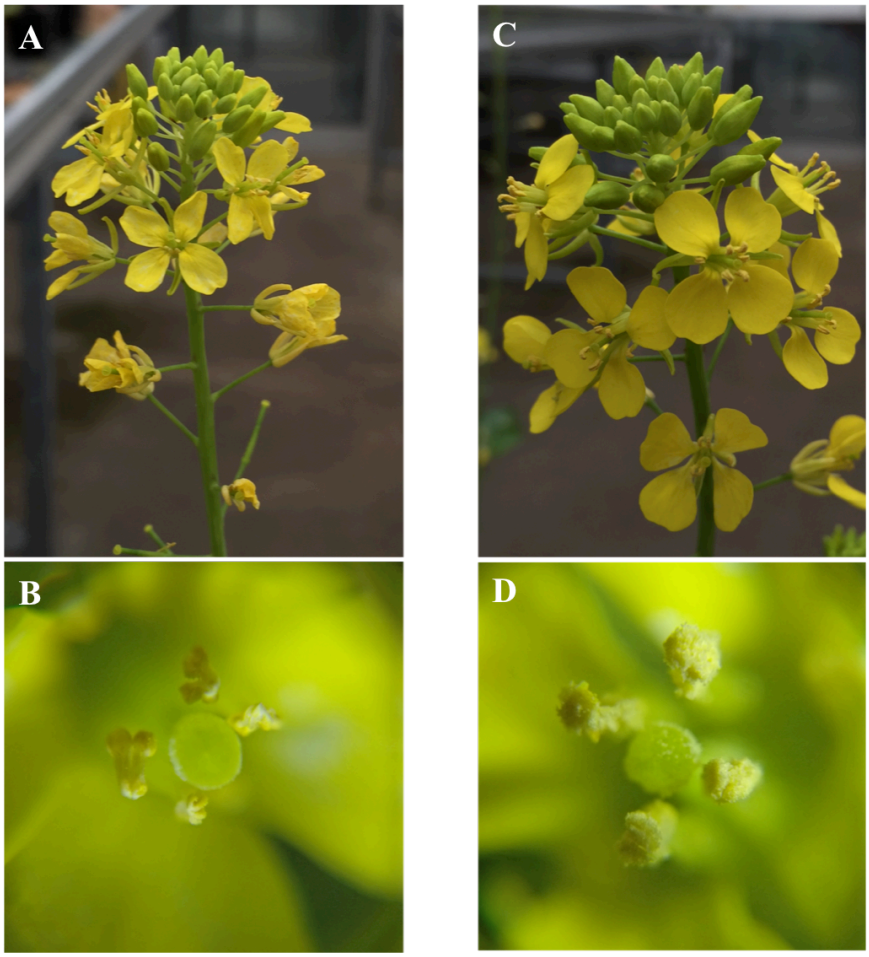


Figure 6.11 − Inflorescences and flowers of resynthesised allodiploid hybrid (**A and B**) and resynthesised tetraploid *Brassica juncea* (**C and D**). Pictures **B and D**, courtesy of Lawrence Bramham, University of Warwick.

As indicated in the experimental procedure shown in Figure 6.2, the resynthesised tetraploid *B. juncea* plants were self-pollinated. Several attempts at bud pollination were carried out, but no selfed seeds were obtained. Following self-pollination, ovules were fertilised and started to develop. However, they aborted towards harvest time and all the siliques were empty.

The resynthesised tetraploid *B. juncea* plants were crossed to the TuMV-susceptible *B. juncea* line 060DH17. This cross-pollination was successful and F_1 hybrid seeds were obtained.

6.3.6 Testing resynthesised *B. juncea* plants for TuMV resistance

Both the resythesised allodiploid hybrids and resynthesised tetraploid *B. juncea*

plants were tested for resistance to the TuMV isolates UK 1 and vVIR24. The results indicated typical *TuRB01b*-based resistance was present in both lines, as they were highly resistant to UK 1, whilst the resistance was overcome by vVIR24 and resulted in the "$+_N$" phenotype (Fig. 6.12). These phenotyping results suggested that *TuRB01b* was present in the resynthesised *B. juncea* plants.

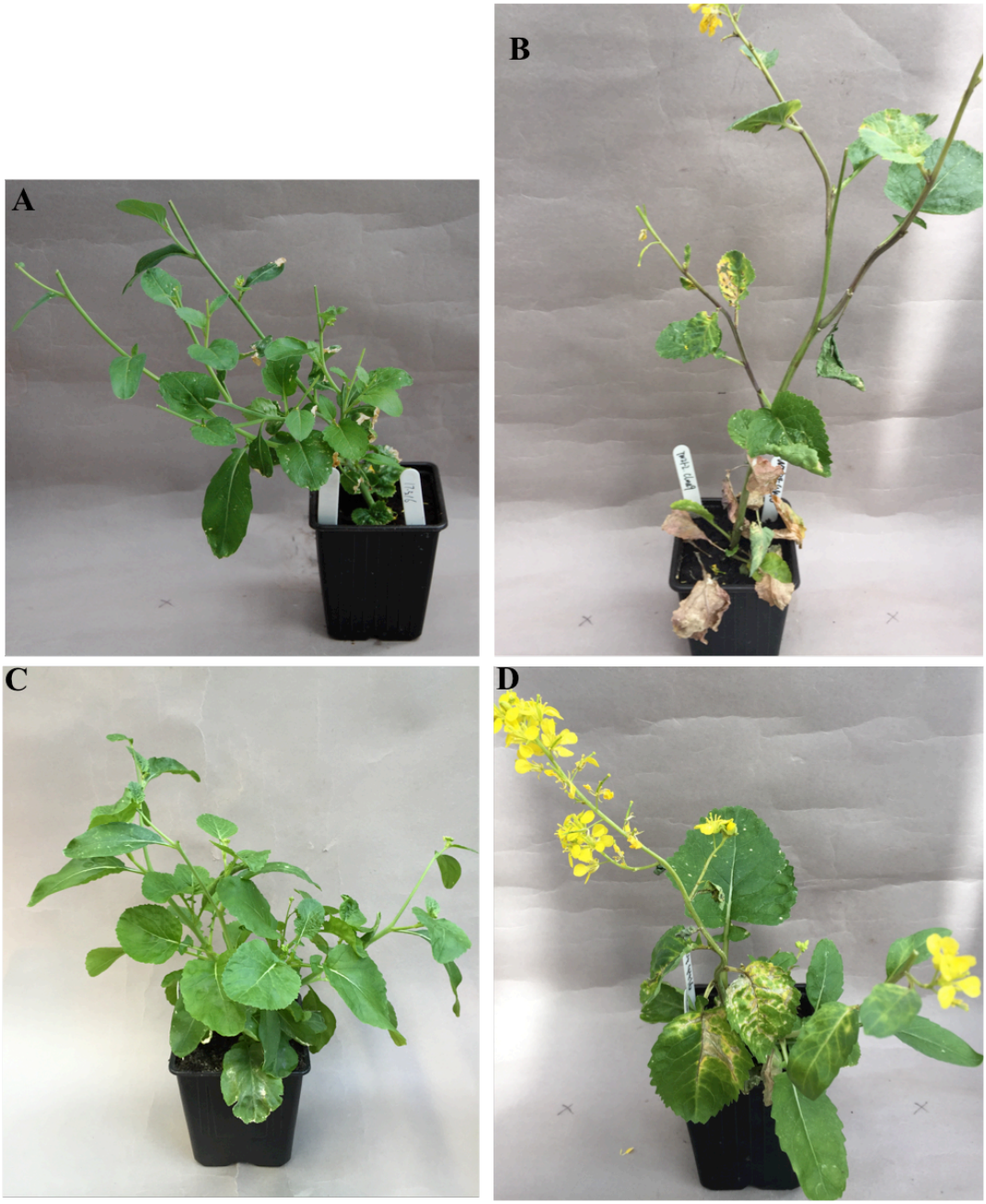


Figure 6.12 – Phenotypes of the resynthesised *Brassica juncea* plants following challenge with Turnip mosaic virus isolates UK 1 and vVIR24. **A,** resynthesised allodiploid hybrid challenged with UK 1 (phenotype 0); **B,** resynthesised allodiploid hybrid challenged with vVIR24 (phenotype $+_N$); **C,** resynthesised tetraploid *Brassica juncea* challenged with UK 1 (phenotype 0); **D,** resynthesised tetraploid *Brassica juncea* challenged with vVIR24 (phenotype $+_N$).

Genotyping using PCR was also performed on the resynthesised *B. juncea* plants, using the primer pair BR211/BR208 (specific for *BORG1* in TuMV-resistant *B. rapa*/*B. napus*) (Fig. 6.13). The result indicated that *BORG1* was present in the resynthesised allodiploid hybrids and resynthesised tetraploid *B. juncea* plant.

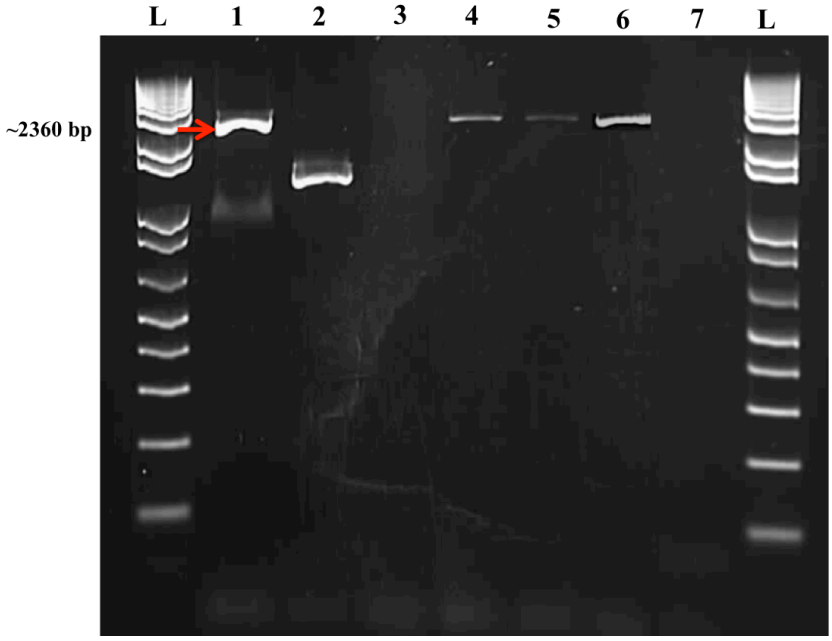


Figure 6.13 – PCR amplification of *BORG1* in parental lines and resynthesised plants, using specific primer pair BR211/BR208.
L, 1 Kb Plus DNA ladder; **1,** *Brassica rapa* line TD-R (R); **2,** *Brassica nigra* line al-1-3 (S); **3,** *Brassica juncea* line 060DH17 (S); **4,** resynthesised allodiploid hybrid plant No.1 (R); **5,** resynthesised allodiploid plant No. 2 (R); **6,** resynthesised tetraploid *Brassica juncea* plant (R); **7,** ddH_2O (negative control). (R), resistant to Turnip mosaic virus isolate UK 1; (S), susceptible to Turnip mosaic virus isolate UK 1.

6.4 Discussion

6.4.1 Three approaches of interspecific crossing for introgression of *TuRB01*/*TuRB01b*-based resistance into *B. juncea*

Plant interspecific hybridisation is an important tool for elucidating inter-genomic associations, transferring beneficial characteristics and developing resynthesised amphidiploids (Choudhary *et al*., 2002). There have been extensive investigations on the compatibility and introgression amongst *Brassica* species. *B. juncea* and *B. rapa* are cross-compatible and a few successful interspecific hybridisations between

them have been reported (Ramanujam and Srinivasachar, 1943; Olsson 1960; Mohapatra and Bajaj, 1988; Sharma and Singh, 1992; Choudhary *et al*., 2002). Hybrids can be obtained by bud pollination or other conventional methods without using *in vitro* techniques. These studies suggested that using *B. juncea* as the female parent was more effective than the other direction of cross-pollination. Different studies also suggested that an interspecific hybrid between *B. juncea* and *B. napus* could be easily produced by bud pollination (Rao and Shivanna, 1997; Choudhary and Joshi, 1999; Mason *et al*., 2011; Tsuda *et al*., 2011). Moreover, of all the *Brassica* species, *B. juncea* is recognised as having the second highest crossability with *B. napus*, second only to *B. rapa*. *B. rapa* and *B. juncea* have been the major recipients of introgression from *B. napus* (Tsuda *et al.*, 2014).

This chapter described attempts to introgress the *TuRB01*/*TuRB01b*-based TuMV resistance from *B. napus* and *B. rapa* into *B. juncea*. To achieve this, three approaches of interspecific crossing were used. The first two approaches were crossing TuMV-resistant *B. rapa* (possessing *TuRB01b*) and *B. napus* (possessing *TuRB01*) lines to a TuMV-susceptible *B. juncea* line using bud pollination. Plants were produced for F_1 generations through to BC_2 generations, without using *in vitro* technique. The *TuRB01*/*TuRB01b*-based TuMV resistance has been progressively introgressed into the *B. juncea* background, assisted by the phenotypic and genotypic selection.

The third approach involved producing resynthesised *B. juncea* by crossing a TuMV-susceptible *B. nigra* line to a TuMV-resistant *B. rapa* line possessing *TuRB01b*. In previous studies, *B. rapa* was the cytoplasmic donor in most of the normal *B. juncea* varieties. Also resynthesised hybrids between *B. rapa* and *B. nigra* were obtained at a relatively higher frequency when *B. rapa* was used as the female parent (Bhat and Sarla, 2004; Bansal *et al.*, 2009; Ghani *et al.*, 2014). Based on this, the *B. rapa* line TD-R was used as the female parent for crossing. No hybrid seeds were obtained as embryo degeneration occurred after bud pollination. Therefore, in order to overcome the incompatibility barrier, an embryo rescue technique was used to culture fertilised ovules. Rescued allodiploids (with 'AB' genome) were obtained. The anthers of allodiploids were small and did not produce any pollen. Chromosomal doubling was successfully induced by colchicine

treatment of the roots of allodiploids. The resynthesised tetraploid *B. juncea* plants (with 'AABB' genome) showed well-developed anthers with viable pollen. Both the resythesised allodiploid hybrids and resynthesised tetraploid *B. juncea* plants were highly resistant to TuMV UK 1 (with the phenotype "0") and susceptible to vVIR24 (with the phenotype "$+_N$"), indicating the plants possessed *TuRB01b*-based resistance.

Comparing these three approaches of interspecific crossing, the crossing between *B. juncea* and *B. rapa* seems to be the most efficient one as it has produced six BC_2 populations. As the time taken for each generation (plant growth, flowering, seed set etc.) was considerable, there was not enough time to develop further generations to increase the stability of the *TuRB01*/*TuRB01b*-based resistance in *B. juncea*. However, it reinforced the viability of these approaches for introgression of other TuMV resistance genes into *B. juncea*.

6.4.2 Genetic variation in plant interspecific hybridisation

The interspecific hybridisation has contributed significantly to the genetic enrichment of many crops. It not only incorporates desirable genes, but also at the same time provides great opportunities for generating additional variability and broadening the gene pool. Many novel and valuable variants have been selected from the interspecific hybrids of brassicas (Lee and Namai, 1994; Choudhary *et al*., 2004; Ghani *et al.*, 2014; Zhang *et al*., 2015). In particular, the resynthesised amphidiploid plants significantly increase the range of genetic variability whereby both interspecific hybridisation and genome doubling result in extensive genomic changes (Ghani *et al.*, 2014). The genomic changes during interspecific hybridisation can be generated either by large chromosome fragment rearrangements (genome reshuffling), or minor genomic alterations such as short stretch deletion and insertion (Szadkowski *et al*., 2010; Zhang *et al*., 2015).

Genetic variation is essential for developing new crop varieties, which is very important to global agriculture. However, crop genetic diversity is currently undergoing a sharp decline. One reason for this situation is the highly homogeneous nature of commercial seeds, which hinders new traits from arising or being adopted

to develop new varieties. The use of the interspecific hybridisation has the potential to improve this situation. Using the combination of interspecific hybridisation and backcrossing, Zhang *et al*. (2015) developed resynthesised *B. napus* and its backcross progenies conferring multiple novel phenotypes within the *B. rapa* and *B. olearacea* species.

The cultivars of *B. juncea* arose from a narrow genetic pool, which constricts the improvements of the crop through breeding. The development of resynthsised *B. juncea* enhances the genetic variation and broadens the gene pool. The plant materials developed in this study might be useful in future *B. juncea* breeding programs.

6.4.3 Marker-assisted selection in gene transfer

As described in section 1.6.3, marker-assisted selection is a very important technique in plant breeding. It can track the desirable gene(s) closely and speed up the introgression of beneficial characteristics. An adequate marker system and reliable markers are crucial to a functional marker-assisted breeding program. The molecular markers should have the attributes of simplicity and low-cost of use and analysis, small amount of DNA required, co-dominance and reproducibility of results. In addition, the marker should be located in close proximity to the target gene, or ideally present within the gene. Nowadays, marker-assisted backcrossing is regarded as the most widely and successfully used method in practical molecular breeding (Jiang, 2013).

BORG1 specific primers have been designed according the sequences of *BORG1* and other allele at this locus, and they were within-gene molecular markers. Moreover, in addition to the genetic testing of the specific allele at *BORG1* locus on gDNA samples, cDNA samples were also analysed, which revealed the expression pattern of *BORG1* in different plants. This genetic testing can be easily implemented by PCR. The PCR amplifications using primer pairs BR211/BR208 and BR138/BR210 (used on gDNA samples) produced clear results in terms of either presence or absence of the PCR band. The results were repeatable and showed consistent correlation to the phenotype of individual plants (Table 6.4). In

comparison, the primer pairs BORG1L/BR208 and BORG1L/BR210 (used on cDNA samples) were less efficient. The PCR amplifications using these two primer pairs produced very weak PCR bands in some plants and the results were not always consistent. In Table 6.4, neither of these two primer pairs produced a PCR product for plants No. 7 or No. 9, indicating no expression of alleles at the *BORG1* locus. The result of these genetic tests did not correlate to the phenotype of these two plants. This might have resulted from recombination occurring in this region, otherwise it is likely that the PCR conditions for these two primer pairs needs further optimisation. Further study is needed in this area. Generally speaking, the *BORG1* within-gene specific markers could be suitable and stringent for marker-assisted selection and they could be tested on further populations.

6.5 Conclusions

The main aim of the research described in this chapter was to introgress *TuRB01*/*TuRB01b*-based TuMV resistance from *B. napus* and *B. rapa* into *B. juncea* through interspecific hybridisation. For the crossing between TuMV-resistant *B. napus* / *B. rapa* (possessing *TuRB01*/*TuRB01b*) and TuMV-susceptible *B. juncea*, BC_2 generations were produced. The *TuRB01*/*TuRB01b*-based TuMV resistance has been progressively introgressed into the *B. juncea* background, assisted by the phenotypic and genotypic selection. For the crossing between TuMV-resistant *B. rapa* (possessing *TuRB01b*) and TuMV-susceptible *B. nigra*, resynthesised *B. juncea* (possessing *TuRB01b*) was developed using embryo rescue and polyploidy induction techniques. F_1 progeny of resynthesised *B. juncea* was obtained. *TuRB01*/*TuRB01b* specific within-gene molecular markers were designed and tested. A program of marker-assisted selection for *TuRB01*/*TuRB01b* transfer was established.

Chapter 7

General Discussion

Brassica juncea is an economically important crop. Apart from various vegetable uses, it is a well-known oilseed crop. *B. juncea* is the predominant oilseed crop in India (Chen *et al*., 2013). It was the third brassica oilseed crop to be developed as a canola crop, after *B. napus* and *B. rapa*. It exhibits better drought and heat tolerance and higher pod shatter resistance compared to *B. napus* and *B. rapa* (Woods *et al*., 1991; Burton *et al*., 1999). Because of its superior adaptation to the semi-arid conditions, canola-quality *B. juncea* is considered to be able to extend the commercial canola production where the productivity of *B. napus* and *B. rapa* is limited (Miller *et al*., 2003; Le *et al*., 2014). TuMV was considered to be the second most important virus infecting field vegetables (Tomlinson, 1987). It is particularly damaging to brassicas in parts of Asia, North America and Europe (Walsh and Jenner, 2002). The majority of *B. juncea* cultivars are very susceptible to TuMV, resulting in severe losses (Nyalugwe *et al*., 2014). The exploitation of natural, durable resistance to TuMV in *B. juncea* would be the most cost-effective and reliable approach to disease control. There has been very limited research on the TuMV resistance in *B. juncea* and no robust resistance has been reported yet.

This PhD project attempted to identify and exploit the TuMV resistance in *B. juncea* following two approaches. The first way was to directly seek natural resistance to TuMV in *B. juncea*, followed by characterisation and mapping of such TuMV resistances. The second way was to investigate the introgression of well-characterised resistance genes from closely related *Brassica* species into *B. juncea*.

This study has led to the following conclusions:

1. Natural resistance to TuMV exists in *B. juncea* varieties. The TuMV resistances in *B. juncea* lines TWBJ14 and TWBJ20 both confer broad-spectrum resistance, as they are highly effective against TuMV isolates UK

1, vVIR24 and CDN 1, representing the major pathotypes 1, 3 and 4, respectively.

2. According to the phenotype of the F_1 generations and the phenotypic segregations of the BC_1 and F_2 generations, the TuMV resistances in both TWBJ14 and TWBJ20 fit a Mendelian model based on the action of two recessive genes.
3. There were three phenotypes (0, + and $+_N$) in TWBJ14 BC_1 and F_2 populations, in comparison to the two phenotypes (0 and $+_N$) in TWBJ20 BC_1 population. This suggested there were different resistance genes involved in these two resistant lines. Additionally, the result of the complementation test indicated at least one resistance gene was not shared between TWBJ14 and TWBJ20.
4. For the TuMV resistance in the *B. juncea* line TWBJ14, two QTL were mapped on chromosomes A02 and A06. It was an additive effect between these two QTL and together they accounted for 48.9% of the total phenotypic variation.
5. For the TuMV resistance in the *B. juncea* line TWBJ20, two QTL were mapped on chromosomes A06 and A08. It was again an additive effect between these two QTL and together they accounted for 76.9% of the total phenotypic variation.
6. From the positions of the mapped QTL, three potential candidate *eIF* genes were identified for the TuMV resistance in line TWBJ14. Four potential candidate *eIF* genes were identified for the TuMV resistance in line TWBJ20.
7. For the necrotic hypersensitive response to TuMV infection in the BC_1 population of *B. juncea* line TWBJ14, one significant QTL was mapped on chromosome A06, accounting for 45.3% of the total phenotypic variation.
8. *BORG2*, a candidate for TuMV resistance gene *TuRB01*/*TuRB01b* in *B. napus* and *B. rapa* respectively was ruled out.
9. Introgression of *TuRB01*/*TuRB01b*-based TuMV resistance into *B. juncea* was initiated, using interspecific crosses between TuMV-resistant *B. napus*/*B. rapa* (possessing *TuRB01*/*TuRB01b*) and TuMV-susceptible *B. juncea*. BC_2 generations have been produced to date.

10. Resynthesised *B. juncea* (possessing *TuRB01b*) was produced from interspecific crossing between TuMV-resistant *B. rapa* (possessing *TuRB01b*) and TuMV-susceptible *B. nigra*, using the embryo rescue and polyploidy induction techniques.
11. Within-gene molecular markers specific for *TuRB01*/*TuRB01b* were designed and tested. Molecular markers were developed for marker-assisted selection to introgress *TuRB01*/*TuRB01b* into *B. juncea*.

7.1 TuMV resistance in *Brassica* species

7.1.1 The distribution of TuMV resistance in three *Brassica* genomes

As described in section 1.5.2, both dominant and recessive resistances to TuMV have been found and characterised in *B. rapa* and *B. napus* in the past. Almost all the resistance genes mapped to date are located in the brassica 'A' genome, with the exception of one resistance gene, *TuRB02* (conferring weak and quantitative resistance) mapped to the 'C' genome of *B. napus* (Walsh *et al*., 1999). Previous studies on the testing of *B. oleracea* for TuMV resistance have covered a broad diversity of genotypes, but no major sources of resistance were identified in *B. oleracea* (Walsh and Jenner, 2002).

In earlier studies (Kehoe *et al*., 2010) and my study on TuMV resistance in *B. nigra* in this thesis, no sources of TuMV resistance have been identified in *B. nigra*. Even though the number of *B. nigra* lines tested to date is considerably less than the number of *B. oleracea* lines that were tested, it is tempting to speculate that the TuMV resistance is scarce in the 'B' genome, as it is for the 'C' genome. Although it was reported that TuMV resistance was found in *B. carinata* (Nyalugwe *et al*., 2014), the TuMV isolate used in that study belonged to pathotype 8, which is not a prevalent pathotype.

As the resistance to TuMV isolate UK 1 was found in eight *B. juncea* lines in my study, it is possible that some resistances are from the 'B' genome of *B. juncea*. These sources of TuMV resistance in *B. juncea* are very important. However,

further screening of *B. nigra* lines and initiating resistance testing of *B. carinata* for TuMV resistance could be interesting.

7.1.2 Genetic mapping of TuMV resistance in the resistant *B. juncea* lines

In Chapter 4, the Illumina Infinium Chip designed for *B. napus* ('A' and 'C' genomes) was used for the QTL mapping of TuMV resistance genes in *B. juncea* lines TWBJ14 and TWBJ20. The limitation of this method is that there is no reference 'B' genome information and we cannot interpret the SNPs information for the 'B' genome. However, between the three *Brassica* ancestral genomes, the homology between the 'A' and 'B' genomes is significantly less than that between the 'A' and 'C' genomes (Navabi *et al*., 2013). This was also indicated by my genotypic data in which the call rates of the *B. juncea* lines on the *B. napus* chip were low (around 54%), possibly due to the lack of hybridisation to the 'B' genome. Additionally, during the genetic linkage analysis, there were some markers that could possibly be 'B' genome markers (work not described in results), as they were not linked to any other loci. But these markers were very few and far-between, which compromised the genetic map to some extent. These markers did not contribute to the linkage analysis and were not included.

For each *B. juncea* BC_1 population, two significant QTL with additive effect were mapped by QTL analysis, which fitted the two recessive gene model indicated by the phenotypic segregation. All of these QTL were mapped to the 'A' genome. It is conceivable that there are no significant QTL in the 'B' genome for the TuMV resistance in both *B. juncea* lines (TWBJ14 and TWBJ20). Another genotyping method − Genotyping-by-sequencing (GBS) (Elshire *et al*. 2011) could be used to get more information from the 'B' genome of the *B. juncea* lines.

7.1.3 Candidate *eIF* genes for TuMV resistance in *B. juncea*

The susceptibility factors eIF4E/eIF(iso)4E and eIF4G/eIF(iso)4G protein families are indispensible for potyvirus infection. In a pervious study on *Arabidopsis*, the simultaneous inactivation of *eIF(iso)4G1* and *eIF(iso)4G2* gave rise to the resistance to TuMV isolates UK 1 and CDN 1, indicating TuMV can selectively recruit either of these two factors for infection (Nicaise *et al*., 2007). This finding

might be applicable to my research as the TuMV resistance in both *B. juncea* lines TWBJ14 and TWBJ20 is found to be controlled by two recessive genes. According to the results of the QTL mapping, three candidate *eIF4G*/*eIF(iso)4G* genes (*Bra038615*, *Bra008429* and *Bra020407*) were identified for line TWBJ14 and two candidate *eIF4G*/*eIF(iso)4G* genes (*Bra038615* and *Bra010275*) for line TWBJ20. The annotation is not clear whether these candidate genes are *eIF4G* or *eIF(iso)4G*. Further work to check whether any of these candidates are involved in the recessive resistance would be of great interest.

There are another two candidate genes identified for line TWBJ20, which are *BraA.eIF4E.c* (*Bra021026*) and *BraA.eIF(iso)4E.c* (*Bra035531*). To date, one recessive TuMV resistance gene (*retr01*/*retr02*) and one dominant resistance gene (*ConTR01*) were found to be *eIF(iso)4E* genes in *Brassica*. If either of these two candidates is involved in the recessive resistance in *B. juncea* line TWBJ20, this plant line would be a useful resource to study how TuMV interacts with eIF4E/*eIF(iso)4E* in a *Brassica* amphidiploid species.

7.2 Transfer of TuMV resistance genes in brassicas through interspecific hybridisation

The *Brassica* genus contains many important agricultural and horticultural crops and a number of wild species. These plants are excellent sources of genes for many economically important traits. The high degree of compatibility between some *Brassica* species makes the transfer of desirable genes achievable through interspecific hybridisation.

In this study, BC_2 generations were produced from the interspecific crosses between TuMV-resistant *B. napus*/*B. rapa* (possessing *TuRB01*/*TuRB01b*) and TuMV-susceptible *B. juncea*. The *TuRB01*/*TuRB01b*-based TuMV resistance was introgressed into the *B. juncea* background, assisted by phenotypic and genotypic selection. The cross between *B. juncea* and *B. rapa* appeared to be more compatible than the cross between *B. juncea* and *B. napus*.

For the interspecific cross between the TuMV-resistant *B. rapa* (possessing *TuRB01b*) and TuMV-susceptible *B. nigra*, no seeds were produced through the bud pollinations, due to the breakdown of the embryo (post-fertilisation barrier). Therefore, the *in vitro* method was used to culture the fertilised ovules. Resynthesised *B. juncea* (possessing *TuRB01b*) was developed using embryo rescue and polyploidy induction. As mentioned earlier, the homology between the *Brassica* 'A' and 'B' genomes is notably less than that between the 'A' and 'C' genomes. Thus, the frequency of effective chromosome pairing in meiosis between the 'A' and 'B' genomes is very low, which leads to low success rates for interspecific hybridisations between these two diploid genomes (Navabi *et al*., 2013; Ghani *et al*., 2014).

Interspecific hybridisation is a very important technique for transferring desirable genes between brassica genomes and enhancing the quality and productivity of brassica crops. Additionally, it can contribute significantly to the genetic enrichment and improved variation of the species.

7.3 Suggestions for future work

7.3.1 Investigation of the TuMV resistance in the resistant *B. juncea* lines

The TuMV resistances discovered in *B. juncea* lines TWBJ14 and TWBJ20 were effective against the TuMV isolates representing pathotypes 1, 3 and 4. Further TuMV resistance test using different isolates would be useful. This will provide a further useful knowledge about the spectra and specificities of the TuMV resistances in these two lines.

The potential candidate *eIF* genes identified for the TuMV resistances in both TWBJ14 and TWBJ20 BC_1 populations require further detailed study. Sequencing the candidate genes from the resistant and susceptible parental lines and making alignments of the sequences is one approach. If candidates do not show any sequence differences between parental lines, then they may not be involved in the TuMV resistance. However, expression studies would also be needed to confirm this. If candidates show any sequence differences between parental lines, the

candidate is of interest and further experiments on the BC_1 plants would be needed to check the correlation between the genotype and phenotype. Furthermore, site-directed mutagenesis could be used. For example, knocking out candidate genes in TuMV-susceptible *B. juncea* plant lines using CRISPR/Cas9 technology (Bortesi and Fischer, 2015) or TILLIING population (Slade *et al.*, 2005) would be useful.

If the GBS genotypic data for TWBJ14 and TWBJ20 BC_1 populations could be obtained, data analysis would be needed. The filtering and quality control of any markers generated would be required, followed by the genetic linkage analysis and QTL mapping. Such mapping could be compared to the mapping done by the Illumina Infinium Chip.

Apart from lines TWBJ14 and TWBJ20, another six sources of TuMV resistance were identified in *B. juncea*. It would be interesting to characterise and map the resistances in some of these lines. Efforts could be preferentially focused on lines TWBJ23, TWBJ03, TWBJ04 and TWBJ15.

7.3.2 Identification of the TuMV resistance gene *TuRB01*/*TuRB01b*

In this study, *BORG2*, a candidate gene for the TuMV resistance gene *TuRB01*/*TuRB01b* was ruled out, leaving *BORG1* as the only current candidate *R* gene left. There is an ambiguity in the involvement of the second gene (*RCH1*-like gene) in this dominant resistance. Further research is needed to determine whether *BORG1* is the only gene conferring the resistance. To achieve this, techniques of site-directed mutagenesis could be used. Recently, the CRISPR/Cas9 technology has been increasingly used and it provides many advantages over other targeted genome editing methods (Bortesi and Fischer, 2015). This technology could be applied as it is said to be simple and accessible. In addition, it would be of interest to investigate the signal transduction pathways upstream and downstream of *BORG1*.

7.3.3 Investigations on the interaction between *B. nigra* and TuMV

Little is known about the interaction between *B. nigra* ('B' genome) and TuMV. Further investigation of this interaction could help to identify new and additional

sources of TuMV resistance for mapping and introgression into *B. juncea*. Further screening for TuMV resistance in *B. nigra* or testing *B. carinata* would be helpful.

In this study, of 27 *B. nigra* lines tested, only one line showed uniform "+" phenotype, whereas the other lines all showed "$+_N$" phenotype. The necrotic hypersensitive response (HR) could be mapped in *B. nigra*. To accomplish this, a segregating mapping population could be developed by crossing *B. nigra* line 'TWBN05' (phenotype +) and the well-charaterised line 'Ni-100'/'al-1-3' (phenotype $+_N$) for which there is genomic information.

7.3.4 Introgression of *TuRB01*/*TuRB01b* into *B. juncea*

From the interspecific cross between the TuMV-resistant *B. rapa* (possessing *TuRB01b*)/*B. napus* (possessing *TuRB01*) line and a TuMV-susceptible *B. juncea* line, several BC_2 seed lines were obtained. The phenotyping and genotyping (genetic testing using within-gene specific markers) of these BC_2 plants could be done. Further backcross generations need to be developed in order to increase the stability of *B. juncea* lines possessing *TuRB01*/*TuRB01b*-based resistance. For the evaluation of the markers based on primer pairs BORG1L/BR208 and BORG1L/BR210, the PCR conditions require further optimisation.

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Appendix

Table A.1 − PCR condition of primer pair BR183/BR184 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	30
Annealing	63°C	30 s	
Extension	72°C	1 min 30 s	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.2 − PCR condition of primer pair BR185/BR186 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	35
Annealing	68°C	30 s	
Extension	72°C	1 min 30 s	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.3 − PCR condition of primer pair BR191/BR192 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	35
Annealing	66°C	30 s	
Extension	72°C	1 min 30 s	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.4 − PCR condition of primer pair BR196/BR195 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	35
Annealing	60°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.5 − PCR condition of primer pair BR138/BR158 using Elongase.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	4 min	1
Denaturation	95°C	30 s	5
Annealing	55°C (one degree reduction per cycle)	30 s	
Extension	68°C	4 min	
Denaturation	95°C	30 s	30
Annealing	50°C	30 s	
Extension	72°C	4 min	
Final extension	72°C	10 min	1
Hold	12°C		

Table A.6 − PCR condition of primer pair BR190/BR186 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	35
Annealing	72°C	30 s	
Extension	72°C	40 s	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.7 − PCR condition of primer pair qRT2080 using Taq.

Step	Temperature	Time	Cycles
Initial denaturation	94°C	4 min	1
Denaturation	94°C	30 s	35
Annealing	58°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.8 − PCR condition of primer pair BR205/BR206 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	35
Annealing	72°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.9 − PCR condition of primer pair BR164/BR165 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	
Annealing	72°C	30 s	35
Extension	72°C	3 min	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.10 − PCR condition of primer pair BR138/BR139 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	
Annealing	71°C (one degree reduction per cycle)	30 s	5
Extension	72°C	2 min	
Denaturation	98°C	10 s	
Annealing	66°C	30 s	30
Extension	72°C	2 min	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.11 − PCR condition of primer pair BR211/BR208 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	
Annealing	60°C	30 s	38
Extension	72°C	1 min	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.12 − PCR condition of primer pair BR138/BR210 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	
Annealing	55°C	30 s	35
Extension	72°C	1 min	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.13 − PCR condition of primer pair BORG1L/BR208 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	38
Annealing	60°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.14 − PCR condition of primer pair BORG1L/BR210 using Phusion.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	35
Annealing	55°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	7 min	1
Hold	12°C		

Table A.15 - Detailed result of ploidy level testing (using flow cytometry) of F_1 plants generated from interspecific crossing between *B. rapa*/*B. napus* and *B. juncea*.

Plant line	Relative DNA ratio with internal standard
B. rapa line TD-R	0.60
B. juncea line 060DH17	1.25
B. napus line Westar	1.37
F_1-1 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.95
F_1-2 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.94
F_1-3 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.93
F_1-4 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.90
F_1-5 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.96
F_1-6 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.95
F_1-7 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.93
F_1-8 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.94
F_1-9 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.93
F_1-10 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.92
F_1-11 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.94
F_1-12 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.96
F_1-13 from *B. juncea* (060DH17) × *B. rapa* (TD-R)	0.95
F_1-1 from *B. juncea* (060DH17) × *B. napus* (Westar)	1.31
F_1-2 from *B. juncea* (060DH17) × *B. napus* (Westar)	1.31
F_1-3 from *B. juncea* (060DH17) × *B. napus* (Westar)	1.31
F_1-4 from *B. juncea* (060DH17) × *B. napus* (Westar)	1.28
F_1-5 from *B. juncea* (060DH17) × *B. napus* (Westar)	1.31
F_1-6 from *B. juncea* (060DH17) × *B. napus* (Westar)	1.30
F_1-7 from *B. juncea* (060DH17) × *B. napus* (Westar)	1.31
F_1-8 from *B. juncea* (060DH17) × *B. napus* (Westar)	1.27
F_1-9 from *B. juncea* (060DH17) × *B. napus* (Westar)	1.27

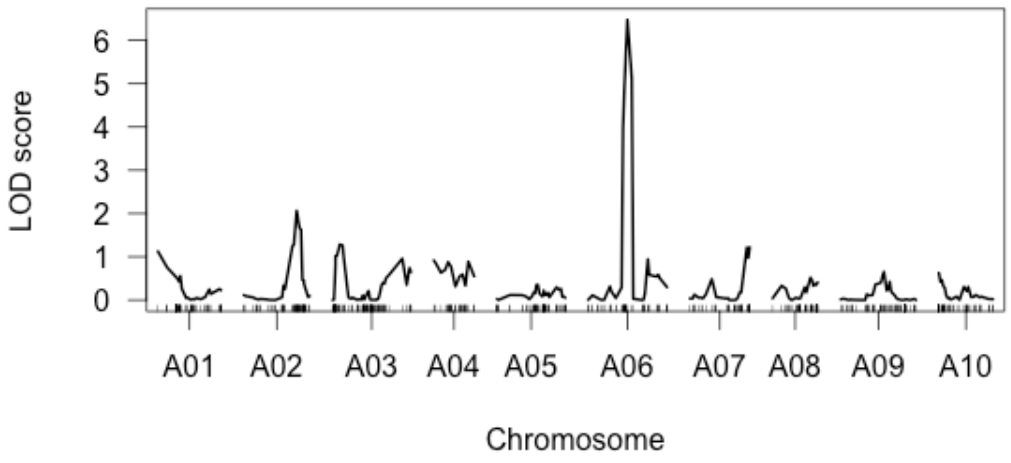

Figure A.1 – Detection of QTL for Turnip mosaic virus resistance in *Brassica juncea* line TWBJ14 using the composite interval mapping method in R/qtl.

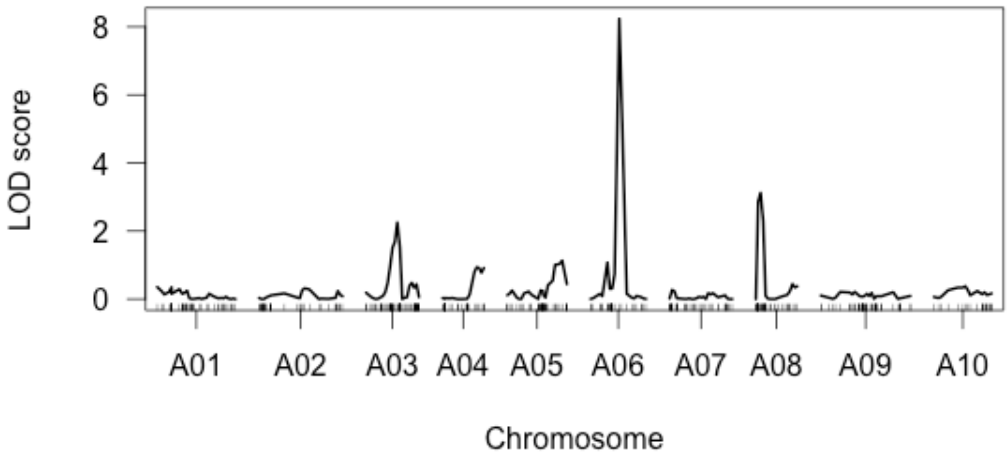

Figure A.2 – Detection of QTL for Turnip mosaic virus resistance in *Brassica juncea* line TWBJ20 using the composite interval mapping method in R/qtl.

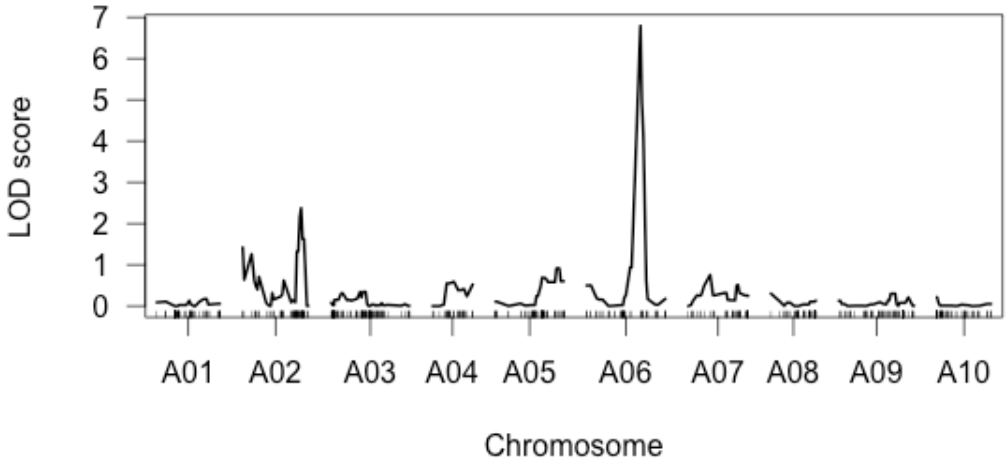

Figure A.3 − Detection of putative QTL controlling the necrotic response to Turnip mosaic virus in *Brassica juncea* line TWBJ14 using the composite interval mapping method in R/qtl.