Characterisation of temperature-sensitivity of *Brassica* resistance against *Leptosphaeria maculans*

Katherine Louise Noel, B.Sc.,

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School of Life and Medical Sciences, University of Hertfordshire,
Hatfield, Herts,
AL10 9AB

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Abstract

Variety resistance is an important tool in controlling against plant pathogens. As temperatures increase due to global warming, temperature-resilient resistance may play an important role in crop protection. However, the mechanisms behind temperature-sensitivity of the resistance response is poorly understood in crop species, and most of our knowledge comes from studies on the model plant *Arabidopsis thaliana*. This project aims to further understand the temperature-sensitivity of *Brassica napus* resistance against *Leptosphaeria maculans*.

Field and controlled environment experiments were done to investigate the effects of cultivar *R* gene-mediated and quantitative resistance and weather on the severity of phoma stem canker. Higher maximum June temperatures were found to be related to phoma stem canker severity; this effect was reduced in cultivars with good quantitative resistance. The hypothesis that *R* genes operate in the stems of adult winter oilseed rape plants, providing some protection against *L. maculans* was supported by a controlled environment stem inoculation assay. A constantly elevated temperature of 25°C appeared to reduce this effect.

Suppressor of NPR1, constitutive (*BrSNC1*) (an ortholog of an *R*-gene that mediates high temperature inhibition of resistance in *A. thaliana*), and *FocBr1*, which confers resistance to *Fusarium oxysporum* in *Brassica rapa*, were investigated for their roles in the temperature-sensitivity of resistance in *B. rapa*. TILLING mutations in the genes showed phenotypic differences in *L. maculans* inoculation assays at 20°C and 25°C. *FocBr1-1* mutants displayed higher susceptibility at 20°C compared to the wild-type, whereas *Brsnc1* mutants showed what is hypothesised to be an autoimmune response at 20°C and 25°C. It is postulated that, analogous to the mechanism in *A. thaliana*, *BrSNC1* is a temperature-sensitive component in the *B. napus* resistance response.

B. napus R-gene introgression lines Topas-*Rlm7* and Topas-*Rlm4* were found to be temperature-sensitive and -resilient, respectively, from cotyledon inoculation assays at 20°C and 25°C. Differences in defence related gene expression, including pathogenesis-related protein 1 (*PR1*) (a salicylic acid inducible marker gene) were explored using qPCR and RNA-seq. Lower levels of *PR1* expression were found in Topas-*Rlm7* lines compared to the temperature-resilient Topas-*Rlm4* line at 25°C. Further identification of other genes expressed differentially in Topas-*Rlm4* compared to Topas-*Rlm7* may provide useful molecular markers that could be used by breeders to guide their selection of *B. napus* cultivars with temperature-resilient resistance to *L. maculans*.

These three studies provide greater understanding of different aspects of *Brassica* defence at elevated temperatures; specifically in how quantitative resistance is affected by high temperatures, the roles of two specific defence related genes *BrSNC1* and *FocBr1* and gene expression differences in temperature -sensitive and -resilient *R* genes. These findings provide valuable information that increases the scope for developing oilseed crops better suited to a warming climate due to greater temperature-resilient resistance to phoma stem.

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Abbreviations

ABA	abscisic acid
ANOVA	analysis of variance
BLAST	basic local alignment search tool
cDNA	complementary DNA
CE	controlled environment
Ct	threshold cycle
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dpi	days post inoculation
ETD	effector triggered defence
ET	ethylene
ETI	effector triggered immunity
HR	hypersensitive response
IAA	indole-3-acetic acid
IL	introgession line
JA	jasmonic acid
KASP	kompetitive allele specific PCR
L	litre
Log	logarithm
NBS-LRR	nucleotide-binding site leucine-rich repeat
NB-ARC	nucleotide-binding adaptor shared by APAF-1, certain *R* gene products and CED-4
NLR	nucleotide-binding domain leucine-rich repeat
NO	nitric oxide

MAPK	mitogen-activated protein kinase
Mol	molar
mRNA	messenger RNA
PAMP	pathogen-associated molecular pattern
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
PTI	PAMP-triggered immunity
PR1	pathogenesis-related protein 1
qRT-PCR	quantitative reverse transcription
R gene	resistance gene
RLP	receptor-like protein
RLK	receptor-like kinase
ROS	reactive oxygen species
RNA	ribonucleic acid
SA	salicylic acid
SAR	systemic acquired resistance
SE	standard error
SNC1	suppressor of npr1-1 constitutive 1
SUMO	small ubiquitin-like modifier
TILLING	targeting induced local lesions in genomes
TIR-NB-LRR	toll interleukin1 receptor - nucleotide binding site - leucine-rich repeat
WAKL	wall-associated kinase-like

1 General introduction

1.1. Climate change impacts on crop diseases

Climate change presents a great threat to agricultural production systems and food security. Alongside more direct outcomes, such as quickening of plant development, climate change will impact greatly on plant diseases (Bebber et al., 2013; Newbery et al., 2016). Current models forecast an increase in weather variability and a greater incidence of extreme weather events. Temperatures are predicted to increase in Europe, with the most substantial changes seen at high latitudes (Northern Europe), at night and during the winter (Rosenzweig et al., 2001; Siebold and von Tiedemann, 2012).

1.1.1. Influence of temperature on crop diseases

Weather is known to be a primary factor influencing plant disease epidemics; plant pathogens are highly responsive to humidity, rainfall and temperature (Anderson et al., 2004; Siebold and von Tiedemann, 2012). Temperature is predicted to be the most significant environmental factor influencing pathogen spread and development (Siebold and von Tiedemann, 2012). Climate change impacts on crop diseases will differ between pathosystems and geographic locations, while many factors, from molecular to global scales, will interact to determine the amount of damage. Thus, the overall impact on a pathosystem will be unique (Boland et al., 2004; Garrett et al., 2006). For these reasons, it is becoming increasingly important to understand how the relationship between hosts and pathogens is affected by the weather in general and by temperature in particular.

1.2. Resistance against crop pathogens

Plants recognise pathogens through receptors that set off a signalling network cascade controlled by calcium fluxes, oxidative bursts and MAPK (mitogen-activated protein kinase) cascades (Bigeard et al., 2015) that lead to a defence response. Following the activation of the defence response, a great number of cellular processes occur. These include the activation of protein kinases, a rapid influx of calcium ions, an oxidative burst, leading to the production of reactive oxygen species (ROS) and changes in hormone and gene expression (Nováková et al.,

2015). The defence-related genes that are expressed include those involved in encoding enzymes, strengthening cell walls and the biosynthesis of antimicrobial compounds.

Current understanding of the plant immune system separates it into two branches; a primary basal defence response known as PAMP (pathogen-associated molecular pattern) triggered immunity (PTI) and effector-triggered immunity (ETI). PTI recognises conserved molecules common across many classes of microbe whilst ETI recognises and responds to effectors produced by the pathogen to suppress PTI. The Zig-zag model, presented by Jones and Dangl (2006), describes the relationship between ETI and PTI in the plant immune system. The Zig-zag model splits the resistance response into four consecutive phases; phase 1 (PAMPs detected by pattern recognition receptors (PRRs)); phase 2 (pathogens deliver effectors to interfere with PTI); phase 3 (effectors recognized by NB-LRRs, ETI) and phase 4 (loss/gain of effectors over evolutionary time). However, since this Zig-zag model was proposed, advances have been made in our understanding of plant immunity revealing limitations to this model.

Six of these limitations were set out by Pritchard and Birch (2014). 1) Limited molecular scope; it excludes both other potentially significant molecules (e.g. DAMPS) and other interactions (e.g. nutrient exchange). 2) Absence of environmental context; past and current biotic and abiotic experiences can affect a plant's immune response (e.g. whether there was prior exposure to pathogens or sterile growth and temperature). 3) Ordering of events; the four phases described by the Zig-zag model may be misleading as it suggests a highly ordered resistance response, whereas later studies point to various signals occurring concurrently. 4) Timescale; the model encompasses molecular processes taking place in a single plant-pathogen interaction (phases 1 – 3) and those occurring over a much longer timescale as selection pressure cause the loss/ gain of effectors (phase 4) which is potentially a source of confusion. 5) Physical scale; the model may be misinterpreted as it is ambiguous about the physical scale it describes, whilst the evolutionary events of phase 4 take place at the population level, the molecular processes described occur only in specific cells that are challenged by the pathogen. 6) The model is qualitative; it is unclear what measurements the scale of amplitude represents.

Further issues with the Zig-zag model arose from confusion over the classification of resistance against apoplastic pathogens as PTI or ETI (Thomma et al., 2011; Jones and Dangl, 2006), Stotz et al., 2014). The classification of effector triggered defence (ETD) as an additional form of resistance, separate from ETI and PTI was first proposed by Stotz et al. (2014). ETD replaces ETI when extracellular apoplastic pathogens are encountered. As well as involving different receptors (ETI is triggered by intracellular NBS-LRR (nucleotide-binding site leucine-rich repeat

receptors)), ETD differs from ETI on several additional points. The ETD response is delayed relative to ETI, which is associated with fast, hypersensitive host cell death. Furthermore, with ETD the pathogen is not killed and may resume growth following the onset of host senescence, or if host resistance response is otherwise compromised (Stotz et al., 2014).

1.2.1. Temperature and resistance

Temperature affects many plant growth and developmental processes, such as germination and flowering. Resistance to pathogens is also influenced by temperature; an increase in temperature can lead to inhibition of defence responses modulated by many *R* genes (Mang et al., 2012). Exposure of cereals to low temperatures has also been found to increase their resistance to several pathogens (Gaudet et al.,2011; Kuwabara et al., 2012).

1.2.2. Molecular mechanisms underlying temperature-sensitivity of resistance

There are limited studies on the mechanistic components that determine how pathogen-induced defence responses are altered in response to ambient temperature changes in crop plants. Most of the work investigating the molecular mechanisms behind temperature-sensitivity have focused on the pathways in the model organism *Arabidopsis thaliana*. Several factors have been found to play a key role in temperature-sensitivity of *R* genes in *A. thaliana*. These *A. thaliana* studies provide us with the closest understanding of the temperature-sensitivity of the defence network. A summary of key papers is given in Table 1. The inhibition of the plant defence response by increased temperature is likely to be mediated by a pathway made up of many temperature-sensitive components of defence signalling, downstream from pathogen recognition.

1.2.2.1. Resistance genes

It has been postulated by Zhu et al. (2010) that NLRs (nucleotide-binding domain leucine-rich repeat) act as the temperature-sensitive component in plant defence responses. Suppressor of *npr1-1*, constitutive 1 (*SNC1*) is a TIR-NB-LRR (toll interleukin1 receptor - nucleotide binding site-leucine-rich repeat) resistance (*R*)-like gene in *A. thaliana* which activates the immune response via an associated protein, a transcriptional co-repressor called Topless related 1 (TPR1). TPR1 represses the expression of negative regulators of immune responses (Zhu et al., 2010). *SNC1*

Table 1.1 Summary of key studies providing information on the pathway behind temperature-sensitivity of defence responses against pathogens.

Author	Title	Key information on temperature-sensitivity pathway
Hua et al., 2001	Plant growth homeostasis is controlled by the Arabidopsis *BON1* and *BAP1* genes	• As *bon1-1* mutants have a miniature phenotype at 22°C but a wild-type appearance at 28°C, it was shown homeostasis of *A. thaliana* growth requires the *BON1* gene. • Expression of *BON1* and a *BON1*-associated protein (*BAP1*) is modulated by temperature, which suggests they play a direct role in regulating cell expansion and cell division at lower temperatures. • Findings suggest that the *BON1* gene family may function in the pathway of membrane trafficking in response to external conditions.
Yang and Hua., 2004	A haplotype-specific resistance gene regulated by BONZAI1 mediates temperature-dependent growth control in Arabidopsis	• *BON1* negatively regulates *SNC1*; mutant *bon1-1* activates defence responses, leading to reduced growth in a *SNC1*-containing accession but not in accessions without a functional *SNC1*. Thus, *SNC1* is epistatic to *BON1* and acts downstream in the pathway. • *SNC1* is under a positive feedback regulation involving SA temperature modulation. • Plant growth homeostasis, controlled by *BON1*, involves repression of an *R* gene and modulation of defence responses by temperature.
Li et al., 2007	The TIR-NB-LRR gene *SNC1* Is regulated at the transcript level by multiple factors	• When introduced to *A. thaliana* as a transgene *SNC1* is expressed at a much higher level than an endogenous gene, this confers a dwarf phenotype caused by upregulation of defence responses. • Three independent modes of regulation on the *SNC1* transcript level were identified: a repression by the chromosomal structure, a feedback amplification from *SNC1* on its promoter sequences, and a repression by *BON1*.

		• Regulation of *SNC1* is suggested to demonstrate a universally complex control of *R* genes, causing a repression of *R* activation under non-stress conditions and a robust activation of defence responses once the *R* gene is induced.
Wang et al., 2009	Analysis of temperature modulation of plant defense against biotrophic microbes	• Effects of a moderate temperature increase on resistance to bacterial pathogen *Pseudomonas syringae* and two viral elicitors in *A. thaliana* and *Nicotiana benthamiana* were characterised. Basal and *R* gene-mediated defence responses were both inhibited, as well as the hypersensitive responses induced by *R* genes against two viruses, suggesting that temperature modulation of defence responses to biotrophic and hemibiotrophic pathogens might be a general phenomenon. • The roles of SA and JA in temperature modulation were tested using mutants, it was found that neither can induce temperature-sensitivity by itself. • Temperature regulation of the transcript levels of *EDS1* and *PAD4* is not sufficient to cause temperature-sensitivity of the defence responses
Zhu et al., 2010	Temperature modulates plant defense responses through NB-LRR proteins	• The NB-LRR type of R or R-like proteins are temperature-sensitive components of plant defence responses. • Alterations in *SNC1* (*A. thaliana*) and the tobacco *R* gene, *N*, both showed changes temperature-sensitivity of defence responses. • An elevated temperature reduces the nuclear accumulation of SNC1, which likely contributes to the inhibition of disease resistance at high temperatures.
Wang et al., 2011	BON1 interacts with the protein kinases BIR1 and BAK1 in modulation of temperature-	• *BON1* interacts with the protein kinases *BIR1* and *BAK1* to modulate temperature-dependent plant growth and cell death in *A. thaliana*. • *BIR1* functions as a negative regulator of plant resistance.

	dependent plant growth and cell death in Arabidopsis	• Findings suggest *BON1* and *BIR1* might modulate both PAMP- and R protein-triggered immune responses.
Mang et al., 2012	Abscisic acid deficiency antagonizes high-temperature inhibition of disease resistance through enhancing nuclear accumulation of resistance proteins SNC1 and RPS4 in Arabidopsis	• A mutant screen with an *A. thaliana* temperature-sensitive autoimmune mutant *bonzai1* showed the ABA-deficient mutant *aba2* enhances resistance mediated by the *R* gene *SNC1* at high temperature.
Gangpappa et al., 2017	PIF4 coordinates thermosensory growth and immunity in Arabidopsis	• *PIF4* negatively regulates plant immunity, and modulation of its function changes the balance between growth and defence. • Modulation of *PIF4* signaling alters temperature-sensitivity of disease resistance • Ecotypes of *A. thaliana* show variations of *PIF4* signaling underlying growth-defence balance
Hammoudi et al., 2018	The Arabidopsis SUMO E3 ligase SIZ1 mediates the temperature dependent trade-off between plant immunity and growth	• At normal and high temperatures *SNC1*-dependent resistance responses are supressed by *SIZ1*. • *SIZ1* also amplifies dark and high temperature growth responses, likely through *COP1* and upstream of gene regulation by *PIF4* and *BRZ1*.

was identified by as a central component in temperature-sensitivity through a genetic screen for *A. thaliana* heat-stable mutants. These mutants displayed the ability to retain the defence response under elevated temperatures, which normally inhibited the defence response. Furthermore, this finding was repeated in tobacco, when a similar mutation was created in the *N* gene, which confers resistance against tobacco mosaic virus. This suggests that this mechanism is a widespread phenomenon across all NB-NLR *R* genes. Zhu et al. (2010) proposed that a conserved SUMOylation (Small Ubiquitin-like Modification) motif may operate in distant taxa as a common mechanism for regulating temperature-sensitivity across various types of resistance to pathogens. SIZ1, a SUMO E3 ligase operates as an important positive regulator of growth and as a negative regulator of the SNC1-dependent immune response at high temperature (Hammoudi et al., 2018). SIZ1-mediated SUMOylation of TPR1 was found to repress its activity in *A. thaliana* (Niu et al., 2019).

1.2.2.2. PIF4 transcription factor

SNC1 auto-immunity is suppressed at 28°C by a transcription factor PIF4; the PIF4 transcription factor has been found to coordinate thermosensory growth and immunity in *A. thaliana* (Gangpappa et al., 2017). Several assays done on various mutant *A. thaliana* strains found that PIF4 acts as a negative regulator of plant immunity, and modulation of its function alters the balance between growth and defence. Furthermore, the study identified a natural variation of PIF4 signalling in the *A. thaliana* ecotypes serving to underlie growth-defence balance. However, as this study focused solely on *A. thaliana*, it cannot be certain that the PIF4-mediated thermosensory signalling module acts in the same way in other plant species, including *B. napus*. Therefore, there is a need to conduct further experiments to determine whether what has been observed in *A. thaliana* also applies to other *Brassica* species.

1.2.2.3. Abscisic acid

Several plant hormones, including abscisic acid (ABA), salicylic acid (SA), ethylene (ET), cytokinin (CK) and auxin (AUX) have been proposed to play important roles in plant thermo-tolerance (Mang et al., 2012). A study into the effect of temperature on the role of ABA in the defence response by Mang et al. (2012) found that ABA antagonises resistance at increased temperature, by decreasing the accumulation of resistance proteins SNC1 and RPS4 in the nucleus. The group determined that the ABA deficient mutant *aba2* counteracted the inhibition of defence response

against *P. syringae* caused by increased temperature, leading to enhanced resistance at elevated temperatures and accumulation of RPS4 in the nucleus.

1.2.2.4. Salicylic acid

The SA pathway plays an important role in plant defence responses against biotrophic pathogens. During the hypersensitive response (HR), SA is up-regulated, leading to the induced expression of pathogenesis-related proteins, including *PR1* (Wang et al., 2009). It has been suggested by Wang et al. (2009) that the temperature-sensitive pathway in disease resistance is mediated by SA. A study by Zhao et al. (2016) on the temperature-dependent defence of *Nicotiana tabacum* against cucumber mosaic virus, found that lower temperatures (≤ 24°C) promoted SA-dependent responses, whilst elevated temperatures (> 24°C) upregulated genes associated with the jasmonic acid (JA) pathway. When activated, *SNC1* induces SA accumulation and defence responses. A positive feedback system exists between *SNC1* and SA which is supressed by high temperatures (Yang and Hua, 2004).

1.2.2.5. EDS1 and PAD4

Enhanced disease susceptibility 1 (EDS1) and phytoalexin deficient 4 (PAD4) are two important signalling components in the SA pathway of the defence network (Carstens et al., 2014). Both PTI and TIR-NB-LRR (but not CC-NB-LRR) resistance protein-mediated ETI against biotrophic and hemibiotrophic pathogens are known to be centrally regulated by the EDS1-PAD4 node (Carstens et al., 2014). Full accumulation of SA requires the formation of the EDS1-PAD4 complex (Carstens et al., 2014). The mobilisation of this pathway is less effective at temperatures > 25°C (Alcázar, 2011). The roles of EDS1 and PAD4 in temperature modulation were investigated by Wang et al. (2009). Their findings suggest that these defence regulators act as components in the temperature-sensitive pathway of the defence response, being modulated by temperature and/or are involved in temperature regulation.

1.2.2.6. SGT1 and RAR1

SGT1 (suppressor of G2 allele of *skp1*) and RAR1 (required for *Mla12* resistance) have been identified as mediators of various *R* genes in response to pathogen effectors (Zhang et al., 2010; Yang et al., 2010). SGT1 and RAR1 act as co-factors, functioning in the Heat Shock Protein 90

(HSP90)-mediated stabilisation of pre-activated NB-LRR protein complexes (Bieri et al., 2004). SGT1 has been found to interact with the LRR domains of certain NB-LRR proteins, potentially aiding proper folding (Bieri et al., 2004; Yang et al., 2010). An investigation was done by Yang et al. (2010), using the *A. thaliana* mutant chilling sensitive 3, which exhibits arrested growth and constitutively activated defence responses at lower temperatures (16°C) and not at a higher temperature (22°C). These defence phenotypes were found to be suppressed by eds1, sgt1b and rar1, but not by pad4. These findings suggest that there is a mutual interaction between cold signalling and the defence responses.

1.2.2.7. Heat-shock protein 90

In a study on temperature-dependent seedling growth in *A. thaliana*, Wang et al. (2016) demonstrated that TIR1 (an auxin co-receptor protein) is in a complex with both HSP90 and the co-chaperone SGT1. Furthermore, it was found that the HSP90-SGT1 chaperone system is required for the plant's response to temperature increase. HSP90 is required for stabilisation of the TIR1 at 29°C, a process associated with increased seedling growth. HSP90 has also been found to be involved in plant defence responses, probably through performing chaperone functions with SGT1 (Wang et al. 2009).

1.2.2.8. H2A.Z nucleosomes

Histone proteins are responsible for the nucleosome structure in eukaryotes. Nucleosomes consist of approximately 146 bp of DNA wrapped around a histone octamer composed of pairs of the four core histones (H2A, H2B, H3, and H4). Kumar and Wigge (2010) found that the histone variant H2A.Z-containing nucleosomes present a key node of regulation of the temperature transcriptome in plants. By wrapping DNA more tightly, H2A.Z nucleosomes influence the ability of RNA polymerase (Pol) II to transcribe genes in response to temperature, thus presenting a mechanism by which the transcriptome can be thermally regulated.

1.2.3. Temperature-sensitivity of the *B. napus* resistance response against *L. maculans*

Whilst these molecular studies outline the situation in *A. thaliana*, and serve as a good reference for our understanding, the molecular pathway for temperature-sensitivity in crop plants may differ. The pathosystem involving the pathogen *Leptosphaeria maculans* (phoma stem canker)

and the host *Brassica napus* (oilseed rape) provides an increasingly well-understood model in the genetic study of host-pathogen interactions. As *B. napus* also contains several well-characterised resistance genes, some of which display temperature-sensitivity, it is a good subject for this study.

1.3. Oilseed rape (*Brassica napus*)

B. napus L (2n=38, genome AACC) is a member of the Brassicaceae. Derived from a naturally occurring intraspecific cross between *B. oleracea* (2n=18, CC) and *B. rapa* (2n=20, AA), *B. napus* is allopolyploid, containing both parental sets of diploid chromosomes (Chaloub et al., 2014). A genetic relationship between *B. napus* and the other five most widely grown *Brassica* species (*Brassica nigra, Brassica rapa, Brassica oleracea, Brassica juncea* and *Brassica carinata*) was first established by Woo Jang-Choon (U, 1935). The triangle of U (Figure 1.1.) describes how three *Brassica* species are derived from the interspecific crossing of three species with ancestral genomes. The model plant species *A. thaliana* is also in the *Brassicaceae* family (Snowdon and Iniguez Luy, 2012).

Winter and spring cultivars of oilseed rape differ both genetically and agriculturally (Becker et al., 1995). Winter oilseed rape is an annual plant, sown in the autumn and requiring vernalisation before flowering in the spring of the following year. In Europe, most winter oilseed rape crops are sown in late August and are harvested the following year in July (Butterworth et al., 2010). Spring cultivars are sown and harvested in one calendar year (Butruille et al., 1999; Cruz et al., 2007). Oilseed rape is the most commonly grown and most profitable break crop in Europe (Finch et al., 2014). In 2019, 547 000 hectares of oilseed rape were grown in England (Department for Environment, Food and Rural Affairs, 2019)). *C*. 38-40% of the harvested seed is oil, which is extracted by crushing. Oilseed rape cultivars are in two categories, those that produce low levels of erucic acid and glucosinolates (double low cultivars); and those high in erucic acid (HEAR cultivars) (Finch et al., 2014). Double low cultivars are suitable for use in the food industry, such as for spreads, dressings and cooking oil, while HEAR cultivars are grown for specialist industrial uses and biodiesel. Plant material remaining after extraction of oil is also used for animal fodder (Finch et al., 2014).

1.3.1. Pests and diseases of oilseed rape

UK oilseed rape crops are at risk from many pests and diseases that can, in some seasons, lead to losses of > £80 million (Gladders, 2005). The five most important fungal diseases of oilseed

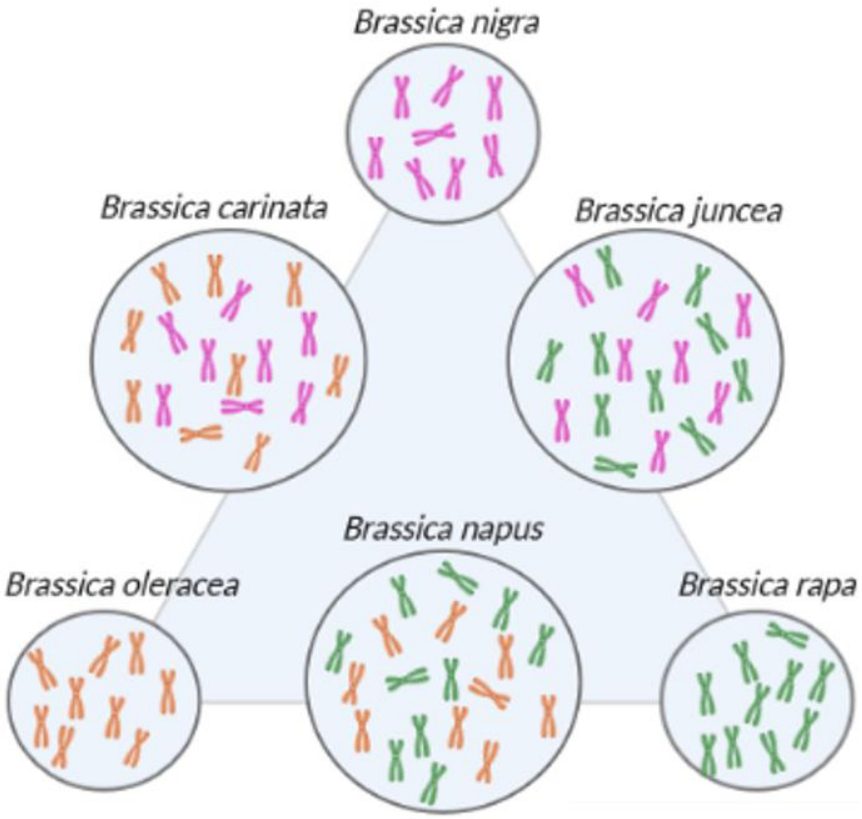


Figure 1.1 Triangle of U depicting the genetic interconnection between Brassica species. *B. rapa* (AA), *B. nigra* (BB) and *B. oleracea* (CC) are diploids, while *B. juncea* (AABB), *B. carinata* (BBCC) and *B. napus* (AACC) are allotetraploids, each containing two diploid sets of chromosomes (U, 1935). Created with BioRender.com.

rape in Europe at present are phoma stem canker (*L. maculans* and *Leptosphaeria biglobosa*), stem rot (*Sclerotinia sclerotiorum*), verticillium stem striping (*Verticillium longisporum*), black spot (*Alternaria brassicae*) and light leaf spot (*Pyrenopeziza brassicae*) (Siebold and von Tiedemann, 2012).

1.4. The phoma stem canker (*L. maculans*) - oilseed rape (*B. napus*) pathosystem

1.4.1. Significance of phoma stem canker

Phoma stem canker of *B. napus* is caused by two related filamentous dothidiomycete fungi, *L. maculans* and *L. biglobosa.* A major disease of worldwide importance, each year phoma stem canker disease is responsible for serious crop losses in countries where oilseed rape is widely grown, including Europe, Canada, Australia and China (where *L. biglobosa* and not *L. maculans* is currently widespread) (Zhang et al., 2013; Cai et al., 2017; Fitt et al., 2006a). *L. maculans* and *L. biglobosa* both have a global distribution, probably due to their ability to be transmitted on debris of several brassica crops (Fitt et al., 2006a). Twenty-five European countries, eight African countries, sixteen Asian countries, five countries in Oceania (including Australia) and regions across America have noted the occurrence of one of the two species (Fitt et al., 2006a). Both species are found, in differing relative frequencies, in the UK, France and Germany (West et al., 2001). *L. maculans* has spread globally, into Canada and eastwards across Europe into Poland (Fitt et al., 2006a; Stonard et al., 2009). China faces a significant threat to its oilseed rape production from *L. maculans* entering via imports, as the cultivars grown are highly susceptible to *L. maculans* (Fitt et al., 2008). Losses to phoma stem canker have been estimated by Eckert et al. (2009) to total c. £695 million globally. In the UK, using Crop Monitor's annual survey data, the disease was estimated to result in losses of *c.* £80 million in 2014, despite fungicide application (Cropmonitor.com). These losses occur when phoma stem canker lesions form on stems, leading to lodging or early senescence before harvest (West et al., 2001). Whilst phoma stem canker is associated with both *L. biglobosa* and *L. maculans*, the symptoms caused by the two pathogens are somewhat different in the UK, most identifiably, stem cankers from *L. maculans* are often located lower on the stem than the upper stem lesions associated with *L. biglobosa* infection (Figure 1.2.) (Fitt et al., 2006a).

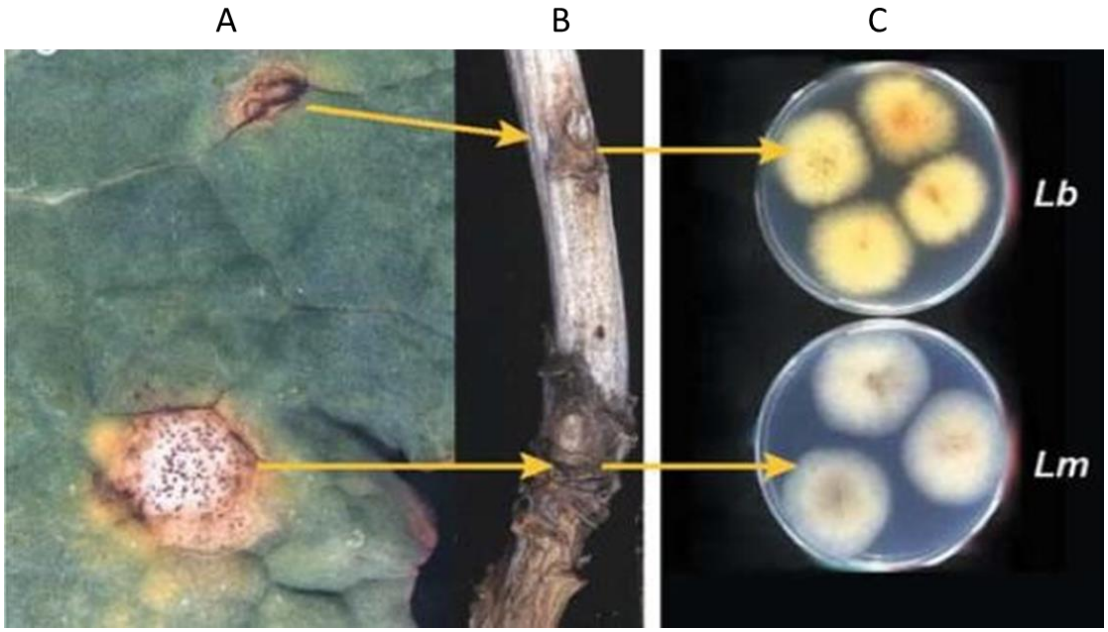


Figure 1.2 Differences between *L. biglobosa* and *L. maculans* (A) Symptoms on leaves: lesions from *L. biglobosa* are small and dark whilst *L. maculans* lesions are large and pale with pycnidia, (B) formation of stem cankers: the *L. biglobosa*-associated lesions are generally higher up the stem than the *L. maculans* basal stem canker and (C) fungal cultures on potato dextrose agar: the *L. biglobosa* colony has a yellow pigment, whilst the *L. maculans* colony is colourless when grown on potato dextrose agar. Fitt et al. (2006b).

1.4.2. *L. maculans* and *L. biglobosa* species complex

L. maculans is generally considered more damaging than *L. biglobosa*, as it causes severe stem base cankers, which result in crop lodging, early senescence and considerable yield loss (West et al., 2001). The less damaging upper stem lesions associated with *L. biglobosa* generally occur only in the outer cortex (Stonard et al., 2009). In the UK *L. biglobosa* also generally infects the crops slightly later in the season due to later production of ascospores (Huang et al., 2011). Due to their similar life cycles, in the past *L. maculans* and *L. biglobosa* have been classified as a single species. Separation of this species into two types (aggressive and non-aggressive) was first suggested by Cunningham (1927). The terms A-group (Tox+, aggressive) or B-group (Tox-, non-aggressive), were later reviewed (Williams and Fitt, 1999). An RFLP analysis done by Koch et al. (1991) on a global collection of 28 isolates found A group and B group isolates to be genetically very dissimilar. *L. maculans* and *L. biglobosa* were later distinguished as two species due to morphological differences in their pseudothecia (Shoemaker and Brun, 2001).

Unsuccessful *in vitro* attempts to carry out mating between isolates led to the suggestion that there are at least four genetically distinct forms of the pathogen within the former species *L. maculans* (Rouxel and Balesdent, 2005). The *L. maculans-L. biglobosa* species complex was further separated into various sub-groups, determined by their differing host ranges and pathogenic abilities (Grandaubert et al., 2014). A comparative genomic analysis was carried out by Grandaubert et al. (2014), exploring speciation in the *L. maculans-L. biglobosa* species complex and the role of transposable elements in reshaping genomes and the rise of species with improved pathogenic abilities. The subspecies *L. maculans* 'brassicae', the most damaging species on oilseed rape, was found to be the only member of the species complex to have a TE-invaded genome (32.5%). It is thought this has enabled this species to better evolve and adapt to its host. Nanopore sequencing of both species was done by Dutroux et al. (2018), generating a chromosome-level assembly for the *L. maculans* reference isolate JN3. These high-quality assemblies and annotations of *Leptosphaeria* genomes will aid progress in future studies on the interactions between *L. maculans* and oilseed rape.

1.4.3. Epidemiology of *L. maculans*

Living as a saprophyte, *L. maculans* can survive for several years on crop debris (Figure 1.3 -1) (West et al., 2001). During this time, ascospores are produced inside pseudothecia through sexual reproduction. As the primary inoculum, ascospores are produced in large numbers and can be carried many kilometres by the wind (Figure 1.3 -2). Upon landing on host cotyledons or

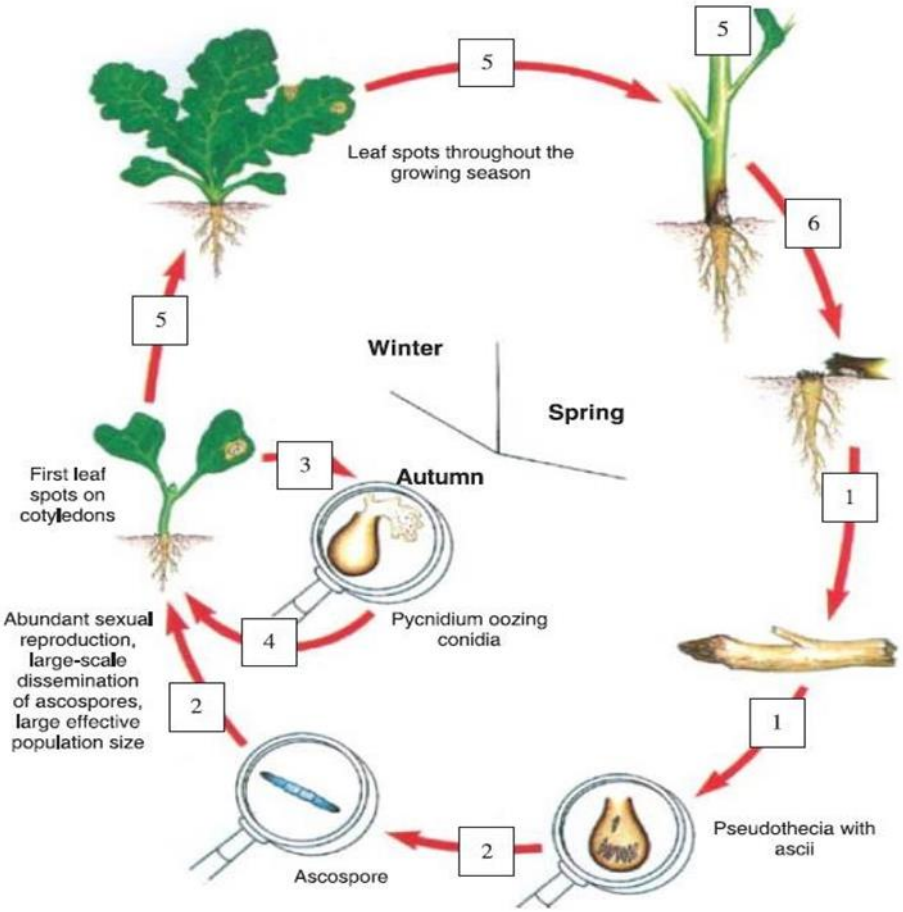


Figure 1.3 The life cycle of *L. maculans* occurring on winter oilseed rape in Europe

1. Saprophytic stage or long-term survival on stubble from the previous crop.
2. Sexual reproduction and production of the primary inoculum, ascospores.
3. Following the establishment of the primary infection, a short necrotrophic phase occurs as lesions develop on host leaves.
4. The asexual cycle occurs, whereby conidia are formed.
5. A long symptomless endophytic stage occurs, during which the pathogen colonises the plant, growing down from leaf tissues into the stem.
6. The pathogen undergoes a necrotrophic shift, and stem cankers develop.

Schematic from Rouxel and Balesdent (2005).

leaves, ascospores germinate, and germ tubes enter through stomata or wounds, proceeding to colonise host tissue asymptomatically. Subsequently, a short phase of necrotrophic growth results in leaf lesions (Figure 1.3 -3), within which the fungus multiplies asexually, and single-celled conidia are produced in pycnidia. These conidia are subsequently transmitted short distances by rain-splash (Figure 1.3 -4). Conidia are believed to be of little importance to the pathogen life cycle in Europe (Rouxel and Balesdent, 2005; West et al., 2001). A protracted period of endophytic colonisation of the host follows the leaf spot stage when the fungus grows endophyticaly from leaf tissues along the leaf petiole towards the base of the stem through intercellular spaces. The fungus moves along the petioles in vascular tissue into the stem. In the stem, hyphae grow in the vascular tissue before growing through the vascular walls and colonising the cortex (Sosnowski et al., 1999) (Figure 1.3 -5). In European winter oilseed rape crops, this phase can last up to 9 months (Rouxel and Balesdent, 2005; West et al., 2001). Towards the end of the growing season, the pathogen switches again to become necrotrophic, destroying the stem base and producing a crown canker, leaving the crop highly susceptible to lodging (Figure 1.3 -6) (Rouxel and Balesdent, 2005; West et al., 2001). It has been suggested that the contrasting stages in the life cycle of *L. maculans* (saprophytic, endophytic and necrotrophic) require the maintenance of a large range of genes. Thus, genes associated with nutrient acquisition and competition with soil flora are required for saprophytic life, genes for producing toxins and degradation enzymes are required for the necrotrophic stage and genes for suppression of host recognition and defence responses are required for the intercellular endophytic growth phase (Rouxel and Balesdent, 2005).

By studying the secreted fungal factors from extracellular apoplastic wash fluids of *Cladosporium fulvum* infected tomato leaves, rather than from media cultured *C. fulvum*, De Wit and Spikman (1982) first concluded that a host plant is required for expression of *Avr* genes. For example, *Avr9* is hardly expressed when *C. fulvum* is grown vitro but is highly expressed during its colonisation of tomato (Van den Ackerveken et al., 1994). Similarly, the *L. maculans*, avirulence genes were repressed during mycelial growth and drastically induced during the early stages of oilseed rape leaf infection (Rouxel et al., 2011). Following primary leaf infection, expression of avirulence genes reaches a peak at 7 days post inoculation (dpi) (Rouxel et al., 2011). How this upregulation is generated in *L. maculans* during *B. napus* infection was investigated by Soyer et al (2014). An epigenetic mechanism, made up of two proteins involved in heterochromatin formation (HP1) and maintenance (DIM-5), was found to modulate this expression, repressing *Avr* expression when the fungus is grown in axenic culture.

The sequencing of the *L. maculans* genome by Rouxel et al. (2011) found it to contain many transposable elements (around 30% of the genome). AT (adenine and thymine)-rich isochores, composed of transposable elements and very few genes were found to be the location of all currently cloned avirulence genes (Rouxel et al., 2011). To date, eight *L. maculans* avirulence genes have been cloned; *AvrLm1-L3*, *AvrLm2*, *AvrLm3*, *AvrLm4-7*, *AvrLm5-9, AvrLm6*, *AvrLm10A/10B* and *AvrLm11* and six others have been identified (Gout et al., 2006; Fudal et al., 2007; Parlange et al., 2009; Balesdent et al., 2013; Ghanbarnia et al., 2015; Van de Wouw et al., 2014).

1.4.4. Effect of climate change on phoma stem canker severity

Several studies have sought to evaluate the future threat of crop diseases under global warming, linking models of disease epidemics to climate change scenarios. Epidemics of phoma stem canker are favoured by higher temperatures and are expected to increase in severity in the UK due to climate change (Butterworth et al., 2010; Evans et al., 2010). A notably informative study was done by Butterworth et al. (2010) on the predicted impacts of climate change on crop yields in the UK. Findings from this study suggested that yield losses from phoma stem canker could be up to 50% in Southern England. These losses are predicted to occur because of increased temperature, the effect of which would outweigh any increase in yield associated with increased CO_2 concentration. Furthermore, the model showed that susceptible cultivars would suffer the greatest yield losses, a result highlighting the importance of successfully breeding new resistant cultivars. Despite the many careful considerations included in these studies, it is of importance to note that future disease risks will probably be affected by both technical developments in areas such as breeding and crop protection and evolutionary developments in the *L. maculans* populations (Elad and Pertot, 2014; Garrett et al., 2006; Juroszek and von Tiedemann, 2015; Siebold and von Tiedemann, 2012).

1.5. Control of *L. maculans* in *B. napus* crops

1.5.1. Chemical treatments

Chemical control options against *L. maculans* available to farmers include seed dressings, coated fertiliser granules and fungicide sprays applied to foliage or stubble (Aubertot et al., 2006; West et al., 2001). Foliar fungicide sprays are commonly used on crops in Western Europe where the potential yields are high, using ergosterol biosynthesis inhibitors (EBIs) (mainly triazoles), methyl

benzimidazole carbamates (MBCs) (mainly consisting of benzimidazoles) and succinate dehydrogenase inhibitors (SDHI) (Eckert et al., 2010; Sewell et al., 2016). While well-timed fungicide applications provide effective control of phoma leaf spot and substantial yield responses, growers now face reduced treatment options. Changes in EU legislation have led to a decrease in permissible treatments that are effective against the disease (Marx-Stoelting et al., 2014; Sewell et al., 2016). Furthermore, applications may become uneconomical if gross profit margins decrease.

1.5.2. Cultural control

The incidence of phoma stem canker can also be reduced through cultural control practices such as stubble removal, field selection and eradication of other susceptible hosts (such as volunteers and certain weeds). The principle aim of cultural control methods is to minimise the numbers of *L. maculans* ascospores landing upon the oilseed rape crop. As the pathogen survives on stubble, one way of achieving this is through effective stubble management. Stubble removal involves the burial of oilseed rape stubble or isolating new oilseed rape crops from the stubble of previous crops through crop rotations (Aubertot et al., 2006). A separation distance of at least 500 m was recommended by Marcroft et al., 2004 to growers of oilseed rape in Australia, beyond which the level of inoculum does not cause enough disease to significantly affect yield

1.5.3. Cultivar resistance

The identification of *R* genes and their use in breeding programmes is an effective approach to controlling *L. maculans* (Hayward et al., 2012). Thus, increasing resistance against the pathogen is a key focus for many oilseed rape breeding programmes (Hayward et al., 2012). Host resistance against *L. maculans* can be made up of quantitative (polygenic) or major *R* genes (Fitt et al., 2006a).

1.5.3.1. Quantitative resistance

Quantitative resistance is described as partial or non-race specific and is generally controlled by several genes (quantitative trait loci). Less well understood in comparison to major *R* gene resistance, quantitative resistance operates in the stem of adult plants during the symptomless growth of *L. maculans,* between the initial leaf spot phase and stem canker development (Figure

1.4) (Delourme et al., 2006; Huang et al., 2009). Quantitative resistance does not prevent the plant from being colonised by the pathogen but is able to reduce the severity of disease symptoms and slow progress of epidemics (Huang et al., 2009). The incomplete and inconsistent nature of this resistance phenotype make it difficult to study, thus the mechanisms controlling quantitative resistance remain poorly understood (Poland et al., 2009). Six hypotheses of the mechanisms underlying were set out by Poland et al., (2009).

1) Quantitative resistance is conditioned by genes regulating morphological and developmental phenotypes. 2) Quantitative resistance loci represent mutations or different alleles of genes involved in basal defense. 3) Quantitative resistance loci are components of chemical warfare. 4) Quantitative resistance loci are involved in defense signal transduction. 5) Quantitative resistance loci are weak forms of R-genes. 6) Quantitative resistance loci are a unique set of previously unidentified genes. It is very likely that several of these hypotheses are valid and that mechanisms for quantitative resistance differ between cultivars of the same species.

In *B. napus*, quantitative resistance against *L. maculans* is known to function in the stem tissue; Huang et al. (2009) used GFP-labelled *L. maculans* to show how the spread of the pathogen was restricted in a cultivar with good quantitative resistance. Quantitative resistance was observed to operate within petioles and stem tissues, preventing or restricting the spread of *L. maculans* and did not operate during the development of phoma leaf spots.

Quantitative resistance is more challenging for breeders to assess and select for than major *R* gene resistance, relying on field assessments of stem canker severity towards the end of the cropping season (Figure 1.5). In these assessments, it is not always clear whether canker symptoms result from colonisation by *L. maculans* or by *L. biglobosa.* Furthermore, this process is made more expensive and time-consuming due to the influence of genotype-by-environment interactions, which necessitate large replicated field plot experiments at various locations over several cropping seasons (Huang et al., 2014; McDonald, 2010). Due to this method of assessing adult plants, quantitative resistance is commonly referred to as ‘adult plant resistance’ (Delourme et al., 2006). It has recently been shown by Huang et al. (2014) that it is possible to assess young plants for quantitative resistance against *L. maculans* in *B. napus* in controlled conditions using leaf lamina or leaf petiole inoculation methods.

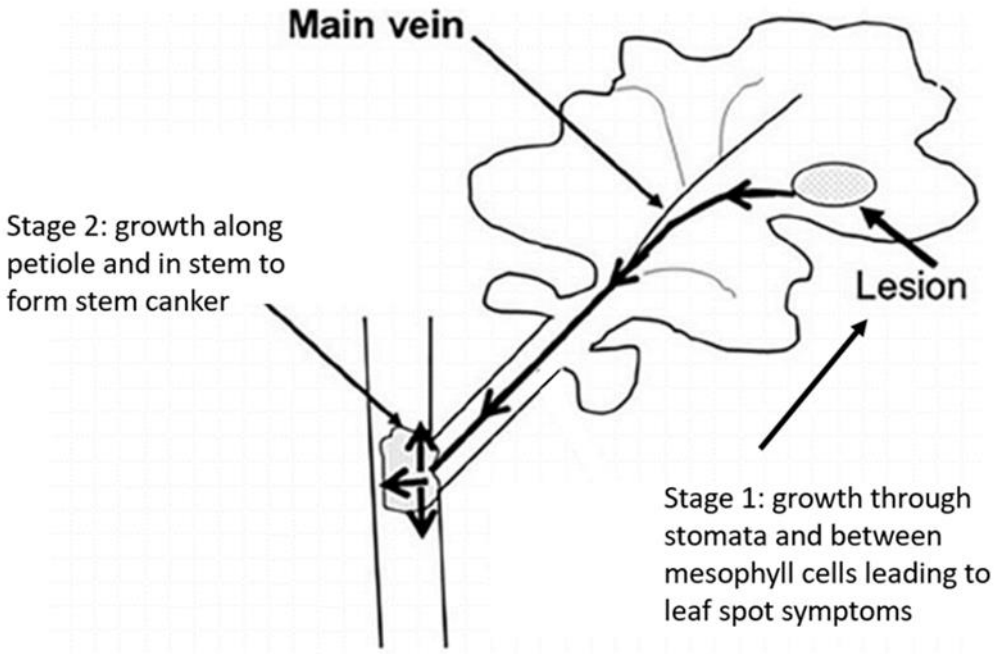


Figure 1.4 The two stages of colonisation of *B. napus* by *L. maculans*; the major *R* gene resistance is expressed during stage 1, in cotyledons and leaves, before the formation of leaf lesions. Quantitative resistance is expressed during stage 2, along the main veins and petioles of leaves and in the stem. Schematic adapted from Huang et al. (2014).

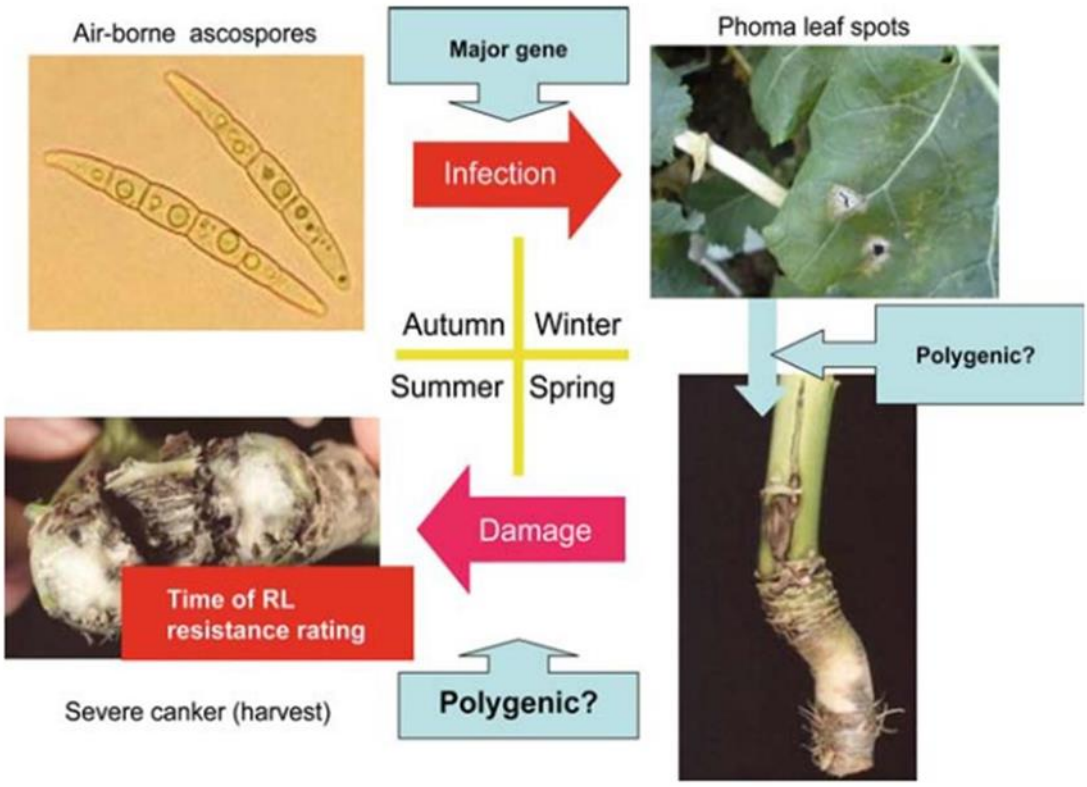


Figure 1.5 The seasonal cycle of phoma stem canker monocyclic epidemics in Europe highlighting the quantitative and major *R* gene components of *B. napus* resistance to *L. maculans*. Disease (phoma leaf spotting) is initiated in autumn by air-borne ascospores; the pathogen spreads along the leaf petioles to reach the stem, where phoma stem base cankers or upper stem lesions develop by harvest. A gene-for-gene specific host-pathogen interaction operates at the leaf infection and stem is not known. Assessments of quantitative resistance traditionally involve examination of the cross-section of the stem damaged by the pathogen in summer before harvest (from Fitt et al., 2006a).

Table 1.2 Major *B. napus* resistance genes and corresponding *L. maculans* avirulence genes identified to date. Adapted from Raman et al. (2013) and Van de Wouw and Howlett (2019).

B. napus resistance gene	*L. maculans* effector (*Avr*) gene	*B. napus* chromosomal location	Reference
Rlm1	*AvrLm1-L3*	A7	Ansan-Melayah et al. (1998)
Rlm2	*AvrLm2*	A10	Ansan-Melayah et al. (1998)
Rlm3	*AvrLm3*	A7	Delourme et al. (2004) Balesdent et al. (2002)
Rlm4	*AvrLm4-7*	A7	Parlange et al. (2009)
Rlm5	*AvrLm5-9*		
Rlm6	*AvrLm6*		
Rlm7	*AvrLm4-7*	A7	Parlange et al. (2009)
Rlm8	*AvrLm8*		
Rlm9	*AvrLm9*	A7	Delourme et al. (2004)
Rlm10	*AvrLm10A/ AvrLm10B*		
Rlm11	*AvrLm11*		
LepR1	*AvrLepR1*	A2	Yu et al. (2005)
LepR2	*AvrLepR2*	A10	Yu et al. (2005)
LepR3	*AvrLm1*	A10	Larkan et al. (2012)
RlmS	*AvrLmS*		Van de Wouw et al. (2009)

1.5.3.2. Major *R* gene resistance

Major *R* gene resistance against *L. maculans* is controlled by single dominant *R* genes and is expressed in cotyledons and leaves during the first symptomless phase following penetration by hyphae from the ascospores (Figure 1.4) (Delourme et al., 2006). As it prevents leaf lesion development, subsequent stem cankers do not develop (Huang et al., 2014). Eighteen *L. maculans* race-specific *R* genes have been identified in the *B. napus* genome, as summarised in Table 1.1. These *R* genes are all located on the *B. napus* A genome; to date only three have been found on the *B. juncea* B genome and none on the C genome (Larkan et al., 2013). *LepR3* was the first *B. napus* disease resistance gene to be cloned and was found to encode a receptor-like protein (Larkan et al., 2013). The *Rlm2* resistance gene, also cloned by Larkan et al. (2015), is co-localized in the same region of chromosome A10. *Rlm2* and *LepR3* were found to be allelic variants of the same LRR-RLP locus.

1.5.3.2.1. Complex gene-for-gene complementarities

The gene-for-gene concept, first described by Flor (1971), states that dominant *R* genes encode receptors that specifically recognise a corresponding effector, encoded by an *Avr* gene of the pathogen, to confer resistance. Whilst this concept remains an important foundation in understanding plant-pathogen interactions, environmental factors (e.g. temperature) and other genetic factors add extra layers of complication. The R / Avr system is quite complex in the *B. napus* - *L. maculans* pathosystem; observed redundancy in *R* genes against an *L. maculans* effector is a clear example that Avr proteins don't always interact specifically with a single R-protein (Larkan et al., 2012). *AvrLm4* and *AvrLm7* are allelic variants of one *Avr* gene (*AvrLm4-7*), corresponding to the two *R* genes *Rlm4* and *Rlm7* (Balesdent et al., 2002; Raman et al., 2013). Furthermore, *AvrLm1* also interacts with two distinct *R* genes, *Rlm1* and *LepR3*, located on separate chromosomes (Larkan et al., 2012). Sequence comparison of *AvrLm5* alleles found a single point mutation to correlate with the *AvrLm9* phenotype. Thus, this gene was renamed as *AvrLm5-9* to reflect the dual specificity of this locus. The recognition of *AvrLm5-9* by *Rlm9* is masked in the presence of *AvrLm4-7* (Ghanbarnia et al., 2018). Two *AvrLm10* candidates were identified as two genes (*AvrLm10A* and *AvrLm10B*) in opposite transcriptional orientation and were both found to be necessary to trigger *Rlm10* resistance (Petit-Houdenot et al, 2019).

Such unconventional relationships have also been observed in other plant-pathosystems. The reaction of barley powdery mildew (*B.g. hordei*) to some *Mla* resistance alleles is controlled by multiple genes. Multiple genes are also involved in the specific interaction of wheat mildew (*B.g.*

tritici), in which *Pm3* alleles and two mildew genes control avirulence on *Pm3* (Bourras et al, 2016). *Fusarium oxysorum f* sp. *Lycopersici* encodes *Avr1* which can be recognised by the tomato *R* gene *I-1* and act to suppress the recognition of *Avr2* and *Avr3* by *I-2* and *I-3*, respectively (Houterman et al, 2008). A long co-evolution of pathogens with their host plant may explain the ability of one *Avr* gene to mask another. The recognition of a single *Avr* gene by multiple *R* genes could be a result of new crops being produced through interspecific hybridization and also in the breeding technique of introgression of *R* genes from related species (Petit-Houdenot et al, 2019). With these factors in mind, Flor's gene-for-gene theory should be expanded upon to consider current knowledge.

1.6. Molecular aspects of plant defence responses in *B. napus* against *L. maculans*

There are hurdles in moving from the model plant *A. thaliana* to crop species like *B. napus* in genetic studies. Although both species belong to the Brassicaceae family, difficulties arise in determining gene function through comparing diploid and an amphidiploid species; *B. napus* has many more genes compared to *A. thaliana*, thus one gene in *A. thaliana* can relate to several in *B. napus*. Several studies have focused on the molecular mechanisms of the defence response of *B. napus* to *L. maculans*, including Sasek et al. (2012) and Haddadi et al. (2015). Becker et al. (2017) used both *B. napus* and *A. thaliana* in an investigation of the ETD pathway. RNA sequencing identified unique genes and plant defence pathways specific to plant resistance in *B. napus* against *L. maculans* (*LepR1* - *AvrLepR1*) interaction over time while the *A. thaliana – L. maculans* model pathosystem was used to functionally characterise the genes involved. A study by Haddadi et al., 2019 provided further insight into the molecular mechanism of host genetic background and *R* gene effect through transcriptome analysis on *B. napus* lines carrying one of four blackleg *R* genes (*Rlm2*, *Rlm3*, *LepR1* or *LepR2*) in Topas or Westar background throughout time following an incompatible interaction with *L. maculans*.

1.6.1. *B. napus R* gene recognition of *L. maculans*

Since *L. maculans* grows between the host's mesophyll cells, releasing effectors into the apoplast, host recognition relies on extracellular recognition of effectors by a class of R proteins known as membrane bound receptor-like proteins (RLPs). These cell surface RLPs are integral to the cell membrane and possess an extracellular LRR domain and a short cytoplasmic tail lacking a signalling motif (Stotz et al., 2014). As RLPs do not possess cytoplasmic signalling domains,

upon ligand detection, RLPs instead stimulate cellular responses through interaction with receptor-like kinases (RLKs). Figure 1.6. presents this mechanism and the possible outcomes for *B. napus* challenged with *L. maculans* describing ETD. The receptor-like kinase suppressor of BIR1-1 (*SOBIR1*) is known to play a key role in RLP-mediated ETD as an essential regulatory RLK, functioning specifically in receptor complexes with RLPs (Liebrand et al., 2013; Stotz et al., 2014). An identified RLK, BRI1-ASSOCIATED KINASE-1 (*BAK1*) plays a key role in the general regulation of LRR-RLP-containing plasma membrane-associated receptor complexes (Liebrand et al., 2013).

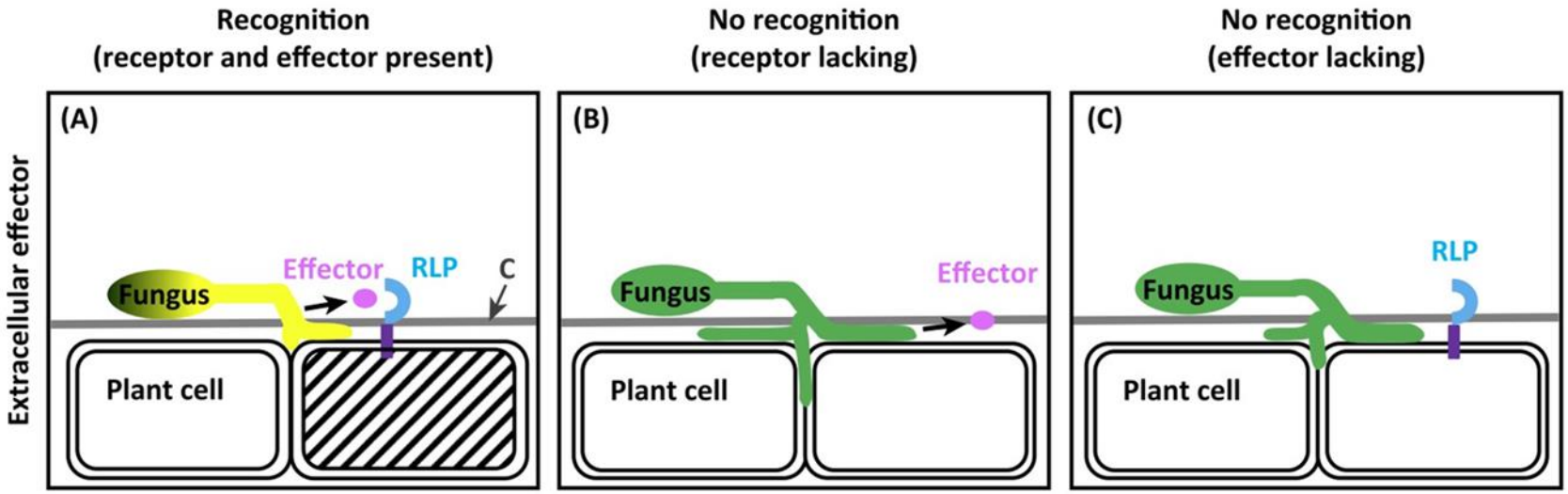


Figure 1.6 Possible outcomes for *B. napus* challenged with *L. maculans* describing effector triggered defence

A. *R* gene-mediated ETD results in incompatible interactions with *L. maculans*. Extracellular recognition of effectors from *L. maculans* growing between mesophyll cells by RLPs can lead to a delayed host cell death, generally occurring several days post-infection in just a few cells.

B. If the *RLP* is absent, a compatible interaction occurs, whereby the host remains alive, and the effectors contribute to pathogen virulence, with the extensive proliferation of *L. maculans*.

C. If the pathogen effector is absent, a compatible interaction occurs. However, the proliferation of *L. maculans* may be less extensive than in (B). Stotz et al. (2014)

1.6.2. Early signalling pathways in the resistance response

Complex signalling pathways mediate early responses in the host plant cells. The initial response to the primary recognition event takes place immediately downstream and involves the activation of ion fluxes and H_2O_2 production. Host recognition of the pathogen is relayed through a series of pathways using G proteins, differences in concentrations of cytosolic Ca^{2+} and protein kinases/phosphatases controlling the activity of key enzymes. This leads several biophysical and biochemical processes to occur before defence-related genes become transcriptionally activated.

Alongside many other cellular processes, plant responses to pathogens are regulated by alterations in concentrations of cytoplasmic Ca^{2+}. Among the earliest cellular responses are Ca^{2+} fluxes, and tight coordination between receptor complex activation and Ca^{2+} fluxes is predicted. BON1 is a regulator Ca^{2+}, interacting with autoinhibited calcium ATPase10 and 8 (ACA10/8) on the plasma membrane to regulate calcium signals in *A. thaliana* (Yang et al., 2017). It is thought that early signal branching at/ just after the receptor level might be coordinated by different Ca^{2+} amplitude and/or temporal differences to stimulate diverse Ca^{2+}-dependent signal flows into various outcomes (Seybold et al., 2014). PAMPs and effectors are both known to induce an influx of Ca^{2+} from the apoplast, causing cytoplasmic Ca^{2+} concentrations to rapidly increase. Free Ca^{2+} ions act to transduce a stimulus to target proteins that guide the cellular response. PAMP recognition rapidly leads to an influx of extracellular Ca^{2+} in the cytosol, occurring at 30 sec - 2 min and reaching a maximum at *c*. 4 – 6 min after recognition (Jeworutzki et al., 2010; Bigeard et al., 2015). High cytoplasmic Ca^{2+} concentrations act as a second messenger to promote the opening of other membrane channels and transporters (Boller and Felix, 2009). In this way, influx of Ca^{2+} results in an influx of H^+ and an efflux of K^+, Cl^-, and NO^{3-}, which causes extracellular alkalinisation (after 1 min) and plasma membrane depolarization (at 1–3 min) (Jeworutzki et al., 2010; Bigeard et al., 2015). Furthermore, high Ca^{2+} concentrations activate calcium-dependent protein kinases (CDPKs). Ca^{2+}-induced CDPKs are required (along with BIK1) for the activation (by phosphorylation) of an NADPH oxidase called respiratory burst oxidase homolog D (RbohD). Calmodulin is also activated by Ca^{2+} and required for the synthesis of nitric oxide (NO) (Bigeard et al., 2015).

Heterotrimeric G proteins (guanine nucleotide-binding proteins) act as molecular signal transducers inside cells, playing a key role in transmitting signals from stimuli outside of the cell to the interior. Their active state is dependent on whether they are bound to GTP (on) or GDP (off) (Zhang et al., 2012). Mitogen-activated protein kinases (MAPKs) are signal transduction

proteins used for transducing extracellular signals from receptors to downstream cellular responses (Bigeard et al., 2015; Innes, 2001). Plant MAPK cascades have been found to play important roles in signalling plant defence against pathogen attack (Meng and Zhang, 2013). MAPK cascades change gene expression by differentially altering phosphorylation status of transcriptions factors in the nucleus (Pearson et al., 2001).

1.6.3. Resistance responses

1.6.3.1. The oxidative burst

The oxidative burst involves the rapid accumulation of ROS, such as hydrogen peroxide (H_2O_2) and NO, and is widely thought to be an early response to pathogens, before HR (Sasek et al., 2012). NO, and its derivatives (reactive nitrogen species) are thought to act both as inducers of defence-related proteins and as toxins causing the death of plant and pathogen cells. Reactive nitrogen species are involved at various steps of signal transduction. Reactive nitrogen species regulate *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*), a key regulator of defence gene expression and RBOHD via cysteine S-nitrosylation (Bigeard et al., 2015). In PTI, the oxidative burst takes place within a few minutes of PAMP perception (Boller and Felix, 2009; Lloyd et al., 2014). Following an incompatible reaction between *L. maculans* and *B. napus,* however, H_2O_2 was found to be produced much later, at 8 dpi (Sasek et al., 2012). Stotz et al., (unpublished) found H_2O_2 after 7 dpi but not at 3 dpi.

A plasma membrane NADPH oxidase enzyme has been found to play a role in the oxidative burst triggered by various plant microorganisms (Torres et al., 2006). During incompatible interactions, NADPH oxidase RBOHD produces superoxides ($O_2^{\bullet-}$) in the apoplast of elicited cells. $O_2^{\bullet-}$ is converted into H_2O_2 by superoxide dismutases (SOD) (Bigeard et al., 2015). NADPH-mediated ROS production is known to be regulated through signal transduction pathways such as calcium influx, phosphatidic acid (PA), 14-3-3 proteins, nitrogen oxide (NO), phosphorylation and G proteins (Bigeard et al., 2015).

1.6.3.2. Changes in pathogenesis-related protein expression

Pathogenesis-related proteins (PRs) are defined as proteins encoded by the host plant but induced specifically in response to a pathogen or related stimuli (van Loon and van Strien, 1999). These PR proteins are currently divided into seventeen different groups, in which classification

is based on biochemical activity, protein sequence similarities and other biological features (Ali et at., 2018). Properties of these PR proteins include chitinase, β-1,3-glucanase, antifungal, proteinase inhibitor, endoproteinase and peroxidase (Sudisha et al., 2012). The PR1 group of PR proteins are accumulated to high levels after pathogen infection (Selitrennikoff, 2001). As expression of *PR1* is SA responsive, *PR1* is commonly used as a SA-responsive marker gene (Sasek et al., 2012).

As *L. maculans* switches from endophytic to necrotrophic growth, an up-regulation of genes associated with JA in the *B. napus* host has been found to correlate with this change in the pathogen life stage (Haddadi et al., 2015). Several transcription factors, including *WRKY* types, are up-regulated in *B. napus* in response to *L. maculans. WRKY70* is a component in the SA-mediated signal pathway. *WRKY70* expression is activated by SA and repressed by JA (Li et al., 2004).

Genes involved in the reinforcement of cell walls and the production of glucosinolates are amongst several plant defence-related genes that are differentially expressed following the detection of *L. maculans.* Genes involved in the early stages of lignin biosynthesis were also found by Haddadi et al. (2015) to be upregulated from 4 dpi in a compatible interaction in a susceptible host. Coordinated lignin deposition was also observed by Becker et al. (2017) in susceptible and resistant *B. napus* host cotyledons following infection with *L. maculans*. While resistant hosts showed prominent and coordinated deposition of lignin proximal to the infection site and surrounding vasculature, lignin deposition in susceptible hosts was uncoordinated and diffuse.

1.6.4. Role of plant hormones in defence responses

Plant hormones are well known to play an important role in regulating signal transduction following pathogen recognition (Pieterse et al., 2012). The principle hormones regulating the plant defence response are SA, JA and ET (Nováková et al., 2015). Other plant growth regulators such as gibberellins, cytokinins, ABA and auxins, also play a role in the modulation of plant defence (Pieterse et al., 2012). SA is an important signal for inducing a local resistance response and systemic acquired resistance (SAR) in plants (Liu et al., 2011). SAR is a mechanism of induced defence conferring long-lasting protection against a broad spectrum of pathogens (Durrant and Dong, 2014).

McDowell and Dangl (2000) first suggested that the plant defence response varies from pathogen to pathogen and that biotrophic pathogens activate the SA pathway while the JA and ET pathways defend against necrotrophs (Glazebrook, 2005). However, plant responses against hemibiotrophic pathogens, such as *L. maculans*, are less understood (Bohman et al., 2004; Liu et al., 2008).

Findings by Sasek et al. (2012) and Haddadi et al. (2015) both suggest that in *B. napus*, both SA and ET are upregulated in response to infection by *L. maculans*. However, the upregulation of hormone biosynthetic genes does not occur immediately following a molecular stimulus. For instance, genes associated with ET biosynthesis were found to be upregulated after 1 hour, reaching a maximum after 4 hours, following an flg22 stimulus in *A. thaliana* seedlings (Liu and Zhang, 2004). SA production in *A. thaliana* seedlings is induced between 3 and 6 hours after flg22 treatment, only reaching a maximum by 9 hours (Tsuda et al., 2008). Thus, there is a time lag of several hours between the initial Ca^{2+} and MAPK signalling and hormone signalling following pathogen recognition.

1.7. Cross-talk between signalling pathways

Induced defence mechanisms are also used to protect plants against herbivore damage as well as for defence against pathogens. Mechanical wounding of leaf tissue is associated with increases in ET, JA, and ABA, collectively triggering cellular senescence at the wounding site. Thus, a host's response against herbivorous insects, dependant on the JA pathway, may act in conflict with SA pathways inducing local HR and SAR responses against many plant pathogens (Stotz et al., 2000; Maleck and Dietrich, 1999). Infection of *B. napus* by *L. maculans* has been found to be enhanced by the concurrent feeding of weevils (*Ceutorhynchus pallidactylus* and *C. napi*) producing more entry sites on the host plant (Alford et al., 2003; Taylor et al., 2004). The effect of wounding on gene expression is also a concern when doing *L. maculans* inoculation experiments for investigations into the pathogen-induced defence response, as commonly used inoculation methods involve wounding *B. napus* cotyledons.

1.8. The durability of disease resistance

Durable resistance was first defined by Johnson (1981) as 'a resistance that remains effective during its prolonged and widespread use in an environment favourable to the disease'. This type of resistance, whether quantitative or *R* gene-mediated, is commonly used to predict the

durability of a cultivar against disease. While quantitative resistance is commonly associated with durable resistance, *R* gene-mediated resistance is often associated with resistance breakdown after growth of the same cultivars over successive growing seasons. This is because single *R* gene-mediated resistance is isolate-specific, therefore causing pathogen populations to rapidly adapt and increase the percentage of virulent isolates through selection and multiplication (Brun et al., 2010).

Large-scale growth of oilseed rape cultivars with single *R* gene resistance exerts strong selection on populations of *L. maculans* to adapt. Examples of this was seen following the widespread growth of cultivars with *Rlm1* resistance in France, which led to a rapid decrease in the proportion of *L. maculans* isolates carrying *avrLm1* (Rouxel et al., 2003; Zhang et al., 2015). Effective *LepR3* resistance in *B. napus* material was also rapidly lost in some parts of Australia in 2002, shortly after it was deployed in 2000 (Sprague et al., 2006).

Another instance of a single *R* gene being overcome is in the case of *Rlm3* in Canada, where it was introduced in *B. napus* cultivars in the early 1990s providing good resistance against *L. maculans*. However, by early 2000s, several moderate to severe outbreaks were observed as the pathogen evolved to overcome *Rlm3* (Zhang and Fernando, 2017).

1.9. Aim and objectives

This project aims to further understand the temperature-sensitivity of *B. napus* resistance against *L. maculans*. A combined approach was chosen to explore different elements of this topic, with three separate lines of study. The first approach explores the relevancy of effect of increased temperature on phoma severity in agriculture and how different quantitative resistance backgrounds and single *R* genes can affect this. The second study takes a more mechanistic approach to understand temperature-sensitivity by investigating *BrSNC1*, hypothesised to play a role, using reverse genetics with a TILLING population of the ancestral diploid species *B. rapa*. The third approach explored differences in defence-related gene expression to compare temperature-sensitive and -resilient introgression lines at 20°C and 25°C. For the purpose of this study, temperature-resilience is defined as maintaining a constant resistance phenotype at 20°C and 25°C, whilst temperature-sensitive resistance is lost at 25°C. Together the findings from these approaches may be utilised by breeders in the development of oilseed varieties that are better adapted to overcome the impact of climate change on diseases. The three main objectives are:

1. To investigate the effects of temperature on *R* gene-mediated and quantitative resistance against *L. maculans* in *B. napus* plants through field and controlled environment experiments.

2. To investigate the contributions of *SNC1* and *FocBo1* homologs in the temperature-sensitivity of *R* genes in *B. rapa* against *L. maculans* by conducting inoculation assays on TILLING mutant lines at 20°C and 25°C.

3. To investigate the defence response through time of temperature-sensitive and -resilient *R* genes by comparing the responses of *B. napus* single *R* gene introgression lines (ILs)inoculated with *L. maculans* at 20°C and 25°C.

2 Influence of increased temperature on phoma stem canker

2.1. Introduction

In the defence of *B. napus* against *L. maculans*, single *R* genes are reported to function during the first colonisation phase in cotyledons and leaves. Thus, in the first few months after drilling in September, single *R* genes are known to be very important in defending the crop against the phoma stem canker pathogen. Quantitative resistance has been described as operating after the initial leaf colonisation, during the second symptomless growth phase of the pathogen, before the formation of stem cankers (Pilet et al., 1998; Delourme et al., 2006; Huang et al., 2009). The mechanisms behind quantitative resistance are poorly understood and the potential role of *R* genes in defending against the pathogen during later colonisation stages has not been explored.

Increased temperature is thought to be linked to more severe phoma stem canker in winter oilseed rape crops. Previous studies have agreed that in seasons experiencing elevated temperatures and increased rainfall, the efficacy of *R* genes is negatively affected, and canker severity is greater (Huang et al., 2018; Evans et al., 2007; Huang et al., 2006). Cotyledon assays show clear differences between *R* genes in their resilience to maintain efficacy under elevated temperatures. Less is known about how these *R* genes respond individually to temperature in crops. Some work has been done in determining which months are most significant in affecting phoma severity; Huang et al. (2018) found phoma leaf spotting and canker severity to be linked to October and June average temperatures, respectively. This severity analysis did not explore the impact of maximum monthly temperatures. There is some evidence that maximum temperatures may influence canker severity. A multiple linear regression analysis, on 40 datasets by Evans et al. (2007), indicated that mean maximum daily temperature and total rainfall (between 15 July and 26 September) produced the best prediction of the start date of the phoma leaf spotting epidemic for all sites and growing seasons included.

Conclusions drawn from investigations into the response of quantitative resistance at increased temperatures are somewhat conflicting. Huang et al. (2009) found, by analysing stem cross-sections, the efficacy of quantitative resistance to be reduced when a cultivar with good quantitative resistance was exposed to an elevated temperature of 25°C compared to 15°C. Whilst more severe cankers were observed on the cultivar without quantitative resistance at 15°C, no significant difference between the two cultivars canker severity at the higher temperature were observed, suggesting that temperature affects the operation of quantitative

resistance to *L. maculans*. An experiment by Hubbard and Peng (2018) subjected *L. maculans* inoculated *B. napus* cultivars containing quantitative resistance to a temperature regime designed to mimic a heat wave, rising to 32°C daytime temperature for 7 hrs before dropping to 18°C for 7 hrs overnight. No difference in disease severity was found compared to plants grown at a moderate temperature regime of 22°C daytime/ 16°C overnight; suggesting quantitative resistance can maintain efficacy at increased temperatures. How temperature affects the operation of quantitative resistance remains poorly understood. Furthermore, although quantitative resistance has been commonly described as occurring at later stages in a plants development, even being called 'adult plant resistance', results of a study into the potential operation of quantitative resistance in young plants by Huang et al. (2014) found that quantitative resistance appears to also be expressed at early stages of plant development.

2.1.1. Objectives

The work in this chapter aims to determine how quantitative resistance and different *R* genes impact upon the severity of phoma stem canker of winter oilseed rape cultivars in crops, specifically in relation to June temperature. A cotyledon experiment was done on the same set of cultivars to determine if field results could be predicted, to see if quantitative resistance can have any effect on the resistance response at the cotyledon stage. A selection of *B. napus* lines with genotypes possessing a combination of good or little quantitative resistance and *Rlm4*, *Rlm7* or *LepR3* were used in field and controlled environment (CE) experiments. The second experiment in this chapter examines the effect of elevated temperature on the quantitative resistance response during the second symptomless phase in the colonisation process of *L. maculans* on *B. napus*. It also investigates whether *R* genes play a role in defence against the pathogen in the stem, and how it may be affected by increased temperature from 20°C to 25°C. Specifically, objectives were:

1. To analyse field and weather data to see if there is a correlation between canker severity in different cultivars and maximum monthly temperature throughout the growing season.
2. To assess selected *B. napus* lines for temperature-sensitivity and resilience in CE cabinets see if there is a correlation between phoma canker severity scores in crops.
3. To see if increasing the ambient temperature from 20°C to 25°C reduces efficacy of the quantitative resistance response by doing stem inoculation assays on *B. napus* cultivars with different levels of quantitative resistance, using a virulent *L. maculans* isolate.

2.2. Materials and methods

2.2.1. Winter oilseed rape field experiments

2.2.1.1. Experimental set up

To investigate the effects of cultivar resistance and weather on the severity of phoma stem canker, field experiments were run for three growing seasons (2016-2017, 2017-2018 and 2018-2019) at various sites in England and France. Locations for 2016-2017 were Impington, Cambridgeshire, UK (52.253824, 0.125801) and Châteauroux, France (46. 5319, 1.3758). Only one site was included for 2017-2018 Wisbech, Cambridgeshire, UK (52.695707, 0.081937458) as at the other site in Châteauroux, France (46.5319, 1.3758) the crop failed to establish due to severe flea beetle damage. The field site did not establish again for 2018/19; two sites were used in the UK; Callow, Herefordshire (51.994688, -2.756194) and Wisbech, Cambridgeshire (52.619527, 0.16128927).

Thirteen winter oilseed rape cultivars were selected for field experiments (Table 2.1). The rationale for the choice of cultivars was to include current breeding lines containing different combinations of 'good' or 'little' quantitative resistance and *R* genes *Rlm4, Rlm7* or *LepR3* (as advised by breeders). Current UK cultivars and breeding lines were included in the study to determine if temperature-resilient characteristics are present in available oilseed cultivars. Although cultivars were classified as having 'good' or 'little' quantitative resistance, the mechanisms of two 'good' cultivars may be completely different (as discussed in section 1.5.3.1). Furthermore, other differences in the genetic backgrounds of these cultivars may also influence their response to the environment and impact on the severity of phoma stem canker. Thus, there are clear limitations for this study. Nevertheless, in the absence of a set of oilseed lines differing only in their quantitative resistance trait loci, this set of cultivars provide a good start in investigating the effect of temperature on the quantitative resistance response.

Eight of the cultivars possess *R* genes with a quantitative resistance background; DK Exception (*Rlm7* + quantitative resistance), Cultivar A (*Rlm7* + quantitative resistance), Adriana (*Rlm4* + quantitative resistance), JetNeuf (*Rlm4* + quantitative resistance), Cultivar C (*Rlm4* + quantitative resistance), Cultivar D (*Rlm4* + quantitative resistance), Cultivar F (*LepR3* + quantitative resistance) and Cultivar G (*LepR3* + quantitative resistance). Three of the cultivars possess *R* genes without a quantitative resistance background; Cultivar B (*Rlm7*), Cultivar E (*Rlm4*) and Cultivar H (*LepR3*). Cultivar ES Astrid contains no *R* genes but has a quantitative resistance background. Cultivar Incentive, which has no known *R* genes or quantitative resistance, was used as a susceptible control.

Table 2.1 Winter oilseed rape cultivars used in this chapter with information of any known single *R* gene or quantitative resistance as indicated by breeders. Cultivars were categorised into eight groups, depending on their combination of *R*-gene and quantitative resistance.

***R*-gene resistance**	**'Good' quantitative resistance**	**'Little' quantitative resistance**
Rlm7	**Group 1** DK Exception[1], Cultivar A[1]	**Group 2** Cultivar B[1]
Rlm4	**Group 3** Adriana[1], Jet Neuf[1,2], Cultivar C[1], Cultivar D [1]	**Group 4** Cultivar E [1,2]
LepR3	**Group 5** Cultivar F [1], Cultivar G [1]	**Group 6** Cultivar H [1]
None	**Group 7** ES Astrid[1,2]	**Group 8** Incentive[1], Cultivar I [2]

Numbers in superscript refer to experiment in which the cultivar was used; Winter oilseed rape field experiment and temperature-sensitivity assay [(1)], adult plant CE temperature-sensitivity assay [(2)].

NPZ cultivars are Cultivar A, Cultivar B, Jet Neuf, Cultivar C, Cultivar D, Cultivar E, Cultivar F, Cultivar G, Cultivar H and Cultivar I. DK Exception is from DEKALB, Incentive is from DSV, Adriana is from Limagrain and ES Astrid is from Euralis.

The field experiments were arranged in randomised block designs with two or three replicates. Seeds were sown between August and early September, at a density of 45 seeds/m^2 in France and 55 seeds/m^2 in the UK. Plots were 3m x 2m for Châteauroux (2016/17), Impington (2016/17) and Wisbech (2017/18), and 8.6m^2 for Wisbech (2018/19) and Callow in (2018/19).

2.2.1.2. Phoma leaf spotting and stem canker severity assessment

The severity of phoma stem canker was assessed in July, prior to harvest, and fifteen plants were pulled from each plot. The stems were cut at the base, immediately above the root collar and the area of necrotic tissue caused by phoma stem canker in the cross-section was scored using a 1-6 scale (Pilet et al., 1998; Delourme et al., 2008); 1 = no affected tissue; 2 = 1-5% area affected; 3 = 6-50% area affected; 4 = 51-75% area affected; 5 = 76-100% area affected, plant alive, and 6 = 100% area affected, stem broken or plant dead.

2.2.1.3. Weather data at field site

Monthly average maximum temperature and total rainfall data were obtained for the five field site locations to assess their effects on canker severity. Weather data were obtained from the NASA Langley Research Centre Atmospheric Science Data Centre Surface meteorological and Solar Energy (SSE) web portal supported by the NASA LaRC POWER Project (https://power.larc.nasa.gov/data-access-viewer/). Average maximum monthly temperature and maximum June temperature were used for analysis to investigate increased ambient temperatures.

2.2.2. Cotyledon inoculation-assessment of cultivars used in field experiments at 20°C and 25°C

To investigate the temperature-sensitivity of *R* genes in cultivars used in the field experiments (Table 2.1), a cotyledon temperature-sensitivity assay was done using *L. maculans* isolate JN3 (*AvrLm1-4-5-6-7-8*). The assay was repeated twice, each with eight plants per treatment for all cultivars, apart from cultivars Jet Neuf, Cultivar E and Cultivar C, for which the assay was done once.

2.2.2.1. Preparation of *L. maculans* conidial suspensions

To prepare inoculum, selected *L. maculans* isolates were sub-cultured in the centre of V8 agar plates (Appendix A.1). Plates were placed in darkness for four days. Then multiple plugs, taken from the edges of mycelial growth, were placed on new V8 plates. After two more days in darkness, the plates were placed in a CE cabinet (ARALAB, Portugal) set to a repeated cycle of 12 hr light at 20°C and 12 hr darkness at 18°C to induce pycnidial development.

Petri dishes were flooded with distilled water, and a glass rod was used to rub along the surface to release the *L. maculans* conidia. The resulting suspension was then filtered through Mira cloth (Calbiochem, USA) into a Falcon tube. The concentrations of conidial suspensions were measured using a haemocytometer and diluted with sterile distilled water to provide concentrations of 10^7 conidia ml^{-1}. Aliquots of conidial suspensions were stored at -20°C.

2.2.2.2. Plant growth and inoculation of cotyledons

Seeds were sown on discs of filter paper, dampened with distilled water, inside Petri dishes. Petri dishes were placed at 4°C for 1 – 3 days to synchronise germination and growth. Petri dishes were then moved to a windowsill for up to two days for germination at an ambient temperature of 20°C. Light intensity at plant height was measured to be 320 µmol/m^2/s. Seedlings were then transferred into a 1:1 ratio of John Innes number 3 and Miracle-Gro compost in 50-cell trays. Plants were grown in CE chambers set to a 12-hour light/ 12-hour darkness cycle with a constant temperature of 20°C and a relative humidity of 70%. Twenty-four hours prior to inoculation, plants being treated at an elevated temperature were moved into another CE chamber, set to the same program but with an increased temperature of 25°C.

Cotyledons were inoculated 10 days after being placed in soil, by wounding followed by droplet inoculation. A sterilised needle was used to pierce two wounds on the adaxial surface of each cotyledon. A 10µl droplet of 10^7 conidia ml^{-1} suspension was placed directly onto the wound site using an electronic pipette (StarLab, Milton Keynes, UK). Following inoculation, seedlings were kept in the dark at high relative humidity for 24 hours. Plastic lids, misted with water, were placed on trays to ensure high relative humidity while wrapping the trays in black plastic ensured darkness. Following inoculation, true leaves were regularly removed from seedlings to allow full expansion of cotyledons and prevent senescence. Plants were watered from the base.

2.2.2.3. Assessment of symptoms

Phoma cotyledon lesions were scored at 12 dpi using a continuous 0-9 scale adapted from that of Koch et al. (1991) (Appendix A.2). 0 = no darkening around wounds, as in controls; 1 = limited blackening around wounds, lesion diameter 0.5-1.5 mm; 3 = dark necrotic lesions 1.5-3 mm; 5 = dark lesions 3-6 mm, brownish on lower surface; 6 = same as in 5, but less necrotic; 7 = grey-green lesions of limited size with no or few pycnidia; 9 = large grey-green lesions with profuse sporulation.

2.2.3. Effect of quantitative and *R* gene resistance on canker severity at 20°C and 25°C in adult plants

2.2.3.1. *L. maculans* isolate growth at 20°C and 25°C

Growth of *L. maculans in vivo* was assessed at both temperatures to ensure any differences in phenotype were not simply related to differences in pathogen growth rate. The rates of growth of *L. maculans* isolates JN3 and V.23.11.9 were compared at 20°C and 25°C. Mycelial disc inoculum was placed in the centre of Petri dishes of V8 agar that were stored for 24 hours in a dark cupboard at 20°C before transfer to the CE chamber, allowing time to grow into the fresh agar. Six replicates were prepared per treatment.

Photographs were taken daily over a 5-day period using a Sony NEX-5R camera with a 40.4 to 49 mm lens. Photos were taken from a fixed height and under controlled lighting to reduce image distortion and give colour consistency between treatments. Image J software was used to trace, using the freehand tool, the circumference of the isolate colony in each image (Appendix A.3). This method was used to provide more accurate results than measuring fungal radius with a ruler as isolates of *L. maculans* often grow in an irregular shape rather than a perfect circle. The dark orange V8 agar provided a clear contrast to the white mycelia and so the areas of fungal growth were clear to identify.

2.2.3.2. Plant growth and stem inoculation

The effect of temperature on the quantitative response and the role of *R* genes during the second symptomless stage of colonisation was investigated through adult plant *B. napus* stem-base *L. maculans* inoculation assays. Cultivars possessing four different combinations of genotypes were selected; susceptible background with no *R* genes (Cultivar I); quantitative

resistance background no *R* genes (ES Astrid); susceptible background with *Rlm4* (Cultivar E) and quantitative resistance background with *Rlm4* (Jet Neuf) (Table 2.1). Avirulent and virulent *L. maculans* isolates, JN3 (*AvrLm4*) and V23.11.9 (*avrLm4*), respectively, were used. Plants were inoculated with the virulent *L. maculans* isolate to remove any resistance response caused by major *R* gene interactions. Thus, any differences in resistance response were due to differences in the quantitative resistance background. Plants were grown in a 1:1 ratio of MiracleGro and John Innes No 3 compost, in 6 x 6 cm wide and 8 cm deep pots, inside CE cabinets at constant temperature of 20°C (12 hr light / 12 hr dark) for 6 weeks under the same light conditions as above. Plants were divided in two groups 24 hours prior to inoculation, half were transferred to 25°C, the rest remaining at 20°C. Plants were inoculated at 6-weeks by placing a 1 cm^2 square piece of sponge cloth soaked in 10^7 ml^{-1} conidial suspension over a 1 cm cut in the stem, then wrapping with Parafilm to secure it in place.

2.2.3.3. Image-based canker severity assessment and measurement of plant health

Assessment of plant health and canker severity was done at 6-weeks following inoculation. To assess plant health, the following measurements were taken for each plant; leaf number, plant height (stem base to tip of longest leaf) and stem thickness (measured with a digital calliper). To assess stem canker severity, pieces 1 cm long of the stem were cut 1 cm below the inoculation site and photographed as described in section 2.3.3.1. Photos were then batch-cropped into fifteen photos of stem pieces, each measuring 815 by 815 pixels, to improve accuracy before statistical analysis. These were then analysed with Image J (Schneider et al., 2012) to determine the percentage area of necrotic tissue discoloured by the disease to assess the severity of the stem canker (Figure 2.1).

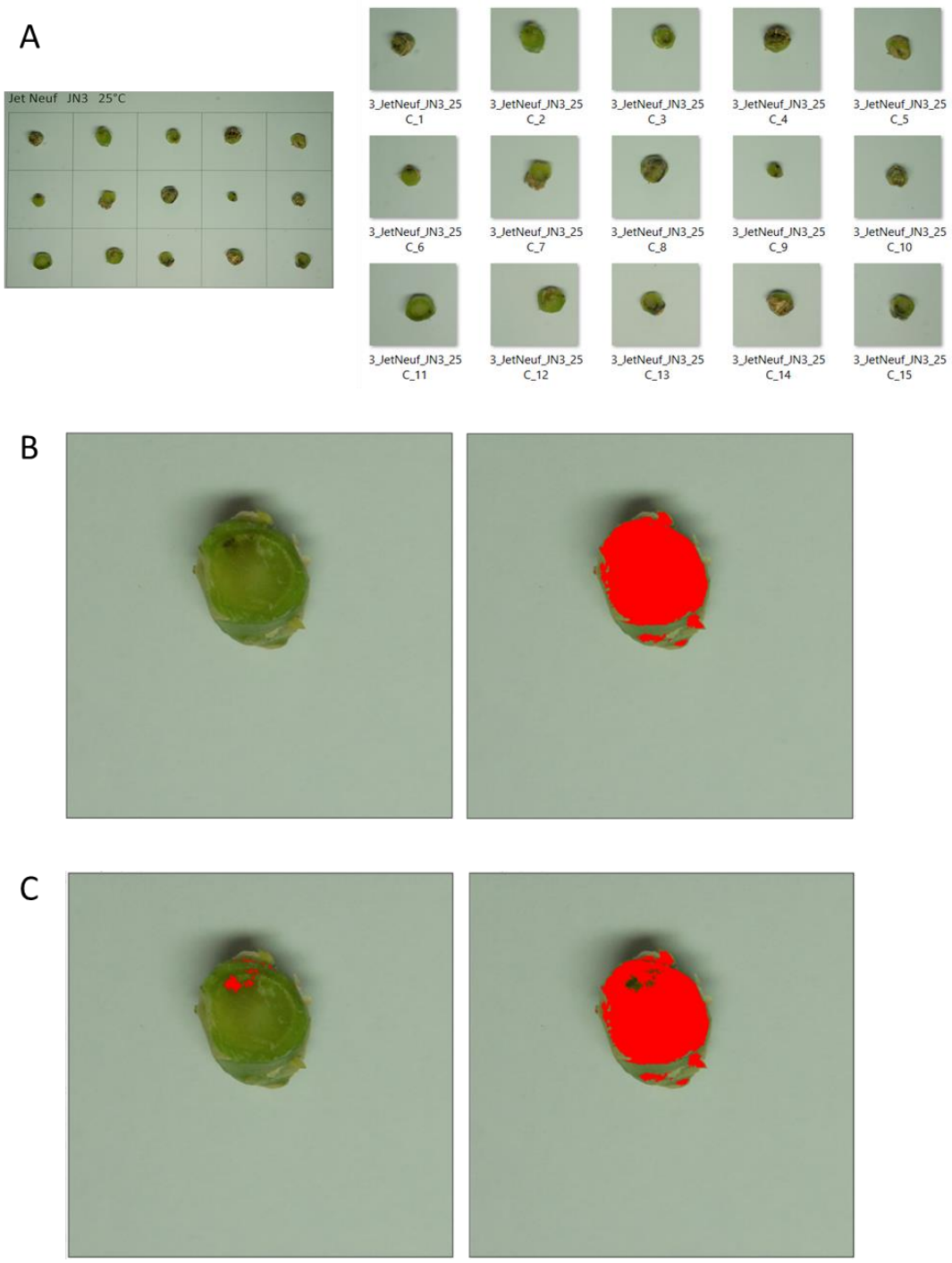


Figure 2.1 Image-based canker severity assessment workflow (A) Photos of stem pieces for each treatment were cropped to isolate each of the individual stems to allow more in-depth statistical analysis. (B) The saturation threshold level was adjusted for each individual image to ensure the cross-sectional area was totally masked, as shown in red on the right-hand side. (C) Brightness and hue threshold filters were applied, identifying necrotic tissue (shown in red on the left) and healthy green tissue (shown in red on the right).

Saturation was adjusted for each image to ensure the cross-section of the stem was fully covered in the analysis. Healthy tissue was identified though setting the colour threshold parameters, in HSB mode to brightness min 82, hue min 42. Settings for necrotic discoloured tissue were brightness max 81 and hue max 41. The Analyze Measure function was then used to measure the pixels in the filtered areas.

2.3. Results

2.3.1. Winter oilseed rape field experiments

2.3.1.1. Stem canker severity on different cultivars

Analysis of cultivar phoma stem canker severity scores (Figure 2.2) found Incentive (little quantitative resistance, no known *R* genes) to have the largest canker severity (3.88), two times greater than the cultivar Astrid with good quantitative resistance only. Cultivar H had the smallest average severity score (0.82). The greatest variance in canker severity was observed in DK Exception and the smallest in Cultivar E.

Cultivars with the same *R* gene and classification of good or little quantitative resistance, provided by breeders, were grouped together to allow cross comparison of cultivars (Table 2.1). A Fisher's least significant comparison test was done to test for significant differences between *R* genes and good or little quantitative resistance on average canker severity (Table 2.2). Large differences were seen in *R* gene effects in cultivars without quantitative resistance; cultivars with *Rlm7*, *Rlm4*, *LepR3* or no known *R* gene were all significantly different to each other. However, in cultivars with quantitative resistance, there were no *R* gene effects (Table 2.2). No significant differences were found between *Rlm7* and *LepR3* cultivars with and without quantitative resistance but were found between cultivars with *Rlm4* and no known *R* gene, with good quantitative resistant cultivars having a significantly smaller score.

2.3.1.2. Phoma leaf spotting on different cultivars in relation to October rainfall

Rainfall was recorded for field experiment locations (Figure 2.3). A correlation coefficient was calculated for total monthly rainfall (months September to December) against the average phoma leaf spotting for each cultivar at each field location. The month in which rainfall had the greatest impact was found to be October had the highest correlation (r = 0.488), followed by November (r = 0.486), September (r = 0.328) and December (r = 0.287) (Appendix A.4).

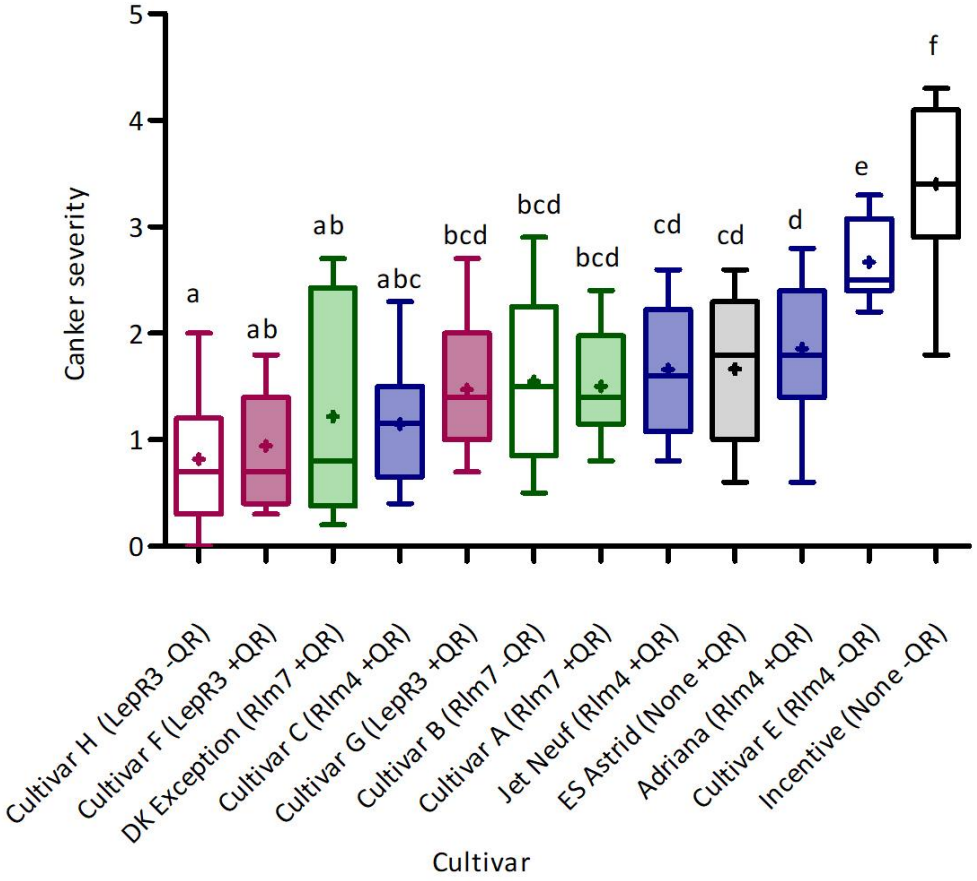


Figure 2.2 Distribution of phoma canker severity scores for winter oilseed rape cultivars grown at five locations over three growing seasons (Chateauroux, France, 2016/17; Impington, UK, 2016/17; Wisbech, UK, 2017/18 and 2018/19; and Hereford, UK, 2018/19). Each box-plot shows the mean (cross) and median (line) scores for each cultivar. Upper and lower box boundaries denote the 25th and 75th percentiles and whiskers indicate minimum and maximum severity scores. Basal stem canker severity (scale 0-6; Lô-Pelzer et al., 2009) was scored on fifteen plant stems sampled from each plot. Colours represent the different *R* genes in cultivars; pink is *LepR3*, green *Rlm7*, blue *Rlm4* and black no known *R* gene. Shaded boxes denote cultivars with higher levels of quantitative resistance as indicated by breeders. Average scores sharing a same letter are not statistically different ($P < 0.05$) in a multiple comparison Fishers least significant difference test.

Table 2.2 Fisher's least significance comparison of average canker severity scores for thirteen winter oilseed rape cultivars grouped by single *R* gene and quantitative resistance. Average scores sharing a same letter were not statistically different at $P < 0.05$ in a multiple comparison Fishers least significant difference test.

R gene	Quantitative resistance		***R* gene mean**
	No	Yes	
Rlm7	1.53b	1.36b	**1.421**
Rlm4	2.66c	1.57b	**1.830**
LepR3	0.82a	1.20ab	**1.072**
None	3.39d	1.66b	**2.520**
Quantitative resistance mean	**2.074**	**1.433**	

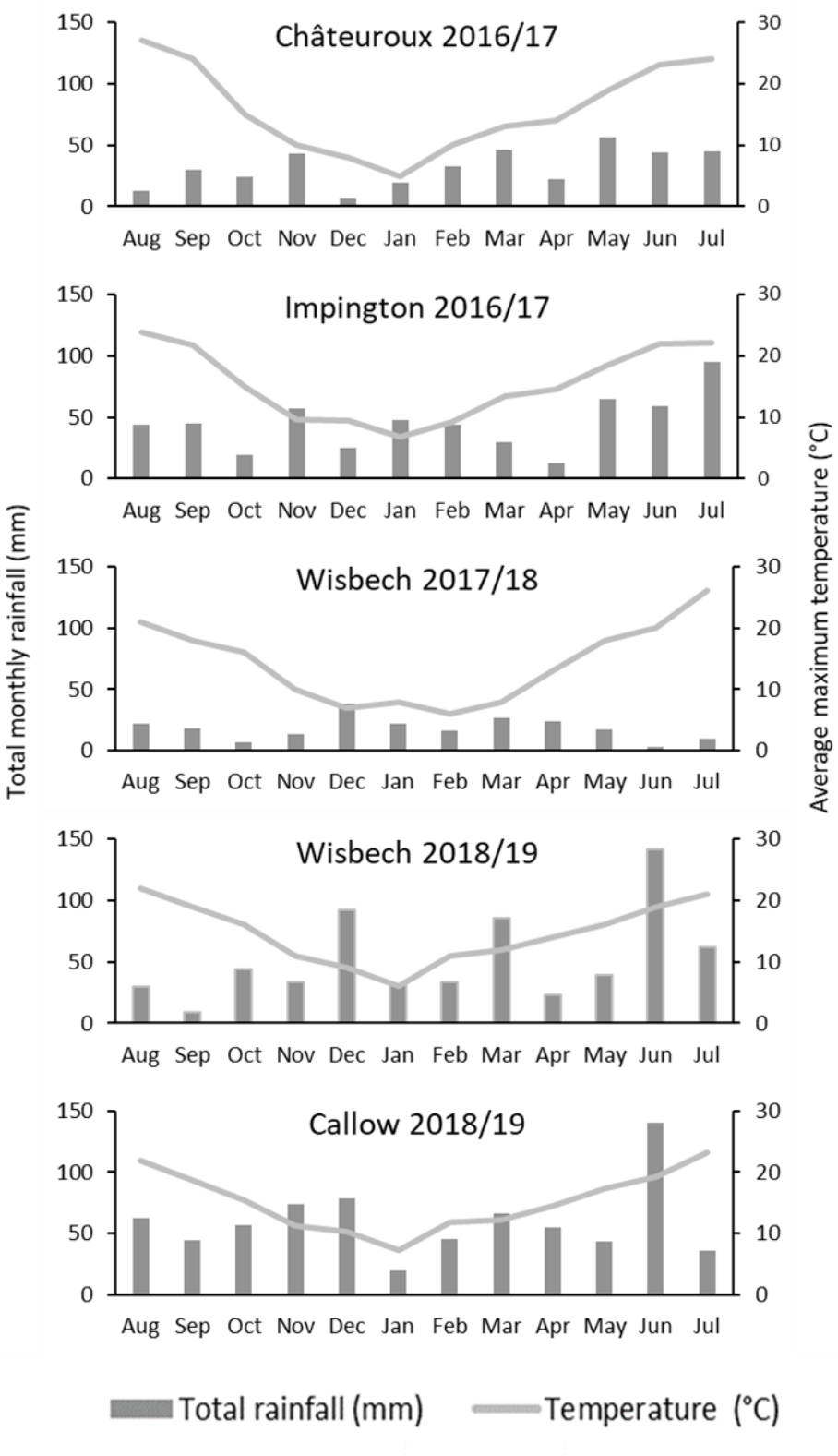


Figure 2.3 Monthly total rainfall and average maximum temperatures at field experiment locations (Chateauroux, France, 2016/17; Impington, UK, 2016/17; Wisbech, UK, 2017/18 and 2018/19; and Hereford, UK, 2018/19).

The total October rainfall was plotted against the mean of the number of leaf spots per diseased leaf for all cultivars, showing a significant relationship with phoma leaf spotting (Figure 2.4). An exponential fitted curve explained 99.8% of the variance in the observed lesion numbers and was found to show significance.

2.3.1.3. Effect of June temperature on canker severity in cultivars with good or little quantitative resistance and *Rlm4*, *Rlm7* or *LepR3*

An initial correlation analysis for canker severity score and mean monthly maximum temperature was done to identify the month with the greatest temperature effect on phoma stem canker severity score. June was found to have the greatest influence, with a correlation coefficient of $r = 0.33$ (Appendix A.5). June is known to be a critical period in the development of phoma stem canker; the most severe stage of the disease, the crown canker, occurs from May to July (West et al., 2001).

Regression analysis of the relationship between the greatest recorded June temperature and phoma stem canker severity score in cropping years 2016/17, 2018/19 and 2018/19 found differences between cultivars (Figure 2.5). The highest maximum June temperature was 35.97°C in Chateauroux, France (22 June 2017) and the lowest maximum June temperature was 23.59°C in Wisbech (25 June 2018).

Groups 1 and 2 (cultivars with *Rlm7* and good or little quantitative resistance) both have a positive correlation, with phoma stem canker scores increasing with maximum June temperature. This correlation was stronger in the cultivars with little quantitative resistance ($R^2 = 0.85$, $P < 0.025$) than what was seen for the cultivars with good quantitative resistance ($R^2 = 0.52$, $P < 0.01$). Groups 3 and 4 (cultivars with *Rlm4,* and good or little quantitative resistance) showed a much weaker relationship with maximum June temperature ($R^2 = 0.31$, $P < 0.01$ and $R^2 = 0.26$, respectively). Group 5 (*LepR3*, good quantitative resistance) (cultivar B) and group 6 (*LepR3*, little quantitative resistance) (Cultivar H) followed a similar trend to groups 1 and 2; both showed positive correlation with phoma stem canker score increasing with maximum June temperature, with a stronger correlation in cultivars with little quantitative resistance ($R^2 = 0.855$, $P < 0.025$ respectively) than good quantitative resistance ($R^2 = 0.29$, $P < 0.05$). Group 7 (good quantitative resistance with no known *R* genes) (cultivar ES Astrid) showed no correlation between canker severity score and maximum June temperature ($R^2 = 0.034$). Group 8 (no known *R* genes, little quantitative resistance) showed a negative

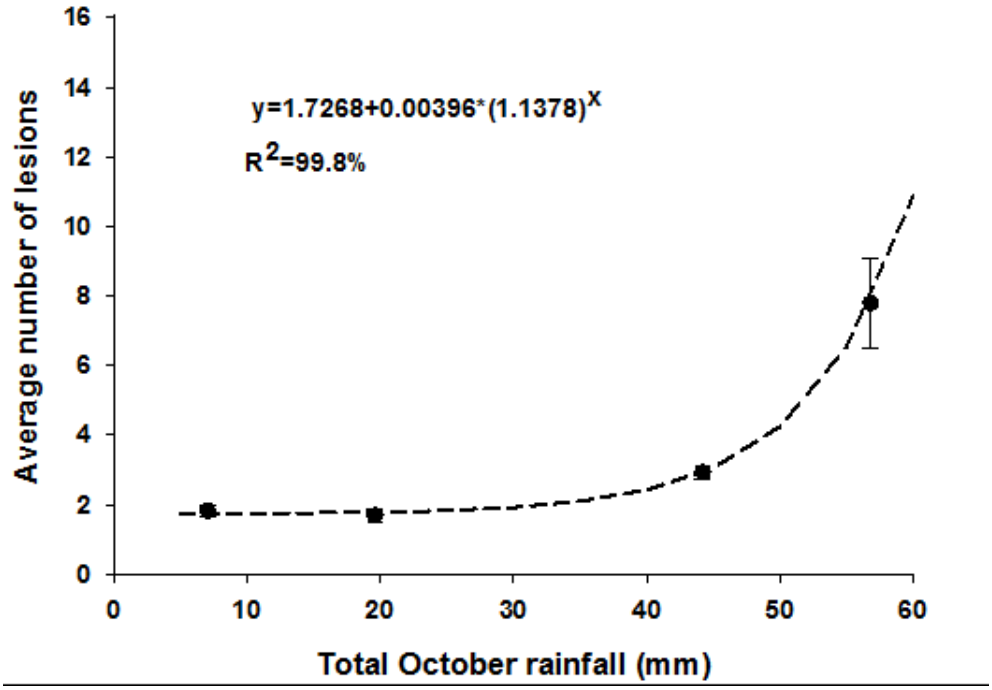


Figure 2.4 Relationship between total October rainfall and phoma leaf spotting incidence on winter oilseed rape plots over three cropping seasons at field experiment locations Impington, Cambridgeshire (2016/17), Wisbech, Cambridgeshire (2017/18 and 2018/19) and Callow, Herefordshire (2018/19). The trendline is an exponential equation in the form $y=A+B*(C^x)$ where y is lesion number and x is the total October rainfall, A, B and C are parameters to be estimated. The fitted curve explained 99.8% of the variance in the observed lesion numbers and shows significance ($P < 0.05$). Ten winter oilseed rape plants were collected from each plot and assessed for incidence of *L. maculans* type leaf lesions. Assessment was carried out in December when plants were around growth stage 1,10 (about tenth true leaf). Average number of leaf lesions per leaf was calculated. Error bars denote standard errors of the means.

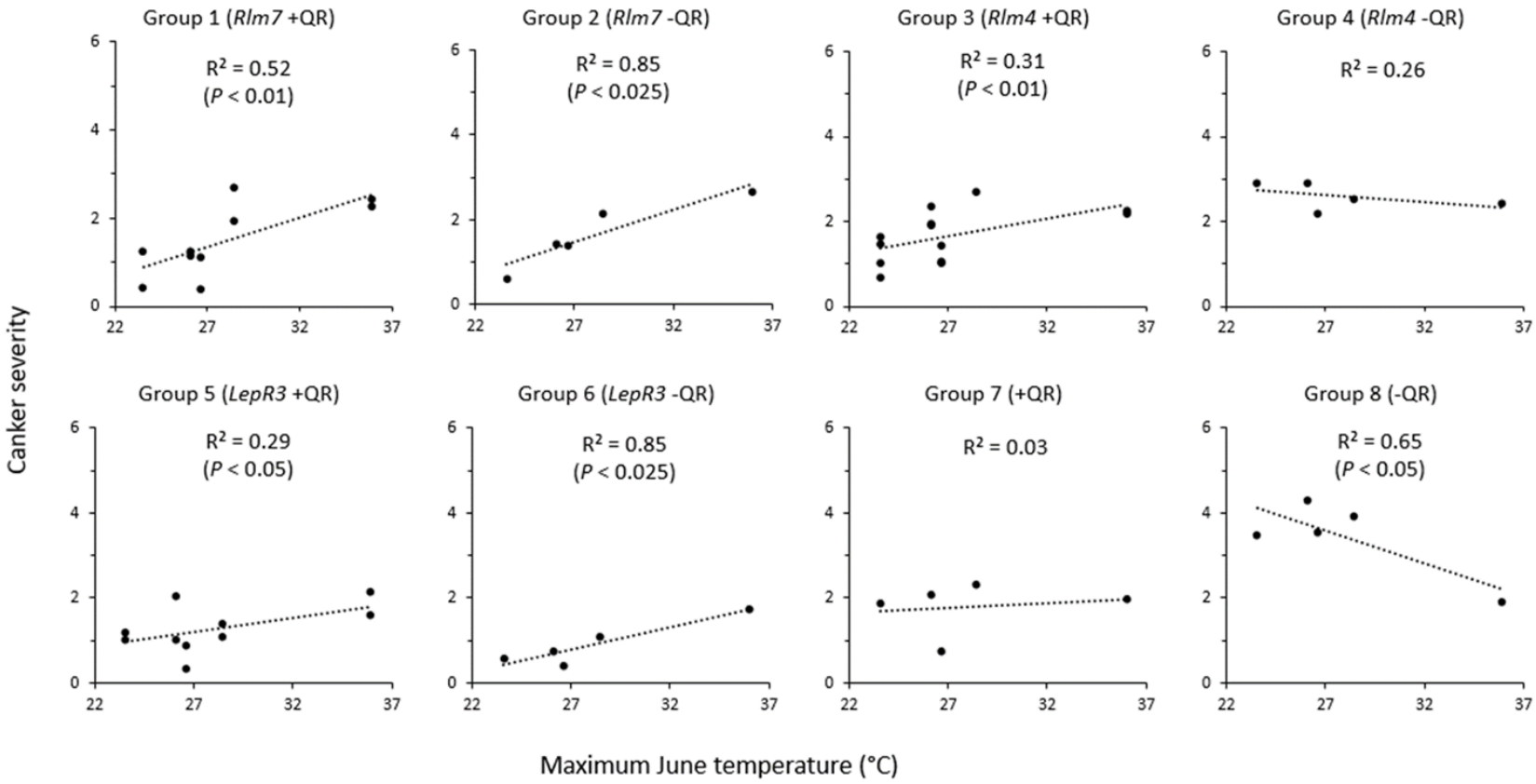


Figure 2.5 Relationship between phoma stem canker severity and maximum June temperatures for experimental winter oilseed rape cultivars. Thirteen cultivars (see Table 2.1) were grown in 2-3 replicate blocks over three growing seasons at five locations in Chateauroux, France, 2016/17; Impington, Cambridgeshire, UK (2016/17); Wisbech, Cambridgeshire, UK (2017/18 and 1018/19); and Callow, Herefordshire, UK (2018/19). Basal stem canker severity (scale 0-6; Lô-Pelzer et al., 2009) was scored on 15 plant stems sampled from each plot. For the equations of the lines, see Appendix A.6.

correlation (R^2 = 0.654, P < 0.05) with reduced phoma stem canker severity at the higher temperatures.

Analysis of position and parallelism based on cultivar *R* genes compared cultivar groups with *R* against susceptible cultivar Incentive. Cultivars with *Rlm7* (P < 0.01) and *LepR3* (P < 0.05) both had significantly different slopes, but not *Rlm4* cultivars (P = 0.061). The intercept was found to be significantly different to Incentive for all cultivars; *Rlm7* (P < 0.001), *Rlm4* (P < 0.05) and *LepR3* (P < 0.01).

2.3.2. Cotyledon inoculation-assessment of cultivars used in field experiments at 20°C and 25°C

Quantitative resistance has been described as being effective only in older plants, although findings that quantitative resistance can also be expressed in younger plants (following petiole inoculation of 22-day old plants) disputes this (Huang et al., 2014). To further investigate if quantitative resistance may play a role in *B. napus* cotyledon defence against *L. maculans* at an elevated temperature of 25°C, a cotyledon assay was carried out. To separate the effect of temperature on the fungal growth to the effect on the resistance response, *L. maculans* growth on agar was compared at 20°C and 25°C (Figure 2.8). All cultivars with *R* genes maintained their resistance to *L. maculans* when inoculated with an avirulent isolate at 20°C and 25°C at the cotyledon stage (Figure 2.6). This suggests that the *R* genes in these cultivars show temperature-resilience. A significant difference between the two temperatures was found for DK Exception (*Rlm7*, good quantitative resistance), Cultivar C (*Rlm4*, good quantitative resistance), Cultivar D (*Rlm4*, good quantitative resistance), Cultivar G (*LepR3*, good quantitative resistance), ES Astrid (good quantitative resistance), Incentive (little quantitative resistance) and in Cultivar I. A susceptible response (interpreted as a score > 5) was observed for cultivars ES Astrid (good quantitative resistance) and Incentive (little quantitative resistance) at both temperatures, and in Cultivar I (little quantitative resistance) at 25°C.

To test if cotyledon assays could be used to predict the impact of high June temperature in crops, a correlation regression analysis was done between R^2 value generated for each cultivar's correlation coefficient for disease severity scores and maximum June temperature against the differences in mean lesion severity scores from cotyledon assays at 20°C and 25°C (Figure 2.7). For the nine cultivars included in this study, no relationship was observed between the R^2 value and the difference in mean (R^2 = 0.00). This data shows that quantitative resistance does not operate at the seedling stage.

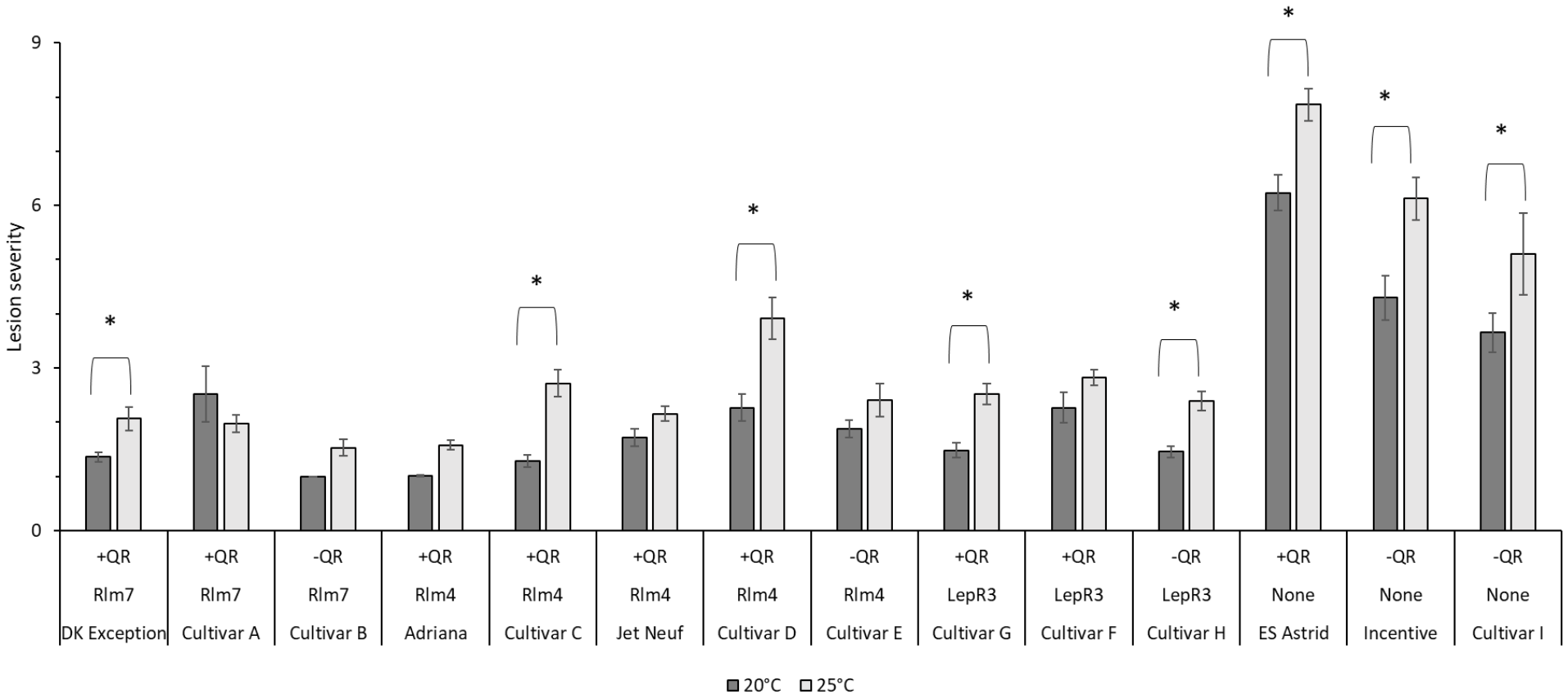


Figure 2.6 Average lesion score (0-9 scale) assessed at 12 days post-inoculation on cotyledons of oilseed rape cultivars included in field experiments assessed in controlled environment experiment at 20°C and 25°C. Twelve-day-old seedlings were inoculated with *L. maculans* isolate JN3 (*Av1-4-5-6-7-8*) by wounding followed by droplet of 10^7 conidial suspension. The assay was repeated twice, each with eight plants per treatment for all cultivars, apart from Jet Neuf, Cultivar E and Cultivar C, for which the assay was done once. Asterisks denote significance between lesion severity at 20°C and 25°C (* $P < 0.05$). Error bars indicate the standard error of the mean.

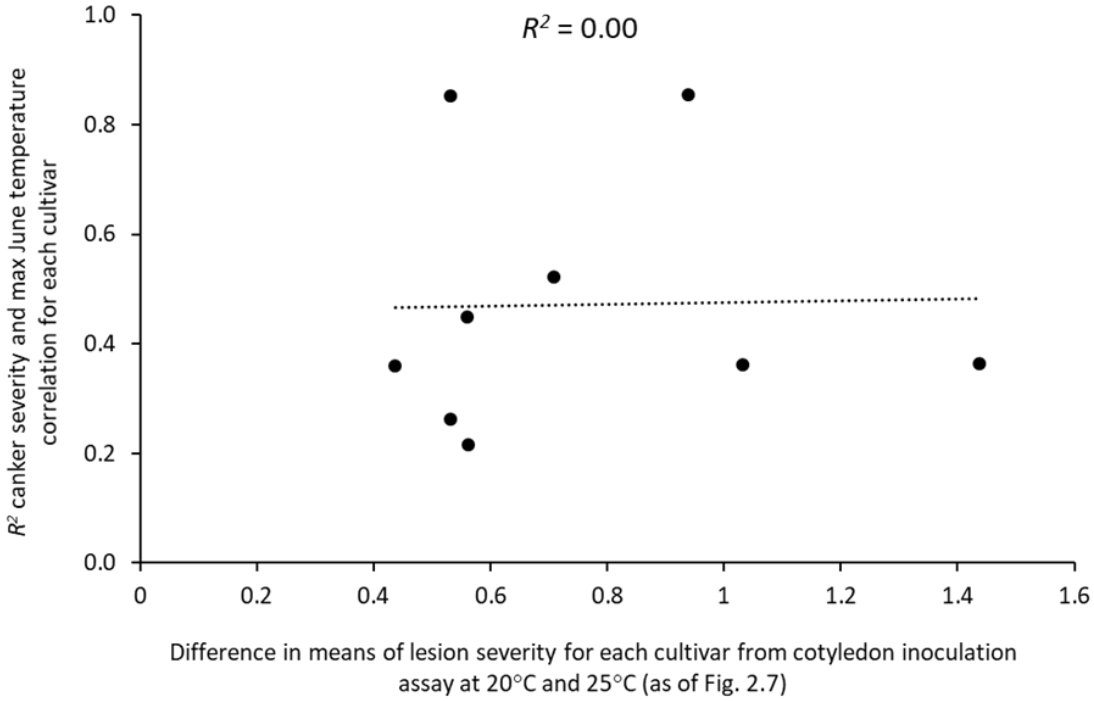


Figure 2.7 Coefficient of determination (R^2) for the relationship between maximum June temperature and canker severity for each cultivar, plotted against the difference in means from the cotyledon inoculation assessment for that cultivar at 20°C and 25°C
For the cotyledon assay, 12-day-old seedlings were inoculated with *L. maculans* isolate JN3 (*Av1-4-5-6-7-8*) by wounding followed by droplet of 10^7 ml^{-1} conidial suspension. To calculate the relationship between phoma stem canker severity and maximum June temperature for each cultivar, field experiment data from five locations over three growing seasons in Impington, Cambridgeshire (2016/17); Wisbech, Cambridgeshire (2017/18 and 1018/19) and Callow, Herefordshire (2018/19) were assessed for correlation with basal stem canker severity. Phoma stem canker severity was scored on 15 plant stems sampled from each plot (scale 0-6; Lô-Pelzer et al., 2009).

2.3.3. Effect of quantitative and *R* gene resistance on canker severity at 20°C and 25°C in adult plants

2.3.3.1. Effects of increased temperature from 20°C to 25°C on growth rate of *L. maculans* isolates JN3 and V.23.11.9.

The two *L. maculans* isolates used in this study, JN3 and V.23.11.9, were grown at 20°C and 25°C and measured every 24 hours for 5 days to determine the effect of temperature on their growth (Figure 2.8). JN3 grew slightly faster at 25°C than at 20°C, whereas V.23.11.9 had a marginally higher growth at 20°C. However, neither of these differences were found to be significant.

2.3.3.2. Image-based canker severity assessment

The effect of increased temperature, from 20°C to 25°C, on canker severity for four winter oilseed rape cultivars with different resistance profiles was determined, six weeks following inoculation with avirulent (JN3) or virulent (V.23.11.9) isolates of *L. maculans* (Figure 2.9). Cultivar Jet Neuf (*Rlm4*, good quantitative resistance) showed the least severe canker at both temperatures for both avirulent and virulent isolates. No significant difference was seen between the temperatures for the treatment with the avirulent *L. maculans* isolate JN3 (11.3% and 24.6% necrosis for 20°C and 25°C respectively); however, the virulent isolate produced significantly greater levels of necrosis at 25°C (54.1%) compared to 20°C (37.1%). Cultivar ES Astrid (quantitative resistance, no known *R* genes) performed well against isolate JN3 at 20°C with an average necrotic area of 31.8%. However, this resistance appeared to lose efficacy at 25°C, with over twice as much necrotic tissue area (83.7%). When treated with isolate V23.11.9, a significant difference was also seen between the temperatures (63.7% and 84.8% necrotic tissue for 20°C and 25°C, respectively). Cultivar E (*Rlm4*, low quantitative resistance) also showed a significant difference between the two temperatures when treated with the avirulent JN3 isolate (46.3% and 60.3% necrotic tissue area 20°C and 25°C respectively). When treated with the virulent isolate, this temperature effect was reversed, with statistically significant greater levels of necrotic tissue area (90.7%) found at 20°C, compared to 69.4% at 25°C. Cultivar I (no known *R* genes and little quantitative resistance) does not exhibited any significant temperature effect. As Cultivar I has no known *R* genes, it is susceptible to both isolates. When inoculated with the JN3, the necrotic tissue area was 66% and 58.4% at 20°C and 25°C, respectively. When inoculated with the V.23.11.9, it was 94.7% and 83.3% at 20°C and 25°C, respectively. Neither of these differences were significant.

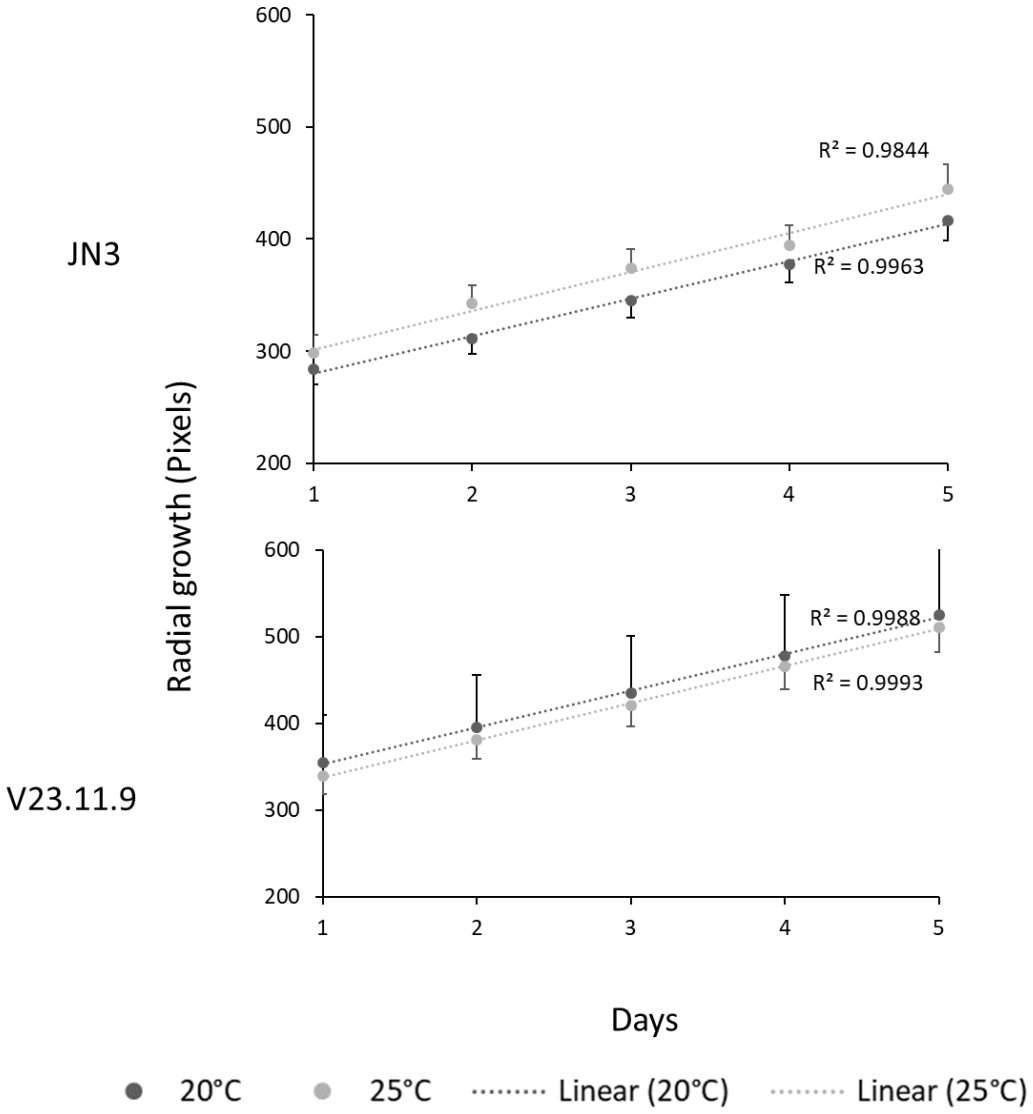


Figure 2.8 Effects of increased temperature from 20°C to 25°C on the radial growth rates of *L. maculans* isolates JN3 and V.23.11.9 Mycelial discs were transferred from fungal colonies onto V8 media Petri dishes and placed in CE cabinets set to a constant temperature of 20°C or 25°C. Photographs were taken daily at regular time points for 5 days, after plants had been acclimatised in cabinets for 48 hrs. An average radius was calculated from the area of each colony at each time point; the area was determined by measuring the area of fungal growth on Image J. Radial growth rate was estimated by linear regression. An ANOVA was performed to determine if temperature affected radial growth rate (P = 0.322) and (P = 0.971) for JN3 and V23.11.9 respectively. Error bars indicate standard error of the mean.

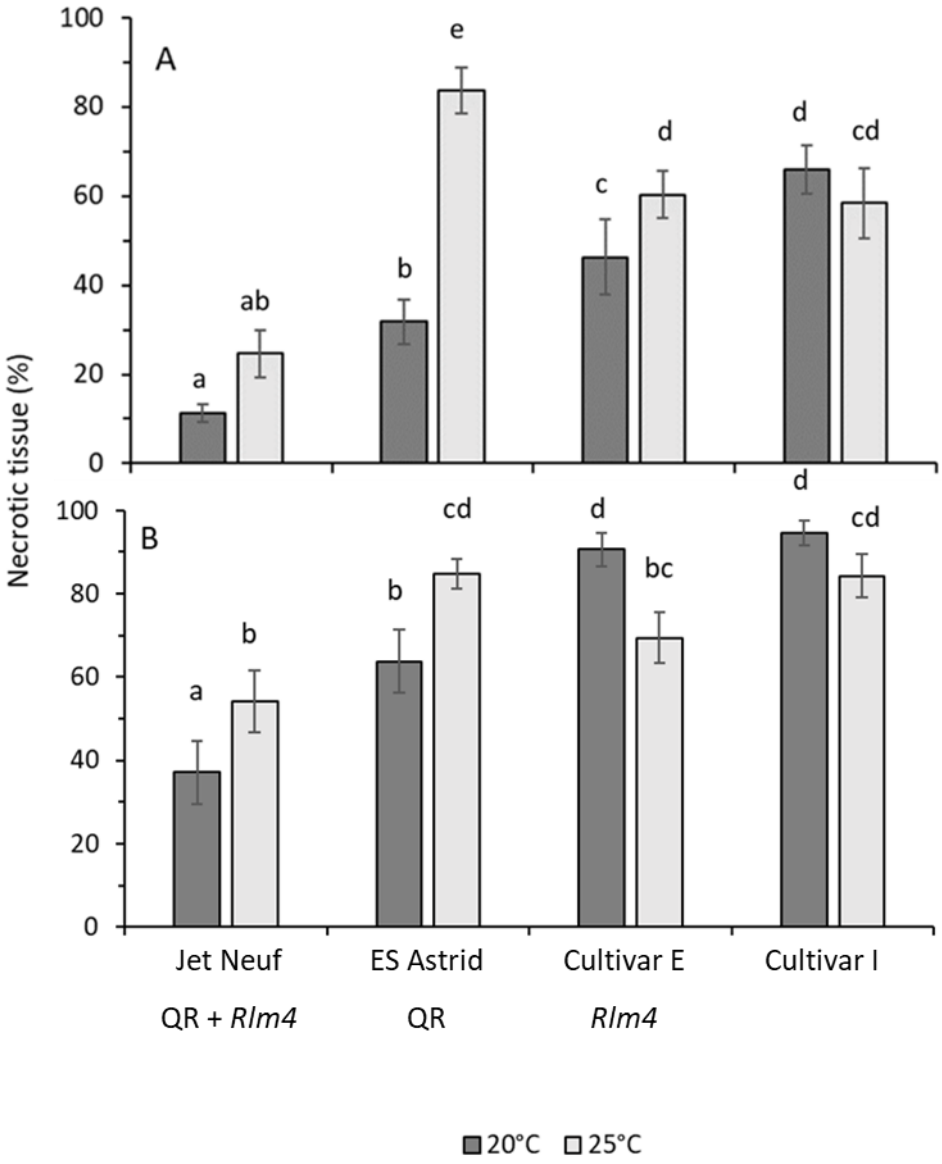


Figure 2.9 Canker severity in four winter oilseed rape cultivars at 20°C and 25°C inoculated with *L. maculans* isolates JN3 (*AvrLm4*) (A) or V23.11.9 (*avrLm4*) (B). Plants were inoculated at 6-weeks by wrapping a sponge soaked in 10^7 ml^{-1} conidial suspension over a 1 cm cut in the stem with Parafilm. Sections 1 cm long of the stem were cut 1 cm above the inoculation site and photographed at 6 weeks post inoculation. Mean percentage area of necrotic tissue in stem sections was calculated from the area of tissue discoloured from the disease and total area analysed on ImageJ. A total of 15 plants per treatment for each cultivar were assessed. Error bars indicate standard error of the mean. Average scores sharing a same letter are not statistically different ($P < 0.05$) in a multiple comparison Fishers least significant difference test.

2.3.3.3. Plant health assessment of four oilseed rape cultivars at 20°C and 25°C

Plant health assessments found very little differences between temperatures (Table 2.3). Although some differences were seen in stem diameters, this will not impact the findings of this study as it does not affect the percentage necrotic area calculated. ES Astrid, susceptible to both JN3 and V23.11.9, grew better at 20°C than at 25°C; plants grew taller and had more leaves. However, the stem diameter was about the same for plants inoculated with V23.11.9 at both temperatures. Cultivar E grew better at 25°C than 20°C when inoculated with JN3 and when inoculated with V23.11.9. All three categories of plant growth were greater at 25°C than at 20°C. Little difference was found for Cultivar I grown at 25°C or 20°C when inoculated with JN3, although plants grew better at 20°C than at 25°C when inoculated with V23.11.9.

2.4. Discussion

2.4.1. Phoma stem canker severity is linked to weather; cultivars containing *Rlm4*, *Rlm7* and *LepR3* respond to maximum June temperatures differently

This study confirms the findings of previous work on the effect of temperature and wetness on phoma stem canker severity. Total October rainfall shows a correlation to leaf spotting, a relationship that has been found in previously (Huang et al. 2018). Whilst warmer average June temperature show correlation to phoma stem canker, a stronger positive relationship was observed for the maximum June temperature recorded and phoma stem canker severity score. This is a new observation that backs the suggestion that more cultivars will require temperature-resilience in order to perform successfully in years experiencing high June temperatures, as are predicted to increase with climate change (Evans et al., 2007; Pullens et al., 2019).

Cultivars with *R* genes *Rlm7*, *Rlm4* and *LepR3* respond differently in their levels of phoma canker severity to maximum June temperatures. Cultivars with *Rlm7* showed a positive correlation with canker severity increasing with temperature. However, this could be due to the higher levels of *L. maculans* isolates with *avrLm7* present in France (in which the field site experiencing the highest maximum June temperature was located), rather than being a temperature-sensitive *R* gene effect. A recent study of the avirulence frequencies present in 30 *L. maculans* isolates sampled in Le Rheu, France found 63% to show virulence against *Rlm7* (Bousset et al., 2020). Cultivars with *LepR3* had the lowest average canker score, but also showed a positive relationship with maximum June temperatures.

Table 2.3 Effect of increased temperature from 20°C to 25°C on average plant height, leaf number and total stem diameter of four winter oilseed rape cultivars, inoculated with avirulent JN3 or virulent V.23.11.9 isolates of *L. maculans*.

Isolate		Jet Neuf		ES Astrid		Cultivar E		Cultivar I	
		20°C	**25°C**	**20°C**	**25°C**	**20°C**	**25°C**	**20°C**	**25°C**
JN3	Height (cm)	30.4	29.3	26.3	25.9	33.4	35.9	27.1	27.9
	Leaf number	6.3	6.4	4.9	4.8	5.9	5.6	7.4	6.9
	Stem diameter (mm)	4.2	4.4	4.3*	3.6	4.6	4.9	5.4	5.3
V23.11.9	Height (cm)	27.9	28.3	26.5	25.5	30.2*	34.6	23.1*	29.8
	Leaf number	5.3	5.7	5.1	4.8	6.0*	5.3	7.9	7.6
	Stem diameter (mm)	4.3	3.8	4.1	4.2	4.7	5.0	4.4	4.9

To compare the differences between variables for JN3, use least significant differences (at 5.0%) between heights = 2.014; leaf numbers = 0.576 and for between stem diameters = 0.467. To compare the differences between variables for V23.11.9, use least significant differences (at 5.0%) between heights = 2.558; leaf numbers = 0.632 and for between stem diameters = 0.598

Cultivar E (*Rlm4* and little quantitative resistance) was found to show an insignificant correlation between maximum June temperatures and canker severity. Cultivar E also had the second highest average canker severity; this could be due to a significant level of *L. maculans* isolates in the fields with virulence against *Rlm4*. Analysis of *L*. *maculans* populations from 13 sites, 11 of which were in the UK by Huang et al. (2018) found mean frequencies of *AvrLm4* to be 41%, less than that of *AvrLm7* which was found to be 100%. This suggests that *Rlm4* gene-mediated resistances would be partially rendered ineffective, explaining the higher canker severity seen in Cultivar E.

2.4.2. In cultivars with low quantitative resistance and no known *R* genes, a lower temperature is more conducive to canker development

A significant negative correlation of phoma canker severity and maximum June temperatures was seen for cultivar Incentive, which lacks any known *R* gene and has low quantitative resistance (Figure 2.5). Cultivar E (*Rlm4*, low quantitative resistance) also had a statistically significant ($P < 0.05$) greater amount of necrosis at 20°C compared to 25°C when inoculated with the virulent isolate of *L. maculans* (Figure 2.9). This could be due to the *L. maculans* growing more rapidly at lower temperatures. Alternatively, it may be a result of a more optimal temperature for PAMP triggered resistance, a lower plant response to the effector triggered HR bought around by *R* genes. Higher temperatures (23°C – 32°C) have been reported to enhance PAMP signalling (Cheng et al., 2013).

2.4.3. Temperature-resilience observed in controlled environment cotyledon assays is not mirrored in the field

No relationship between cultivar resilience to increased temperature at the cotyledon stage and relationship to maximum June temperatures in the field was observed. This is not surprising as the effect of quantitative resistance has not previously been observed at the cotyledon stage. The lack of correlation between seedling cotyledon inoculation assays and field results could also be due to multiple other factors such as differences in avirulence genes in *L. maculans* isolates present in the natural environment to those used in the lab, other climate conditions such as rainfall, greater variation in temperatures and effects of other pathogens and pests.

2.4.4. Single *R* genes also operate in the stems of adult plants

Results from the field experiment suggest that *R* genes are operating alongside quantitative resistance in June to influence the level of phoma stem canker. Through inoculating adult plants directly into the stem, any resistance brought about by *R* genes operating in the leaves was circumvented in the CE adult plant assay. An *L. maculans* isolate, avirulent to *Rlm4*, was found to cause significantly less necrotic tissue in the stem of an adult inoculated and grown at 20°C compared to at 25°C. An isolate virulent to *Rlm4* was also included, thus eliminating any *R* gene effect, caused significantly more necrotic tissue to develop at 20°C compared to 25°C. This suggests that *Rlm4* has a protective effect against the pathogen in the stems of adult plant. This hypothesis contradicts current beliefs, which describe single *R* genes as operating in the leaves of young plants during the autumn (Rimmer and van den Berg, 1992; Fitt et al., 2006a). To confirm this hypothesis, more adult plant stem inoculations could be done using near isogenic lines with or without single *R* genes.

2.4.5. Quantitative resistance may act as a mechanism to protect *R*-gene resistance at high temperatures

Field experiments suggest that quantitative resistance may act to reduce the effect of increasing maximum June temperatures on the phoma stem canker severity when combined with *R* genes. This correlation between maximum June temperature and phoma stem canker severity was weaker for *Rlm7* and *LepR3* cultivars with good quantitative resistance compared to those with poor quantitative resistance. When quantitative resistance is present in a cultivar (ES Astrid) with no known *R* genes, no relationship with maximum June temperature is seen. These findings suggest that quantitative resistance shows temperature-resilience in crops and can buffer to a plant's resistance response against high temperatures, maintaining the efficacy of the plant's resistance response. However, in the CE stem inoculation assays, cultivars ES Astrid (good quantitative resistance) and Jet Neuf (good quantitative resistance and *Rlm4*) were both found to have a significantly lower amount of necrotic tissue at 20°C compared to 25°C. This suggests that, under a sustained temperature of 25°C, the efficacy of quantitative resistance is reduced.

One possible explanation for the difference in performance of ES Astrid in the field and CE cabinets could be the period in which the plant is exposed to elevated temperatures. Whilst quantitative resistance appears to provide a mechanism to reduce the effect of elevated temperatures, this may be broken down if this higher temperature is sustained over a long period of time.

2.4.6. Combining *R* genes and quantitative resistance in cultivars provide the most effective resistance in hot environments

Results from both field experiments and CE assays suggest that by combining temperature-resilient *R* genes, such as *Rlm4*, *Rlm7* and *LepR3*, with a high quantitative resistance background, protection of oilseed rape crops against phoma stem canker disease at elevated temperatures may be increased. Furthermore, the CE assay supports the hypothesis that both quantitative and *R* gene resistance operate in the stem of adult plants, protecting against *L. maculans* infection. Both *Rlm4* and quantitative resistance efficacy can be seen to be reduced when plants are subjected to a prolonged elevated temperature of 25°C. In years experiencing warmer summers, as have been predicted to result from climate change in the UK, a combination of temperature-resilient *R* genes and a high quantitative resistance background will be required to protect oilseed crops from phoma stem canker.

It is not known if higher levels of quantitative resistance are linked with a reduction in fitness. A review by Brown (2002) on yield penalties of disease resistance in crops suggested that plants with high quantitative resistance could suffer from a fitness penalty. The evidence behind this proposal came from the observations of Vanderplank (1984) that quantitative resistance can be lost due to masking by single *R* genes or if not exposed to the pathogen. Quantitative resistance genes could be linked to genes involved in yield, resulting in linkage drag if these resistance genes were to be introgressed. More research is needed in this area to fully understand any potential pay offs in important traits such as yield that may be linked to greater levels of quantitative resistance in oilseed against phoma stem canker.

3 Role of *SNC1* in the temperature-sensitivity of *R* genes in *Brassica rapa* against *Leptosphaeria maculans*

3.1. Introduction

Amongst the combination of factors described in Chapter 1 determining temperature-sensitivity of resistance, NLR proteins are believed to play a key role. NLRs have been proposed to act as a temperature-sensitive component in the plant defence response across several NLR *R* genes in a similar manner, described by Zhu et al. (2010) as a 'widespread phenomenon'. Furthermore, Zhu et al. (2010) suggested that a conserved SUMOylation motif operates in distant taxa as a common mechanism for regulating temperature-sensitivity across various types of disease resistance. In *A. thaliana* a SUMOylation mutant, *siz1*, was found to display *SNC1*-dependent autoimmunity at both 22°C and 28°C, which was EDS1 dependent at both temperatures (Hammoudi et al., 2018). *AtSNC1* is involved in temperature sensitivity of plant defence responses in *A. thaliana*. The *snc1-1* mutant of *A. thaliana* is constitutively activated in defence and has a dwarf phenotype; both phenotypes are lost at higher temperatures (Yang, 2004; Zhang, 2003; Zhu et al., 2010). The *A. thaliana* genome contains an RPP4/ RPP5 cluster with eight NBS-LRR genes on chromosome 4, one of which is *AtSNC1*.

FocBo1 is a single dominant gene conferring resistance to yellow wilt disease, caused by *Fusarium oxysporum,* in *B. oleracea* (Shimizu et al., 2014). *FocBo1* is described as temperature-resilient up to 26 - 28°C, thus it is hypothesised to be a temperature-resilient allele. Following the fine mapping and cloning of *FocBo1,* a candidate orthologue in *B. rapa* (*Bra012688*) was identified and designated *FocBr1* (Shimizu et al., 2014).

In *B. rapa*, two duplicated genes occur; one is believed to be an orthologue of *AtSNC1* and is thus referred to as *BrSNC1* (*Bra012689*) and the other is *FocBr1*. A corresponding locus in *B. napus* is less clear as its genome is more complicated. In the work presented in this chapter, the roles of *BrSNC1* and *FocBr1* are investigated in the defence response of *B. rapa* against *L. maculans* at 20°C and 25°C. It is proposed that *BrSNC1* acts as temperature-sensitive component of the defence response in *B. rapa* in a manner analogous to *SNC1* in *A. thaliana*. *FocBr1* expression is also postulated to be affected by increased temperature. The findings provide knowledge on two genes indicated to be involved in the temperature-dependence of the defence response.

B. rapa (AA, 2*n* = 20), the ancestral species and origin of the A genome in *B. napus*, was used in this study for several reasons. As it is diploid it has simpler genetics and less potential for genetic redundancy than *B. napus* and has a well sequenced genome (reverse genetics requires sequence information on the target gene). A TILLING population for R-o-18, an inbred line of the *B. rapa* subsp. *trilocularis* was produced by RevGen and used in this study. R-o-18 is self-fertile, produces many seeds per plant and has a similar growth pattern to *A. thaliana* and oilseed rape cultivars makes it an attractive system for as well as studies related to yield and quality traits in *B. napus* (Stephenson et al., 2010). *B. rapa* was previously identified as a host for *L. maculans* (Rouxel and Balesdent, 2005) and this study confirms the R-o-18 line to be a good host, this finding will help enable future studies understand the molecular mechanisms behind Brassica resistance.

The NB-ARC (nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4) domain is found at the centre of trimodular R proteins, (van der Biezen and Jones, 1989). This domain is made up of three sub-domains: an NB domain, ARC1 and ARC2 (Albrecht and Takken, 2006). The NB-ARC domain is a functional ATPase module, and its nucleotide-binding state has been found to regulate activity of the R protein. The NB subdomain forms a catalytic nucleotide-binding and nucleotide-hydrolysing pocket, converting ATP to ADP (Tameling et al., 2002). The NB-ARC domain of two tomato (*Lycopersicon esculentum*) R proteins I-2 and Mi-1, and likely of related R proteins, was shown to function as a molecular switch whose state (on/off) depends on the nucleotide bound (ATP/ADP) (Tameling et al., 2002). Two I-2 autoactivating mutants with specific point mutations in the conserved motifs of the NB-ARC domain resulted in autoactivating proteins that induced a pathogen-independent HR. The mutants were defective in ATP hydrolysis, but not in ATP binding, suggesting that the ATP-bound state of the R protein is the active form that triggers defence signaling (Tameling et al., 2006).

3.1.1. Objectives

1. To determine susceptibility and temperature-sensitivity of *B. rapa* R-o-18 wild-type and *BrSNC1* and *FocBr1* TILLING mutants to *L. maculans*
2. To carry out back-crosses for each TILLING mutant to remove unlinked mutations
3. To phenotype and perform molecular characterisation on *B. rapa* TILLING mutants
4. To determine gene expression differences in homozygous *BrSNC1* and *FocBr1* TILLING mutants to explore molecular mechanisms of action

3.2. Materials and methods

3.2.1. Sequence alignments and comparison of deduced protein sequences for hypothesised *SNC1* orthologs in *B. rapa*, *B. napus* and *B. oleracea*

The coding sequences of several *SNC1* candidates in *B. rapa*, *B. napus* and *B. oleracea* were identified using a BLAST search in EnsemblPlants using *FocBo1* and *AtSNC1* templates. The coding sequences were aligned using ClustalW. InterProScan was used to predict protein domains from the amino acid sequences of NLR genes. SMART (a Simple Modular Architecture Research Tool) was used to identify and annotate protein domains in *B. napus* genes of interest.

3.2.2. Phylogenetic analysis of the *SNC1* and *FocBo1* loci

Phylogenetic analysis was done on *SNC1*, its five homologs in *A. thaliana*, candidate *SNC1* homologs in *B. rapa* and *B. napus, FoBo1* in *B. oleracea*, a *FoBo1* candidate gene in *B. rapa* and two candidate *FocBo1* homologs in *B. napus* to compare the sequences and inform how distinct they are from each other. Phylogenetic Analysis by Maximum Likelihood was done using MEGA (version 10.1.5). A 500-bootstrap test of phylogeny was carried out using the Tamura-Nei model of Maximum Likelihood (Tamura and Nei, 1993).

3.2.3. Selection of *B. rapa* TILLING mutants in *FocBr1* and *BrSNC1* genes of interest

The RevGenUK database (www.jic.ac.uk/technologies/genomic-services/revgenuk-tilling-reverse-genetics) was searched for mutations in the *FocBr1* (*Bra012688*) and *BrSNC1* (*Bra012689*) candidate genes in the *B. rapa* line, R-o-18. Two TILLING lines with missense mutations were selected in the *FocBr1* candidate gene; *focBr1-1* (Homozygous, Gly245Arg) and *focBr1-2* (Heterozygous, Val241Met). Both mutations lie in the NB-ARC domain of *FocBr1; focBr1-1* lies in the P-loop region, whereas *focBr1-2* is located just before the P-loop domain (Table 3.1). A TILLING mutant line with a glycine to arginine substitution in the P-loop of the NB-ARC domain of *BrSNC1* was also selected, this genotype will be referred to as *Brsnc1*. In two of the three TILLING mutants (*focBr1-1* and *Brsnc1*) a glycine is substituted for another amino acid in the P-loop. Glycine substitutions have previously been shown to have a significant effect on the phenotype due to its importance in protein function (Perry et al., 2009). Furthermore, it is thought that NB-ARC domains of R proteins function as a molecular switch, the on/off mode of

which is determined by what nucleotide (ATP or ADP) is bound to the P-loop (Tameling et al., 2006).

3.2.4. Genotypic and phenotypic characterisation of TILLING mutant lines

Plants were grown to seed production and phenotypes of each mutant line were observed at various time points throughout the growth stages. Kompetitive Allele Specific PCR ($KASP_{TM}$) genotyping technology (LGC, Middlesex, UK) was used to confirm mutant genotypes and to aid back-crossing. Table 3.2 gives the sequences provided for the SNP-specific KASP by design assay mix. DNA was extracted from young leaves using the DNAMITE Plant Kit (Microzone Ltd., Haywards Heath, UK) following the manufacturer's instructions. Following extraction, samples were analysed for quality and concentration using a Nanodrop ND-1000 spectrophotometer and diluted in PCR water to 25 ng/µL. 25 ng of high-quality DNA was used per reaction. Genotyping reactions were run and read on the Agilent Technologies Mx3000P instrument with Low ROX as per the specific guidelines for that instrument provided by LGC. Positive controls, i.e. samples of known genotype, and non-template controls were included in each assay.

KASP thermal cycling conditions were set to the following: step 1. 94°C for 15 min; step 2. 94°C for 20 sec; step 3. 61°C for 60 sec (repeat steps 2-3 to give a total of 10 cycles, by touch-down PCR, dropping the temperature by - 0.6°C per cycle to achieve a final annealing temperature of 55°C); step 4. 94°C for 20 sec; step 5. 55°C for 60 sec (repeat steps 4-5

Table 3.1 Information on the three mutations in *B. rapa* R-o-18 TILLING lines produced by RevGen UK

Mutant	Gene ID	RevGen ID	Base Change	Amino Acid Change	Information
focBr1-1 Homozygous	Bra012688	JI33099a	GGG>AGG	G>R	Mutation in P-loop motif
FocBr1-2/focBr1-2 Heterozygous	Bra012688	JI31088a	GTG>ATG	V>M	Conserved mutation in NB-ARC domain
BrSNC1/Brsnc1 Heterozygous	Bra012689	JI30143b	TGG>TGA	G>R	Mutation in P-loop motif

Table 3.2 Sequences provided to LGC for KASP primer design containing polymorphisms for mutant and wild-type genotypes

Genotype	Sequence submission for KASP primer design
focBr1-1	AATTCAGTGTTGCGCTTGGATTCTGAGGATGTGAGAATGGTGGGGATTGTG[**A/G**] GGCCTTCAGGGATTGGTAAGAGTATCATAGCGAGAGCTCTTTTCAGCCATC
focBr1-2	TTAGAAGCAATGAATTCAGTGTTGCGCTTGGATTCTGAGGATGTGAGAATG[**A/G**] TGGGGATTGTGGGGCCTTCAGGGATTGGTAAGAGTATCATAGCGAGAGCTC
Brsnc1	GAGGCAATGAATCAATTGTTGTGCATTGAATCTGAGGAAGCTAGAATGGTG[**A/G**] GGATTGTAGGGCCTTCAGGGATTGGTAAGACTACCATAGCAAGAGCTCTTT

25 times (a total of 26 cycles)); step 6. read plate at less than 40°C. After each run, a further KASP recycling cycle was done using the following conditions: step 1. 94°C for 20 sec; step 2. 57°C for 60 sec (repeat steps 1-2 to give a total of 3 cycles).

Seeds of mutant lines *Brsnc1* and *FocBr1-2* were grown to maturity and backcrossed with the R-o-18 TILLING background two times to remove unlinked mutations besides those being studied (Figure 3.1). As the *focBr1-1* seeds provided by RevGenUK were very slow growing and had male sterility, only one backcross was carried out. KASP markers were used to identify and select heterozygous plants produced from the cross which were then used for further backcrossing.

3.2.4.1. Segregation test by KASP marker analysis

To check if seeds produced by selfing *FocBr1-2/focBr1-2* to generate BC_1F_2 segregated in the expected 1:2:1 ratio, KASP marker analysis was carried out on the F2. Seventy *FocBr1-2* BC_1F_2 seeds were sown and leaf samples were removed at two weeks for KASP analysis. A Chi-squared test was then performed on the resulting genotypes.

3.2.5 Assessing temperature-sensitivity of TILLING mutant lines

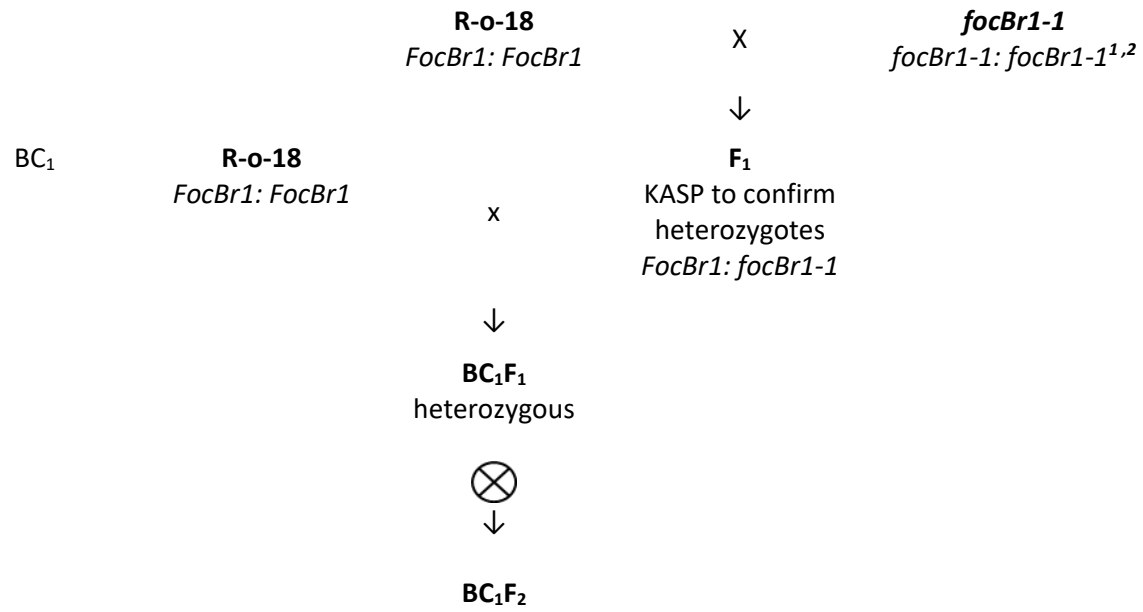
3.2.5.1. Cotyledon inoculation assay at 20°C and 25°C

Inoculation assays were carried out at 20°C and 25°C to determine the temperature-sensitivity of the three genotypes resulting from mutations in the *FocBr1* and *BrSNC1* genes in comparison to the wild-type R-o-18 line. Ten-day old cotyledons of *Brsnc1*, *focbr1-1* and *FocBr1-2/focBr1-2* were inoculated using the wounding and droplet method, described in section 2.2.2.2, with 10^7 conidial spores ml^{-1} of *L. maculans* isolate JN3 (*AvrLm1-4-5-6-7-8*). Twelve days following inoculation, the resulting lesions were assessed as described in section 2.2.2.3.

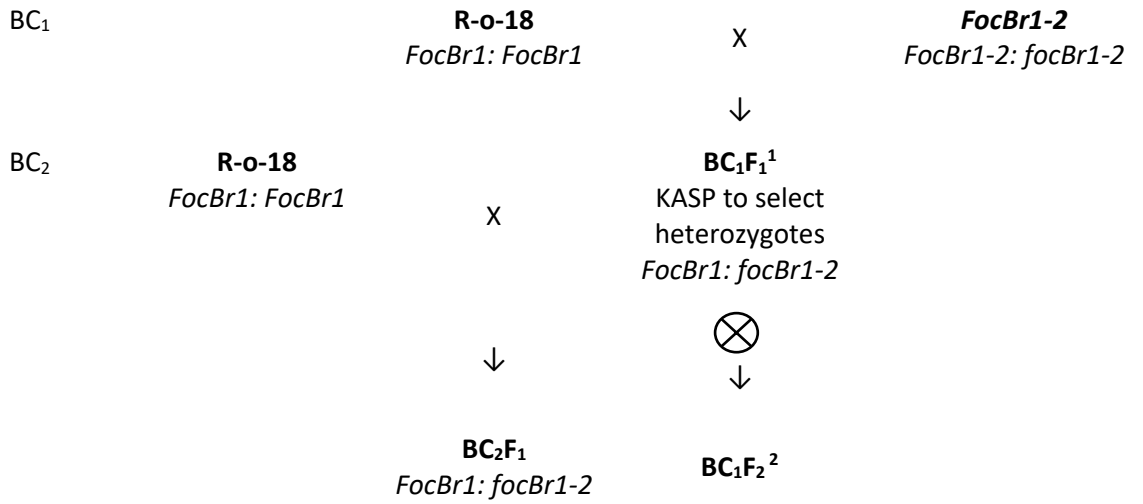
3.2.5.2. True leaf inoculation assay at 20°C and 25°C

For the second assay, 70 seeds of BC_1F_2 *FocBr1-2/focBr1-2* (produced from selfing heterozygous BC_1F_1 plant) were sown. A segregating population for *BrSNC1/Brsnc1* was also planned and a BC_2F_1 plant was selfed. However, the plant was severely overcome by aphid infestation and the seeds produced were found to be of insufficient quantity and unviable.

A.


R-o-18
FocBr1: FocBr1
X
focBr1-1
focBr1-1: focBr1-1[1,2]
BC1
R-o-18
FocBr1: FocBr1
x
F1
KASP to confirm heterozygotes
FocBr1: focBr1-1
BC1F1
heterozygous
BC1F2

B.


BC1
R-o-18
FocBr1: FocBr1
X
FocBr1-2
FocBr1-2: focBr1-2
BC2
R-o-18
FocBr1: FocBr1
X
BC1F1[1]
KASP to select heterozygotes
FocBr1: focBr1-2
BC2F1
FocBr1: focBr1-2
BC1F2[2]

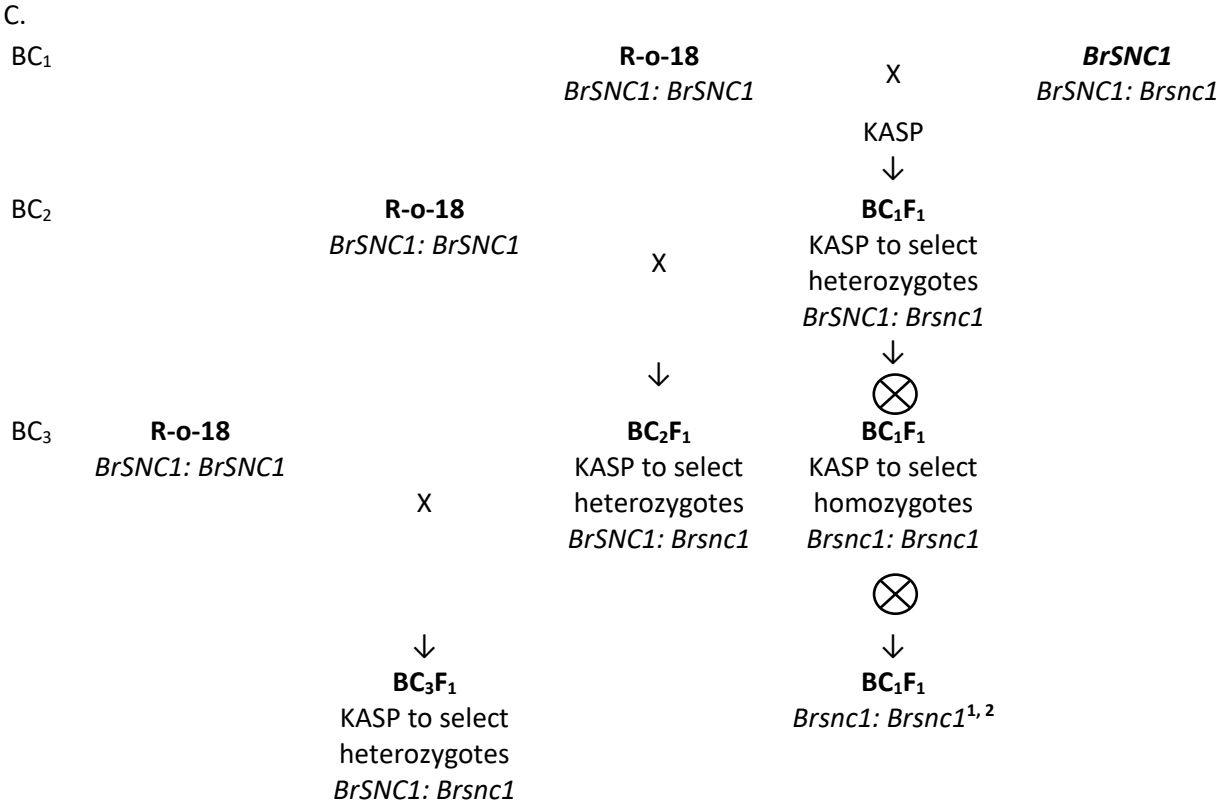


Figure 3.1 Scheme of KASP marker-assisted backcrosses between *B. rapa* TILLING lines *focBr1-1* (A) *focBr1-2* (B) and *Brsnc1* (C) to R-o-18 wild-type to remove unlinked mutations. Superscript denotes assays in which plants were used; [1] cotyledon inoculation assay at 20°C and 25°C; [2] True leaf inoculation assays at 20°C and 25°C.

Once the plants had reached the third true leaf stage (corresponding to 1,3 on the growth stage key for *B. napus* (Appendix A.7) and were 28 days old), *L. maculans* (isolate JN3) was inoculated onto the second and third leaves by wound-droplet inoculation. Inoculation was carried out in the same way as previously described but with a couple of adaptions made for the larger plants. Before wounding, the area surrounding the wound sites were gently rubbed using a damp cotton swab, to prevent the conidial suspension from rolling off the waxy leaf surface. Using a pre-sterilised needle, three to six wounds were made on each of the second and third true leaf depending on the leaf size. At 14 dpi, leaves were photographed, lesions were scored and leaf discs were taken for pathogen DNA quantification.

3.2.6. *L. maculans* quantification

Levels of *L. maculans* DNA were determined for each treatment using quantitative real-time PCR chain reactions. Leaf disc samples (8 mm diameter) taken from the wound sites of infected plants with a Rapid-Core biopsy punch (ProSciTech, Thuringowa Central, Australia) at 14 dpi. Samples were placed in 2 ml skirted screw-cap Eppendorf tubes, along with 3 sterile metal beads. Samples were submerged in liquid nitrogen before storage at -80°C. Frozen tissue was ground into a fine powder using a Retsch Mixer Mill (Retsch, Castleford, UK). From this stage, the DNAMITE Plant DNA Extraction Kit (Microzone Limited, Sussex, UK) was used to extract DNA following the protocol provided. DNA was quantified using a Nanodrop ND-1000 spectrophotometer and diluted to 20 ng/µl with nuclease-free water.

Quantitative real-time polymerase reactions were prepared with *L. maculans* specific primers (Table 3.3) using an Agilent Technologies Stratagene Mx3005P real-time PCR machine (Agilent Technologies). Reaction mixtures were prepared to a total volume of 20 µl containing; 10 µl SYBR green ready mix (Brilliant III Ultra-Fast SYBR Green QPCR Master Mix with low ROX, Agilent Technologies), 6.3 µl nuclease-free water (Qiagen), 0.6 µL (10 µM) forward primer, 0.6 µL (10 µM) reverse primer and 2.5 µl of sample DNA (20 ng/µl). The thermocycling programme was an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 36 sec. A dissociation curve measurement cycle was performed following main thermocycling. All reactions were prepared in duplicate on the plate and each plate was also duplicated. A standard 10-fold dilution series of *L. maculans* DNA (10,000/ 1,000/ 100/ 10/ 1 pg) was included for each plate to give a standard curve. Total DNA (pg) was calculated automatically using the MxPro QPCR software (Agilent).

Table 3.3. Primers used in this chapter

Gene	F primer	R primer	Source
L.mac	CTTGCCCACCAATTGGATCCCCTA	GCAAAATGTGCTGCGCTCCAGG	Liu et al., 2006
BrSNC1 *Bra012689*	AGATGTCGTTGAGAAGTCTGATACC	TTGTTGTTCTGTTATCATGGGATTA	Miyaji et al., 2017
BrPR1	TACCCTTCGAACACGTGCAA	AGCTTTGCCACATCCGAGTT	This work
FocBr1 *Bra012688*	CGATAGGTATTCGAATTTGAGTCT	TCCACACTGTTGTTTGTACTGAGTT	Miyaji et al., 2017
BrActin	CGGTCCAGCTTCGTCATACTCAGCC	AAATGTGATGTGGATATCAGGAAGG	Miyaji et al., 2017

3.2.7. Gene expression analysis

3.2.7.1. RNA isolation

Levels of *PR-1*, *FocBr1* and *BrSNC1* were measured and compared between the mutant lines and R-o-18 wild-type. Five replicates were produced for each genotype. Four leaf discs (8 mm diameter) were taken from young healthy leaves of four-week-old plants with a Rapid-Core biopsy punch (ProSciTech) for each sample. Samples were placed in 2 ml skirted screw-cap Eppendorf tubes, along with a zirconium oxide (5 mm) grinding ball (Retsch). Samples were submerged in liquid nitrogen before storage at -80°C. Frozen tissue was ground into a fine powder using a Retsch Mixer Mill (Retsch). From this stage, the E.Z.N.A. ® Plant RNA Kit (Omega Bio-tek) was used to extract RNA following the protocol provided. An additional step to remove DNA was incorporated into the protocol, with the DNase I Digestion Set (Omega Bio-tek) following the steps provided. RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Labtech International, UK).

3.2.7.2. cDNA synthesis

RNA samples were diluted with DEPC water to produce 8 µl RNA dilutions for each sample containing 1 µg of RNA. The qPCRBIO cDNA Synthesis Kit was used for reverse-transcriptase of mRNA, as per the protocol. TE buffer was used to dilute the cDNA samples by 1:20, producing templates for PCR and qPCR. Samples were stored at -20°C.

3.2.7.3. Polymerase Chain Reaction

Genes were amplified using specific forward and reverse primers (Table 3.3). A master mix was prepared for each gene made up of 10 µL REDTaq (Sigma), 0.5 µL forward primer (10 µM), 0. 5 µL reverse primer (10 µM), 7µL HPLC water and 2µL template cDNA, of which 20 µl was pipetted into sterile 0.2 ml PCR tubes and placed in the PCR machine. The cycle program was an initial denaturation step (2 min at 94°C), 35 amplification cycles (94°C for 45 sec, 55°C for 45 sec and 72°C for 45 sec) and final extension (72°C for 5 min). PCR products containing REDTaq were observed in a 1.4 % agarose gel electrophoresis run at 50 V for 120 min.

3.2.7.4. Quantitative PCR

The expression levels of genes of interest were measured using quantitative PCR, with two technical replicates per sample. Specific primers used are given in Table 3.3. 2 µl of sample cDNA was added to 10 µl of SYBR green mix (Brilliant III Ultra-Fast SYBR Green QPCR Master Mix with low ROX, Agilent Technologies), 5 µl primer mix (150 nM) and 3 µl of HPLC water. A quantitative PCR machine (Agilent Mx3000P QPCR System, Cheshire UK) was set to run the following cycling profile; 1 cycle of denaturation at 95°C for 3 min, 40 amplification cycles of (95°C for 15 sec and 60°C for 1 min) and 1 cycle of (95°C for 1 min, 55°C for 30 sec and 95°C for 30 sec). After each cycle the amplified product was logged automatically by measuring the fluorescence.

3.2.7.5. Analysis of expression data

The expression of *BrPR1, BrSNC1* and *FocBr1* genes was assessed from the qPCR result analysis. The expression profiles for each genotype were calculated from the ΔCt value [ΔCt= (Ct gene - Ct actin)] and -ΔΔCt value [ΔΔCt = (Ct treat. gene - Ct treat. Actin)- (Ct control. gene - Ct control. Actin)] respectively, obtained by MxPro (Mx3005P, version 4.10, Stratagene, United States) software. The data were statistically analysed using GenStat (19th edition) software. The up-regulated genes were defined as a fold-change greater than 2 with $P < 0.05$ and a fold change of 0.5 or less was used to define down-regulated genes when $P < 0.05$. A general ANOVA with Fisher's least significant difference (LSD) procedure was then performed to determine significance between treatments.

3.2.8. Phytohormone analysis

Leaves were taken from 4-week-old wild-type R-o-18 *B. rapa* plants as well as *Brsnc1* and *focBr1*-1. Five plants of each line were sampled, with two young leaves taken from each plant, weighed and then flash frozen in liquid nitrogen. Samples were sent on dry ice to DynaMo MS-Analytics facility (University of Copenhagen, Denmark) where sample extraction was performed as previously described in Großkinsky et al. (2014) and Ionescu et al. (2017), given below.

Frozen ground samples were extracted twice with methanol (1250 µL, 80%) followed by 30 min incubation at 4◦C. The addition of 5 ppm D6-(±)-jasmonate was included as an internal standard. After centrifugation (15 min, 20,000 × g, 4◦C), the supernatant was passed over a C18 column (Phenomex, Strata C18-E, 200 mg/3 ml) using vacuum. The eluate was evaporated to dryness

and samples dissolved in 20% methanol and filtered (0.22 µm, Millipore) by centrifugation (5 min, 3,000 rpm). Samples were subsequently analysed by LC-MS/QqQ to quantify phytohormone levels. Phytohormones were analysed in biological triplicates by UHPLC/TQ-MS on an AdvanceTM-UHPLC/EVOQTMEliteTQ-MS instrument (Bruker) equipped with a C-18 reversed phase column (Kinetex 1.7 u XB-C18, 10 cm × 2.1 mm, 1.7 µm particle size, Phenomenex) by using a 0.05% formic acid in water (v/v), pH 4.0 (solvent A) – methanol (solvent B) gradient at a flow rate of 0.4 ml/min at 40◦C. The gradient applied was as follows: 10–50% B (15 min), 50% (2 min), 50–100% B (0.1 min), 100% B (2.9 min), 100–10 % B (0.1 min), and 10% B (5 min). Compounds were ionized by ESI with a spray voltage of +4,500 and −4,000 V in positive and negative mode, respectively. The heated probe temperature was 350°C and the cone temperature 300°C. Quantification was based on response factors relative to (2H6) JA. The individual hormones were monitored based on the following MRM transitions: (2H6)JA, (−) 215 > 59 [11 V]; ABA, (−) 263 > 153 [7 V]; ACC, (+) 102 > 56 [15 V]; JA, (−) 209 > 59 [11 V]; JA-Ile, (−) 322 > 130 [17 V]; SA, (−) 137 > 93 [20 V]; IAA, (+) 176 > 130 [10 V]; iP, (+) 204 > 136 [10 V]; DHZ, (+) 222 > 136 [15 V]; DHZR, (+) 354 > 222 [15 V]; tZ7G/tZ9G/tZOG, (+) 382 > 220 [17 V]; tZR (+) 352 > 220 [15 V]. tZ7G, tZ9G, and tZOG were distinguished based on retention times in comparison to those of known standards.

3.3. Results

3.3.1. Identification and sequence alignment of hypothesised *SNC1* orthologs in *B. rapa*, *B. napus* and *B. oleracea*

In *A. thaliana* Columbia, *SNC1* is positioned on chromosome 4 (At4), within a cluster of seven TIR-NBS-LRR class *R* genes (Noel et al., 1999). When the *AtSNC1* coding sequence was searched against the *B. rapa* genome sequence, the top two closest matches were *Bra012689* and *Bra012688*, both of which are positioned on chromosome A03. When a BLAST search was done against the *B. napus* genome sequence, most of the syntenic regions were on C07, with several also in the A chromosome. Two of the closest sequences were *BnaC07g34000D* and *BnaC07g33980D*.

The *FocBo1* locus is located on C7 of *B. oleracea* which has collinearity with At4 (Pu et al., 2012). When the *FocBo1* coding sequence was searched against the *B. napus* genome sequence, it was found to be highly syntenic with *BnaC07g33990* and *BnaC07g34000D*. An alignment with DNA

coding sequences of At*SNC1, FocBo1, Bra012689, Bra012688*, *BnaC07g33990* and *BnaC07g33980D* showed synteny between these genes of interest (Appendix B.1)

3.3.2. Phylogenetic analysis of *SNC1*, *FocBo1* and their candidate orthologs

Phylogenetic analysis by maximum likelihood predicted that *FocBo1*, its homolog *FocBr1* in *B. rapa* and partial putative homologs, *BnaC07g34000D* and *BnaC07g33990,* in *B. napus* form a clade. At*SNC1* and it six closely related *R* genes on At4 in *A. thaliana* are shown to be closely related, as would be expected. The candidate homologs of At*SNC1* in *B. rapa* and *B. napus, Bra012689* and *BnaC07g33980D*, respectively are separate from the At4 cluster, but closer to *SNC1* than *FocBo1*. The two branches predicting phylogeny of *SNC1* and *FocBo1* are distinct from each other (Figure 3.2).

The amino acid sequences of *AtSNC1*, *FocBr1* and *BrSNC1* were analysed using InterProScan to predict their domains. All three genes were found to have very similar characteristics (Figure 3.3). All three genes had similar lengths and similar spatial distribution of TIR, NB-ARC and LRR regions. From this study, and previous analysis by Shimizu et al., (2015) it is hypothesised that two *B. rapa* genes; *Bra012689* and *Bra012688* are homologs of *AtSNC1* and *FocBo1* respectively. Figure 3.4 shows how these genes are hypothesised to be *FocBo1* and *SNC1* homologs in *B. rapa*.

The amino acid sequences of *BnaC07g34000D* and *BnaC07g33990* were input into the SMART protein domain annotation web server, to determine their domains. *BnaC07g33990* was found to contain LRR domains, while *BnaC07g34000D* had a TIR-NBS domain. These two genes were hypothesised to together make up a TIR-NBS-LRR *R*-like gene that is a putative ortholog of *FocBo1.* Evidence for this suggestion is threefold: 1) these genes together make up a TIR-NBS-LRR *R*-like gene; 2) *BnaC07g34000D* and *BnaC07g33990* are positioned next to each other on the genome; 3) added together, the sum of the base pairs adds up to 3,957, which is close in length to *FocBo1* (4,420bp). These two genes may have resulted from a single gene being disrupted in the cultivar Darmor bzh, generated through non homologous recombination or an incomplete genome assembly. Alternatively, the gene could encode a protein made up of an amino-terminal and NBS domain with no LRR domain. There are 21 of these TIR-NBS proteins in *A. thaliana*, although their function is unknown, it is suggested that they may act as adaptors or regulators of TIR-NBS-LRR and coiled-coil-NBS-LRR proteins (Meyers et al., 2002; McHale et al., 2006). RT-PCR could be used to resolve this question through comparing the expression levels of *BnaC07g34000D* and *BnaC07g33990* as both would be the same if part of the same gene.

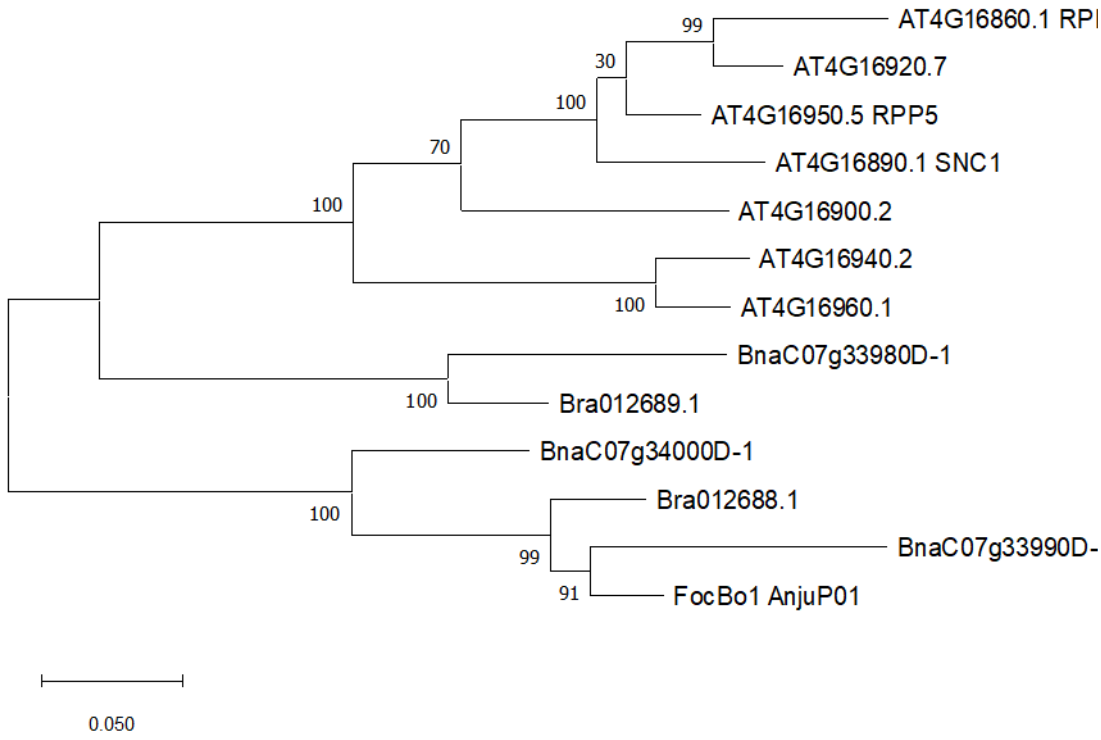


Figure 3.2 Phylogeny of *SNC1* (and closely related genes) in *A. thaliana*, *FocBo1* in *B. oleracea* and candidate homologs of *SNC1* and *FocBo1* in *B. rapa* and *B. napus*
Phylogenetic analysis was carried out by Maximum Likelihood with 500 bootstraps. The phylogeny was inferred through the Tamura-Nei model of Maximum Likelihood (Tamura and Nei, 1993). The tree is drawn to scale with branch lengths showing the expected number of nucleotide substitutions per codon. Numbers represent bootstrap values. Analysis was performed in MEGA.

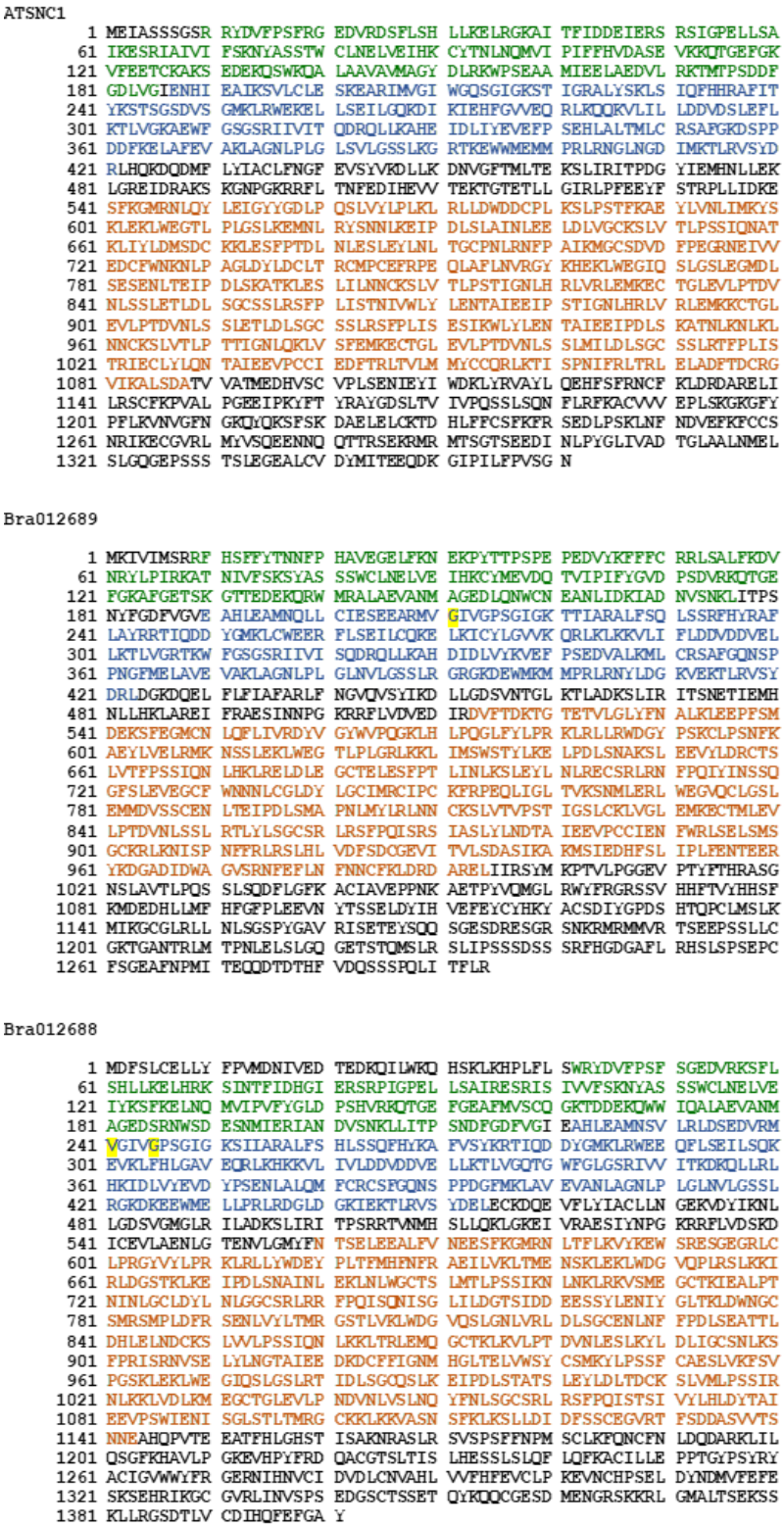


Figure 3.3 Protein sequences of *SNC1* in *A. thaliana* and *BrSNC1* and *FocBr1* in *B. rapa* are similar. TIR regions are in green, NBS-ARC regions are in blue and LRR domains are orange. TILLING mutations in *Bra012689* (*BrSNC1*) and *Bra012688* (*FocBr1-1*) are highlighted in yellow, resulting in changes given in Table 3.1 to the NB-ARC domain.

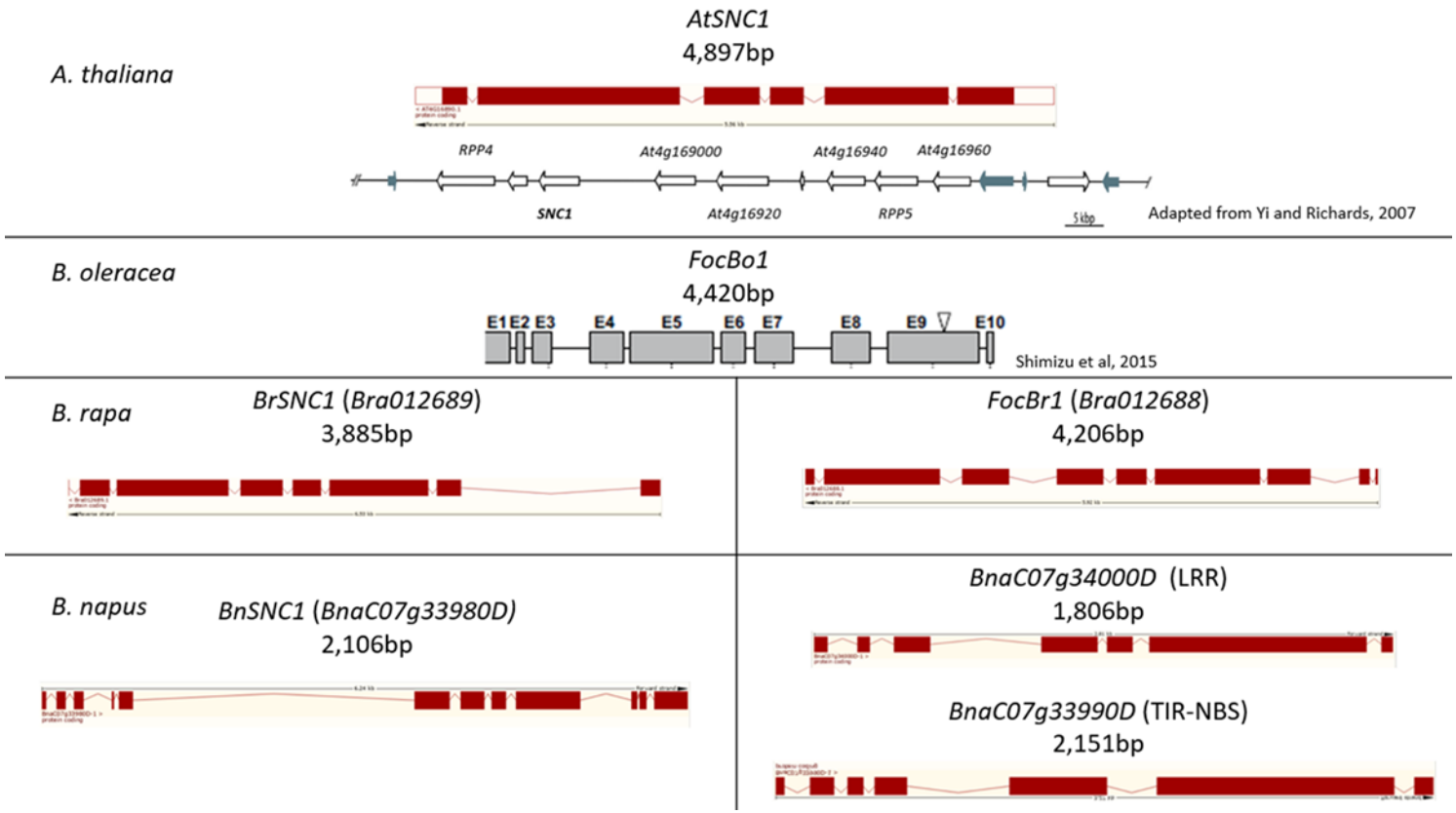


Figure 3.4 Genes observed in *B. rapa* and *B. napus* hypothesised to be separate *FocBo1* and *SNC1* orthologs, identified in *B. oleracea* and *A. thaliana* respectively. Note that *B. napus* gene predictions are much shorter than the gene predictions in the other species.

3.3.3. Genotypic and phenotypic characterisation of TILLING mutant lines

Phenotypes were observed for R-o-18, *focbr1-1*, *focBr1-2* and *Brsnc1* plants (Appendix B.2). *R-o-18*, *Brsnc1* and *focBr1*-2 had the same healthy phenotype. The *focBr1-1* mutant had a much shorter stature, with rounded leaves and a faceted stem. Flowering was much delayed compared to the wild-type, and when flowers were produced by *focBr1-1* plants, they were found to be male sterile. Figure 3.5 shows these dwarf plants alongside R-o-18 wild-type. Analysis of segregation using Chi-squared test on plants produced from selfing a heterozygous *FocBr1-2* BC_1F_2 plant, using genotypes confirmed with KASP marker analysis, found a χ^2 value of 0.7941 (Figure 3.6). As χ^2 is less than the test value 5.99, there is therefore not statistically significant deviations from a 1:2:1 segregation ratio.

3.3.4. Temperature-sensitivity assessment of mutant lines

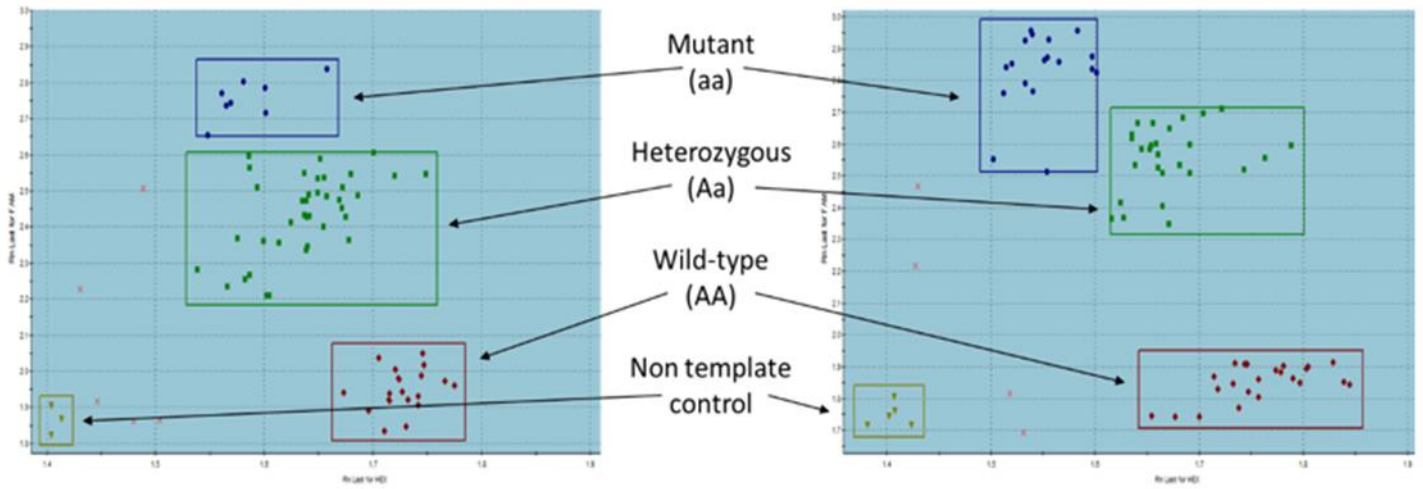
3.3.4.1. Cotyledon temperature-sensitivity assessment

Results from inoculation assays found clear differences in temperature-sensitivity for the three genotypes resulting from mutations in the *FocBr1* and *BrSNC1* genes in comparison to the wild-type R-o-18 line. All four lines showed higher severity scores at 25°C compared to 20°C (Figure 3.7) which is expected as resistance genes often display temperature-sensitivity. R-o-18 showed a resistant phenotype, with an average lesion score of 2.6 at 20°C. This resistance broke down at 25°C, with a significantly higher mean score of 7.3. *Brsnc1* displayed the highest resistance phenotype at both temperatures, with an average score of 1.8 at 20°C and 5.8 at 25°C, significantly lower than the wild-type at the at 25°C, supporting the hypothesis that *BrSNC1* plays a role in temperature-sensitivity of the resistance response. Conversely, *focBr1-1* had a more susceptible phenotype compared to the wild-type, even at 20°C. The mean lesion score at 20°C for *focbr1-1* was 7.7, which rose to 8.9, a significant difference, at 25°C. The heterozygous line for mutant *FocBr1-2* had a mean score of 3.3 at 20°C, not significantly different to the wild-type. At 25°C, the mean score for heterozygous *FocBr1-2/focBr1-2* plants rose to 8.6.

3.3.4.2. True leaf temperature-sensitivity assessment

Lesion phenotypes following inoculation with an avirulent isolate of *L. maculans* at 20°C and 25°C on true leaves of 42-day-old *B. rapa* mutant genotypes *Brsnc1*, *focBr1-1*, *focBr1-2*, segregating *FocBr1-2* wild-type and wild-type (R-o-18) can be seen to differ in Figure 3.8.

Figure 3.5 Phenotypic differences in growth stature of *focBr1-1* compared to R-o-18 wild-type (A) Three *focBr1-1 B. rapa* plants from RevGenUK (right) and two R-o-18 wild-types *B. rapa* plants (left). (B) Wild-type R-o-18 *B. rapa* (left) homozygous TILLING mutant lines *Brsnc1* (centre) and *focBr1-1* (right).



	AA (wildtype)	Aa (Heterozygous)	aa (Mutant)
Observed (*o*)	20	33	15
Expected (*e*)	17	34	17
Deviation (*o* - *e*)	3	-1	-2
Deviation^2 (d^2)	9	1	4
d^2/e	0.5294	0.0294	0.2353

$\chi^2 = d^2/e = 0.7941$

Figure 3.6 Analysis of segregation using Chi-squared test on plants produced from selfing a heterozygous *FocBr1-2* BC_1F_2 plant, using genotypes confirmed with KASP marker analysis As χ^2 is less than the test value 5.99, there is therefore not statistically significant deviations from a 1:2:1 segregation ration on the 5 % level ($P > 0.5$).

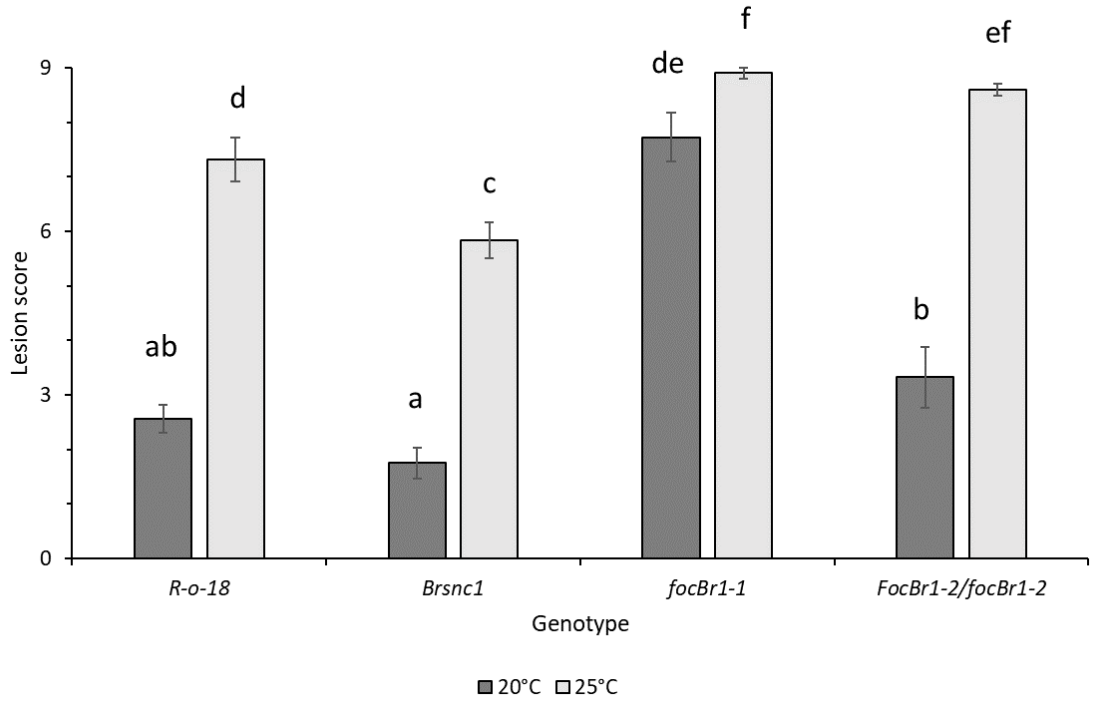
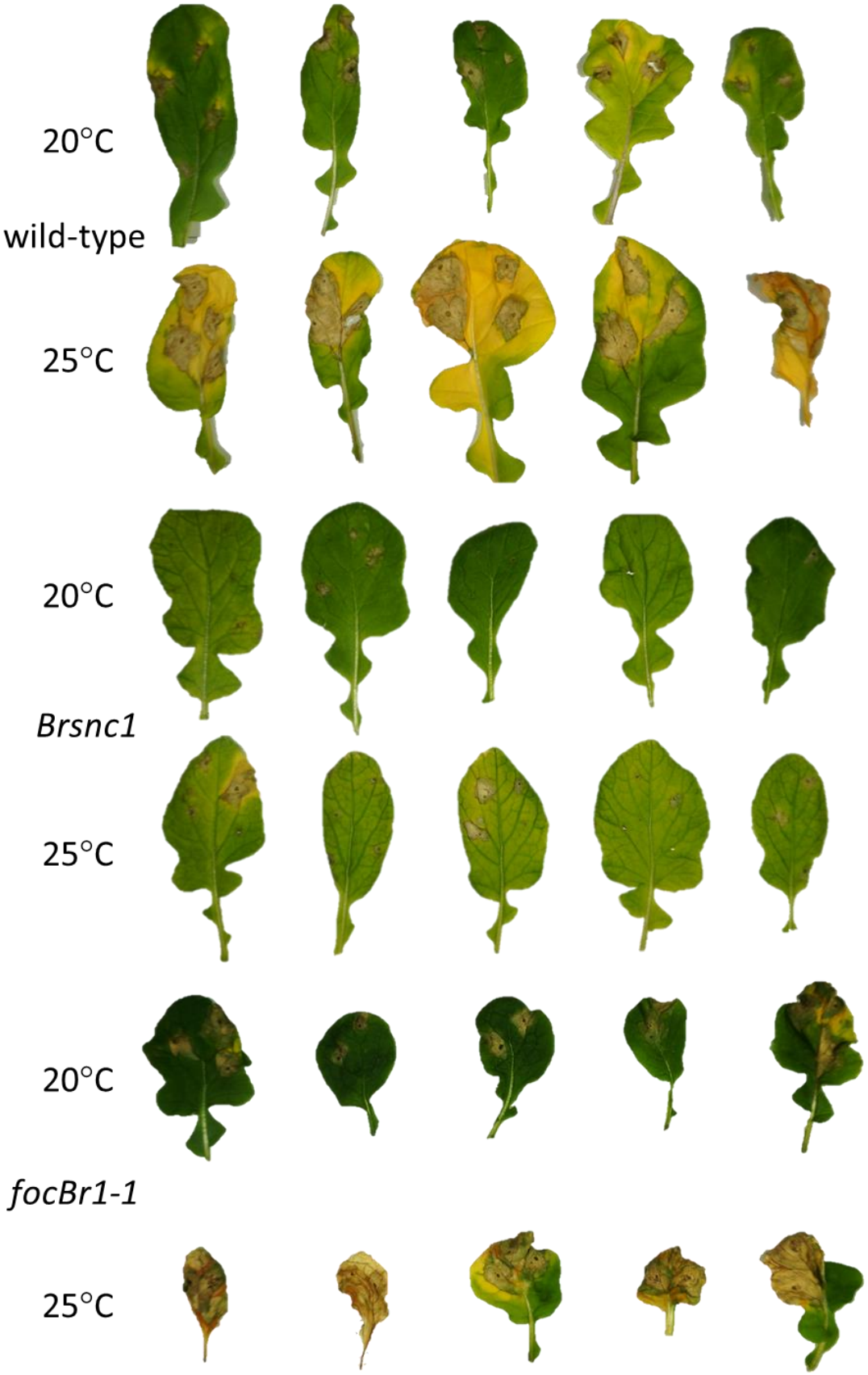


Figure 3.7 Average lesion score (0-9 scale) assessed cotyledons of *B. rapa* TILLING mutants and R-o-18 wild-type, following wound inoculation with *L. maculans* isolate JN3 at 12 days post-inoculation. Error bars indicate the SEM. Plants of *B. rapa* (wild-type R-o-18, TILIIING line mutant genotypes *Brsnc1, focBr1-1* and *FocBr1-2/focBr1-2* were inoculated with 10^7 conidial spores per ml of *L. maculans* isolate JN3 (*AvrLm1-4-5-6-7-8*). Bars represent mean lesion score calculated from ten biological reps. Average scores sharing a same letter are not statistically different ($P < 0.05$) in a multiple comparison Fisher's least significant difference test. Error bars indicate the standard error of the mean.


20°C
wild-type
25°C
20°C
Brsnc1
25°C
20°C
focBr1-1
25°C

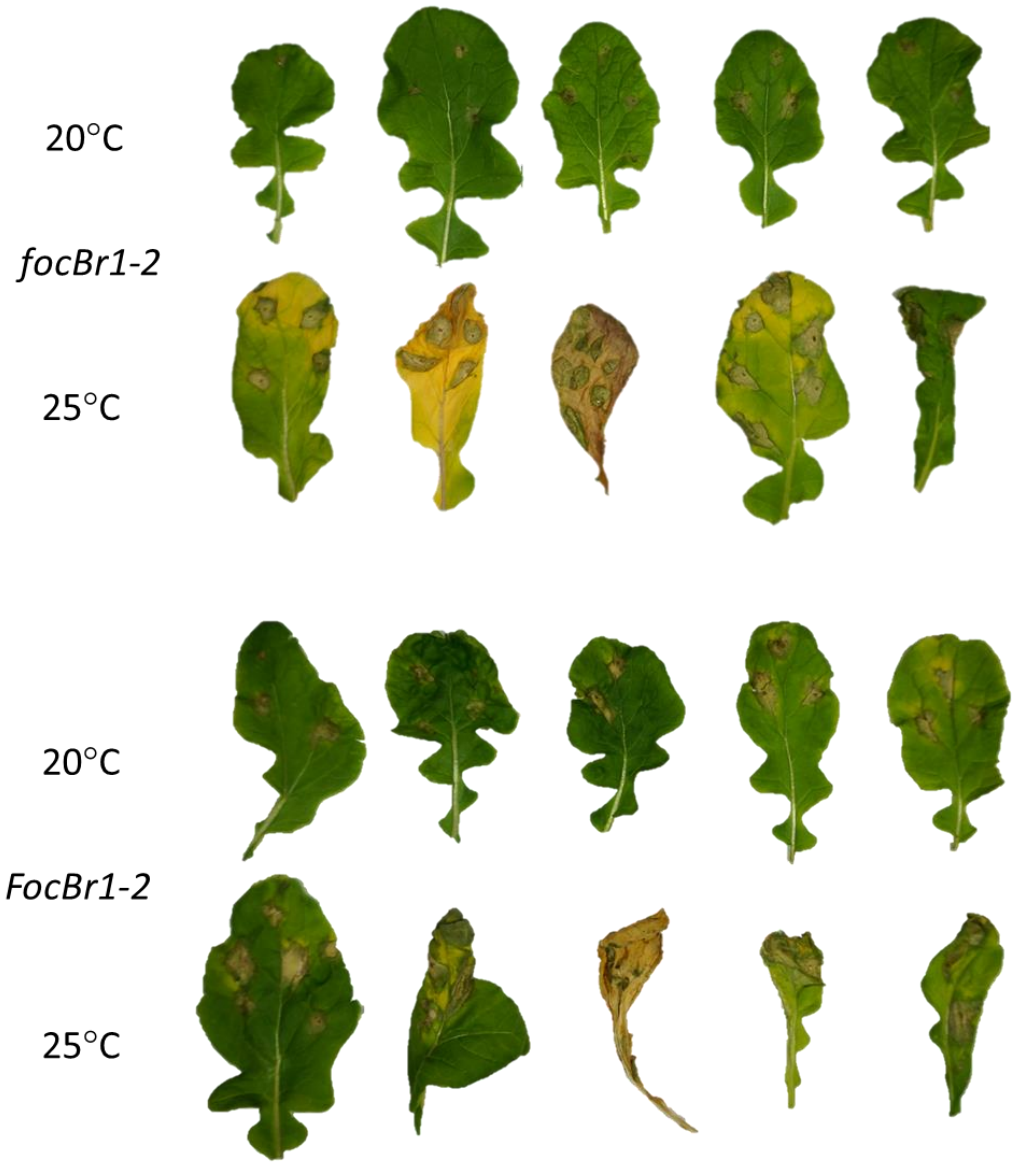


Figure 3.8 True leaves of *B. rapa* mutant lines and the wild-type R-o-18. Photographs were taken 14 days post-inoculation with an avirulent *L. maculans* isolate. Prior to inoculation, plants were grown at 20°C for two weeks, transferred to the assessment temperature for two weeks, wound-inoculated with a conidial suspension of 10^7 ml^{-1} of *L. maculans* isolate JN3 (*AvrLm1-4-5-6-7-8*), before a further two-week period for symptom development. Plants were grown in controlled environment chamber, set to 12 hours light/ dark.

The effect of incubating inoculated plants at an elevated temperature of 25°C had a similar effect on the resistance phenotypes on these older plants as was described previously for the cotyledon assay. However, the differences between *Brsnc1* and the wild-type appear greater in the true leaf assay (Figure 3.9) compared to the cotyledon assay (Figure 3.7), this may be due to age-related resistance in the wild-type. As before, every genotype showed greater severity of the disease at the higher temperature (Figure 3.9). However, the differences observed were more pronounced than in the cotyledon assay. R-o-18 leaves inoculated with JN3 produced an average lesion severity score of 4.6 at 20°C, and 7.5 at 25°C. As was seen in the cotyledon assay, this was found to be significant at the 5% level.

Lesions on the leaves of *Brsnc1* plants were consistently observed to be smaller at both temperatures. These scores were significantly lower than those observed on the R-o-18 wild-type, with an average lesion score of 1.6 and 2.2 at 20°C and 25°C respectively ($P < 0.05$). The *focBr1-1* mutant again showed higher levels of lesion severity compared to the R-o-18 wild-type, with mean scores of 6.9 and 7.9 at 20°C and 25°C respectively. *Focbr1-2* did not display a statistically significant different score in its resistance phenotype compared to the wild-type. However, the *FocBr1-2* segregating wild-type did have a lower level of disease at 20°C compared to the wild-type

3.3.5. Pathogen DNA quantification

L. maculans DNA quantity for each mutant line was determined using qPCR to assess the effect of the mutations on pathogen biomass at 20°C and 25°C and to provide a more quantitative comparison of mutant effect on pathogen growth (Figure 3.10). Whilst the different genotypes were found to contain differing levels of pathogen DNA, only one line, *Brsnc1*, was found to have a significant difference at the 5% level compared to the wild-type. The amount of pathogen DNA in R-o-18 samples were found to be very similar at both temperatures, with 1231 pg at 20°C and 1253 pg measured at 25°C. Neither *focBr1-1* nor *focBr1-2* had a significantly different quantity of pathogen DNA compared to the wild-type at either temperature. Plants containing a mutation in *Brsnc1* had lower amounts of pathogen DNA to the wild-type at both temperatures. At 20°C *Brsnc1* had more than six-fold less pathogen DNA than R-o-18, and almost half as much at 25°C. No significant difference in pathogen DNA was found between the two temperatures for *Brsnc1*. These similarities in DNA quantity could be due to the maximum amount of pathogen biomass being constrained by the size of the leaf disc; the fungal pathogen may be restricted from growing beyond a certain level due to lack of nutrients. Alternatively, there may be similar biomass, but differences in symptom development on different genotypes.

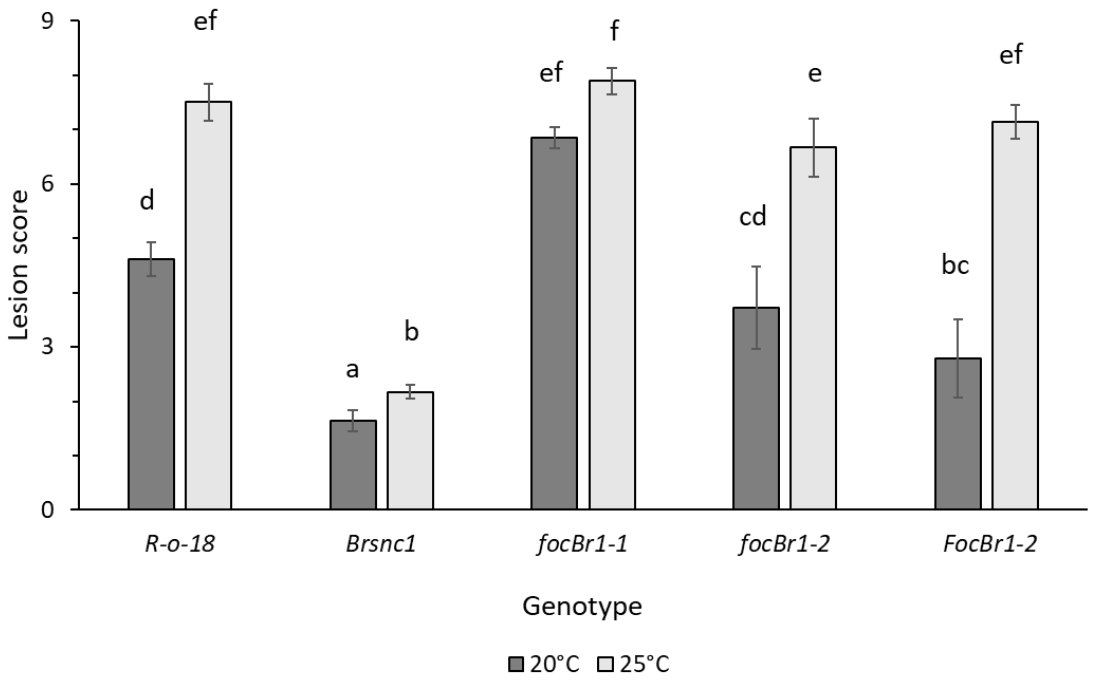


Figure 3.9 Average lesion score (0-9 scale) assessed on 40-day-old *B. rapa* TILLING mutants and R-o-18 wild-type, following wound inoculation with *L. maculans* isolate JN3 at 12 days post-inoculation. Error bars indicate the SEM. Plants of *B. rapa* (wild-type R-o-18, TILIIING line mutant genotypes *Brsnc1, focBr1-1, focBr1-2* and *FocBr1-2* were inoculated with 10^7 conidial spores per ml of *L. maculans* isolate JN3 (*AvrLm1-4-5-6-7-8).* Average scores sharing a same letter are not statistically different ($P < 0.05$) in a multiple comparison Fisher's least significant difference test.

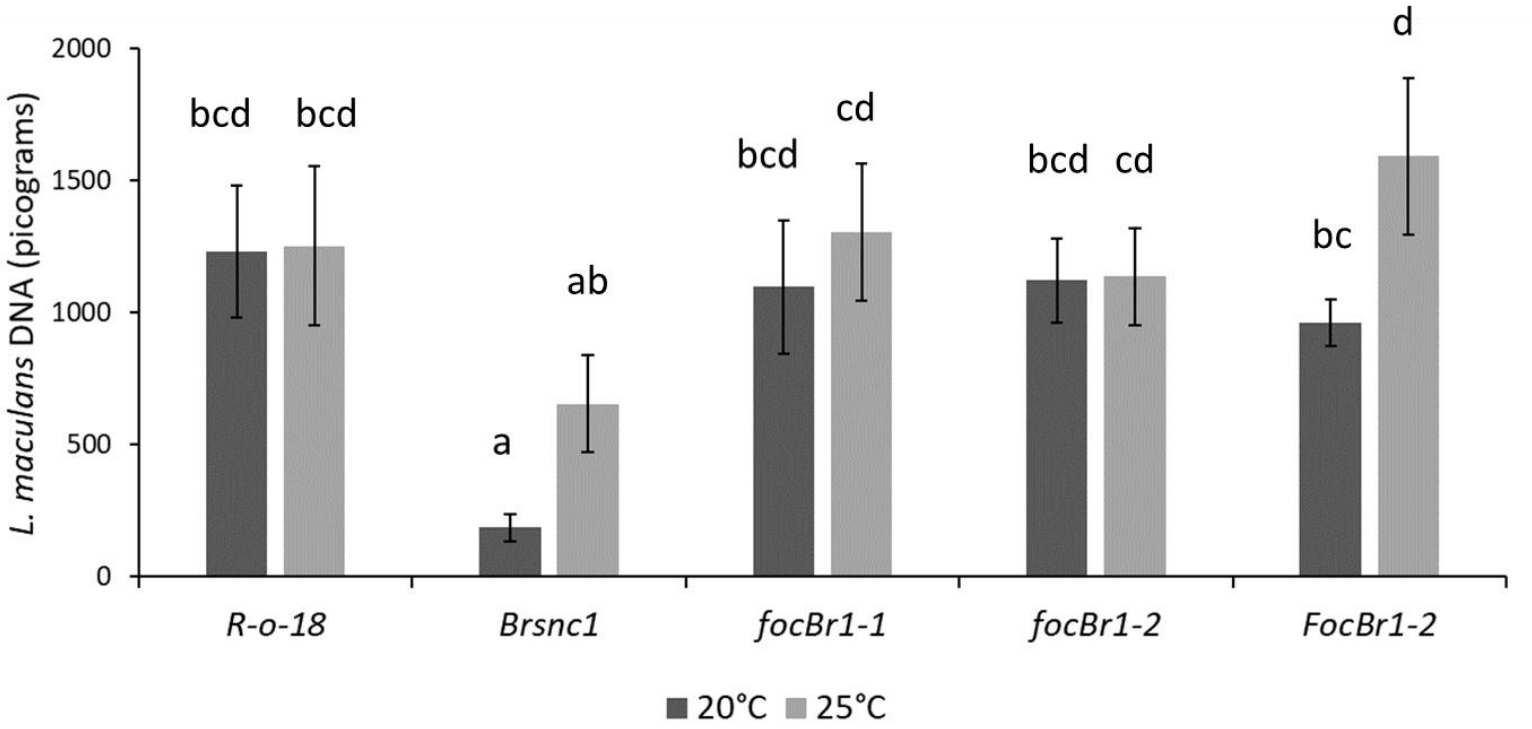


Figure 3.10 Amount of *L. maculans* DNA present in four 8 mm leaf discs taken directly from inoculation sites of *B. rapa* plants 14 days post inoculation. Plants of *B. rapa* wild-type R-o-18 and mutant genotypes *Brsnc1, focBr1-1, focBr1-2* and *FocBr1-2* were inoculated with 10^7 conidial spores per ml of *L. maculans* isolate JN3 (*AvrLm1-4-5-6-7-8*). Average scores sharing a same letter are not statistically different ($P < 0.05$) in a multiple comparison Fisher's least significant difference test.

3.3.6 Gene expression analysis

3.3.6.1 *PR1* gene expression

PR1 is known to be regulated by *L. maculans* (Becker et al., 2017; Haddadi et al., 2018; Sasek et al., 2012) and a correlation between *PR1* expression and SA production following inoculation with an avirulent isolate was shown by Sasek et al. (2012). Therefore, expression of *PR1* was assessed to investigate if the mutations caused an effect on the cotyledons defence response. *PR1* expression was assessed in *Brsnc1*, *focBr1-1* and segregating *FocBr1-2* genotypes, relative to expression in R-o-18 wild-type (Figure 3.11). An ANOVA on -ΔΔCt values found levels of *PR1* expression in healthy plants differed significantly between mutant genotypes and the R-o-18 wild-type (Appendix B3). However, as all the average up-regulated expression fold changes were below a two-fold difference to the wild-type, and all average down-regulated were under a 0.5-fold difference, this difference was not deemed to be biologically meaningful in this study.

3.3.6.2 *BrSNC1* and *FocBr1* expression analysis

SNC1 expression was assessed in *Brsnc1*, *focBr1-1*, *focBr1-2*, *FocBr1-2/ focBr1-2* and *FocBr1-2* genotypes relative to expression in R-o-18 wild-type (Figure 3.12). An ANOVA on -ΔΔCt values found levels of expression of *BrSNC1* was not affected by genotype ($P = 0.6$) (Appendix B4).

FocBr1 expression was also assessed in *Brsnc1*, *focBr1-1*, *focBr1-2*, *FocBr1-2/ focBr1-2* and *FocBr1-2* genotypes relative to expression in R-o-18 wild-type (n = 5) (Figure 3.13). *FocBr1* was significantly affected by genotype ($P < 0.001$) between mutant lines (Appendix B4 and B5 respectively). However, all the average up-regulated expression fold changes were below a two-fold difference to the wild-type, and all average down-regulated were under a 0.5-fold difference, this difference was not deemed to be to be biologically meaningful in this study.

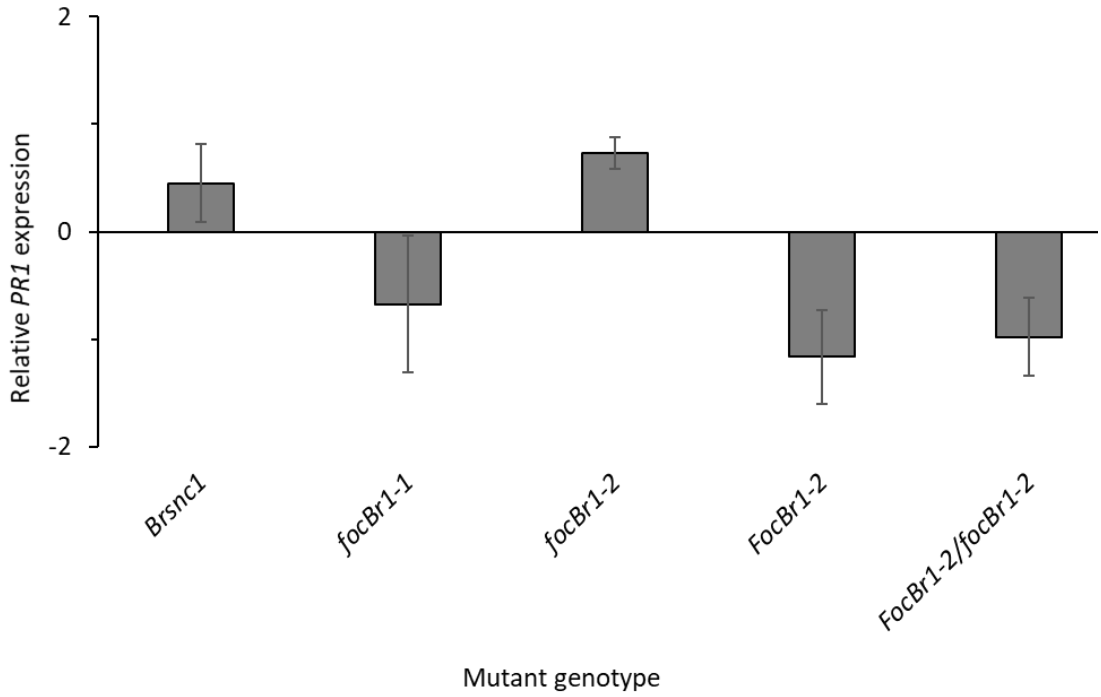


Figure 3.11 *PR1* expression of *B. rapa* R-o-18 TILIIING lines containing mutations in *BrSNC1* and *FocBr1* relative to the R-o-18 wild-type Samples were taken from healthy leaves of 40-days old plants, with five biological replicates per genotype. Bars represent mean expression, relative to expression in the R-o-18 wild-type. Data were normalised to the housekeeping gene actin. -ΔΔCt value (ΔΔCt = (Ct *PR1* - Ct *Actin*) treatment - (Ct *PR1*- Ct *Actin*) control). Error bars indicate standard error of the mean.

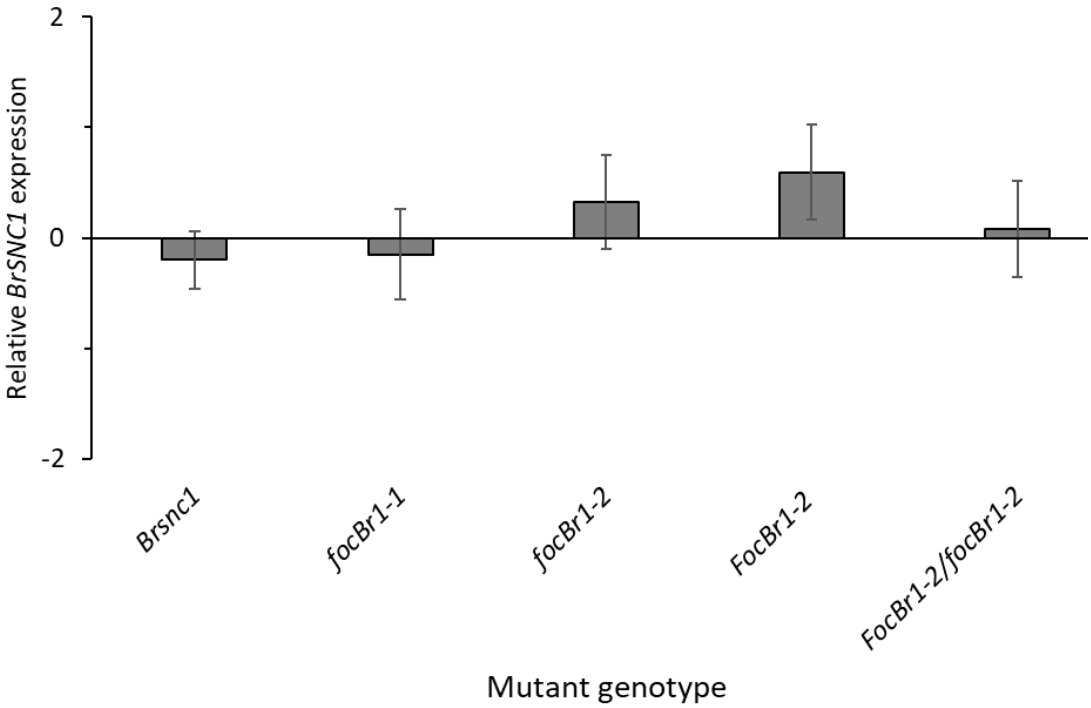


Figure 3.12 Figure 4.13. *BrSNC1* expression of *B. rapa* R-o-18 TILIIING lines containing mutations in *BrSNC1* and *FocBr1* relative to the R-o-18 wild-type. Samples were taken from healthy leaves of 40-days old plants, with five biological replicates per genotype. Bars represent mean expression, relative to expression in the R-o-18 wild-type. Data were normalised to the housekeeping gene actin. -ΔΔCt value (ΔΔCt = (Ct *BrSNC1* - Ct *Actin*) treatment - (Ct *BrSNC1*- Ct *Actin*) control). Error bars indicate standard error of the mean.

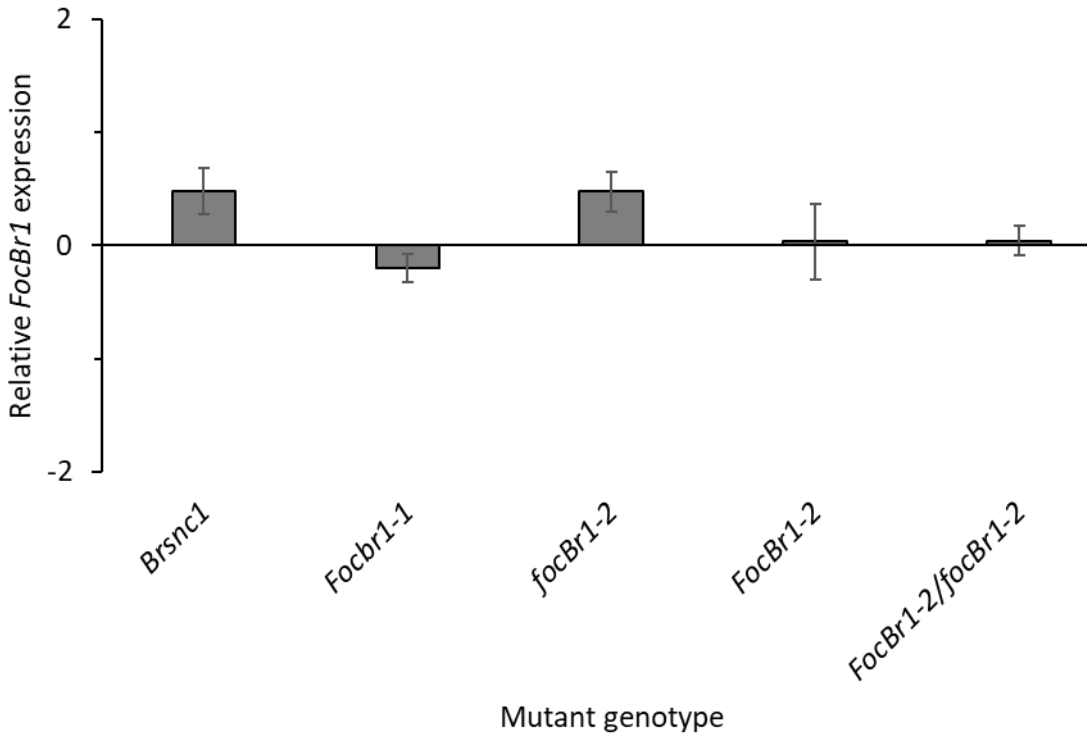


Figure 3.13 *FocBr1* expression in *B. rapa* R-o-18 TILIIING lines containing mutations in *BrSNC1* and *FocBr1* relative to the R-o-18 wild-type. Samples were taken from healthy leaves of 40-days old plants, with five biological replicates per genotype. Bars represent mean expression, relative to expression in the R-o-18 wild-type. Data were normalised to the housekeeping gene actin. -ΔΔCt value (ΔΔCt = (Ct *FocBr1* - Ct *Actin*) treatment - (Ct *FocBr1*- Ct *Actin*) control). Error bars indicate standard error of the mean.

3.3.7. Pant hormone analysis

The quantities of various plant hormones were measured in 4-week old *B. rapa* TILLING mutant lines *Brsnc1* and *focBr1-1*, as well as the R-o-18 wild-type. The three genotypes were found to show clear differences in several phytohormones whilst others were present in similar amounts (Figure 3.14).

Total cytokinins were not found to differ significantly between genotypes ($P = 0.204$), the highest level of total cytokinins were seen in the wild-type, with 1722 nmol/g fresh weight, whilst *Brsnc1* had the lowest level with an average of 962 nmol/g fresh weight. No significant difference was also found for levels of SA between any of the genotypes ($P = 0.423$). *Brsnc1* and *focBr1-1* were found to contain similar levels of SA, with 5757 and 5798 nmol/g fresh weight respectively, whilst R-o-18 had 2365 nmol/g fresh weight. No significant difference was also found for levels of indole-3-acetic acid (IAA) between any of the genotypes ($P = 0.245$), all of which had similar levels 608.9, 610.0 and 683.6 nmol/g fresh weight for *Brsnc1*, *R-o-18* and *focBr1-1* respectively. Similarly, no significant difference was found for 1-aminocyclopropane-1-carboxylic acid (ACC) (the direct precursor of ET), with 15546, 17271 and 19395 nmol/g fresh weight for *R-o-18*, *focBr1-1* and *Brsnc1* respectively.

Brsnc1 had more than a threefold greater volume of indole-3-acetaldehyde (2846 nmol/g fresh weight) compared to the wild-type 854 (nmol/g fresh weight) which was found to be statistically significant ($P < 0.05$). The *focBr1-1* mutant was not found to differ significantly from the wild-type, with an average of 1165 nmol/g fresh weight. *Brsnc1* was also found to contain significantly higher ($P < 0.01$) levels of 3-indoleacetamide, with an average of 2846 nmol/g fresh weight compared to 854 and 1165 nmol/g fresh weight found for the wild-type and *focBr1-1* respectively.

ABA levels were present in much lower levels compared to other hormones analysed. Significantly higher amount of ABA was found in the wild-type plants (90.60 nmol/g fresh weight) ($P < 0.01$) compared to *focBr1-1* and *Brsnc1* mutants, which were found to have averages of 22.96 and 34.71 nmol/g fresh weight respectively.

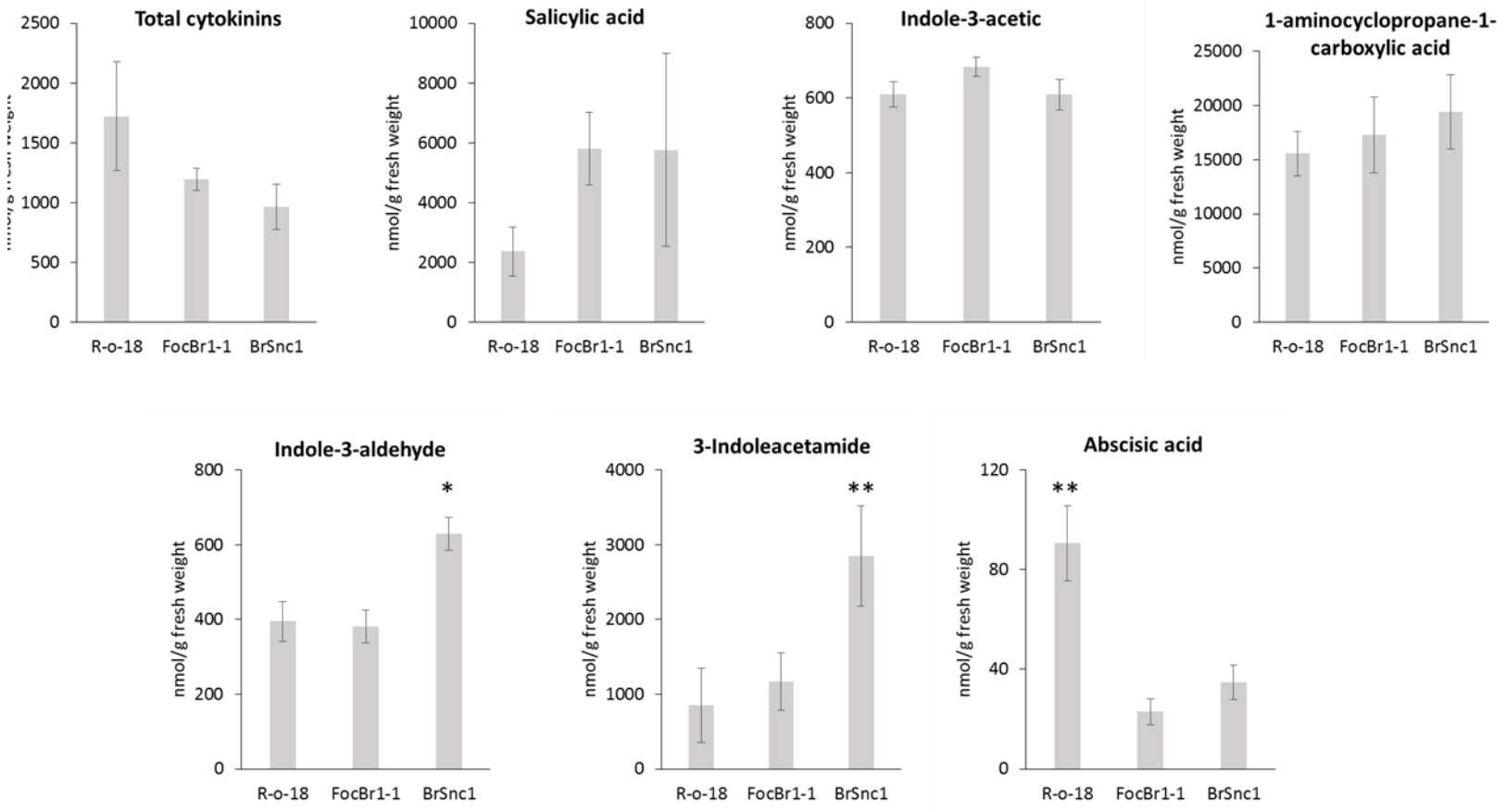


Figure 3.14 Differences in phytohormone levels in 4-week-old *B. rapa* R-o-18 plants with wild-type, *focBr1-1* and *Brsnc1* genotypes. Asterisks denote significance when compared with the wild-type (* $P < 0.05$, ** $P < 0.01$) and error bars denote standard error of the mean.

3.4. Discussion

3.4.1. *SNC1* and *FocBr1* orthologs have been identified in *B. rapa*, the putative orthologous gene of *FocBo1* and *FocBr1* in *B. napus* is disrupted

Findings from sequence analysis and phylogenetic tree construction led to the identification of an ortholog of the temperature-sensitive *SNC1* gene in *B. rapa, BrSNC1*. It was hypothesised that this ortholog of *SNC1* acts as a temperature-sensitive component of the defence response, as *SNC1* has previously been found to behave in *A. thaliana* (Zhu et al., 2010). *FocBr1* was previously identified as a potential ortholog to *FocBo1* in *B. rapa* by Shimizu et al., (2015). Phylogenetic analysis in this chapter reaffirmed this hypothesis. *FocBr1* and *BrSNC1* show high levels of sequence similarity, and it is suggested that they may be duplicated genes, resulting in neofunctionalisation after the duplication event. It is hypothesised that *FocBr1*, is a more recent addition to the genome, equivalent to *FocBo1* in *B. oleracea*, and that it may confer a beneficial temperature-resilient phenotype.

A putative ortholog of *FocBr1* was identified in *B. napus* and appears to be split into two partial genes. *BnaC07g33990* was found to contain LRR domains, while *BnaC07g34000D* has TIR-NB domains. Further analysis of the *SNC1* candidate gene in *B. napus* (*Bnac07g33980*) suggested that it is a truncated pseudogene, merely containing an LRR domain. Gene studies in *B. napus* are more complicated than in *B. rapa*; *B. napus* is an allotetraploid species with a complex genome (Song et al., 2010). The *B. napus* genome is the product of a hybridization between *B. rapa* (2n = 20, A genome) and *B. oleracea* (2n =18, C genome) (U, 1935). Brassica ancestors are also known to have undergone two duplication and two triplication events. Consequently there are many duplicated regions in the *B. napus* genome (Jenczewski et al., 2013). More analysis will need to be carried out in the *B. napus* genome to identify *BrSNC1*, future investigations could look instead to the A genome for *BrSNC1*.

3.4.2. Gain of function *Brsnc1* has a temperature-resilient autoactivated defence phenotype; loss of function *focBr1-1* mutants show increased susceptibility

A previous study by Tameling et al. (2006) showed that mutations to the P-loop region of the NB-ARC domain in *R* genes can impair ATP hydrolysis, leading to autoactivation and induction of the defence response. Therefore, it is not surprising that the two TILLING lines with mutations lying in the P-loop (*Brsnc1* and *focBr1-1*) have a significantly distinct resistance phenotype to the wild-type. The hypothesised effects of *Brsnc1* and *focBr1-1* mutants in the NB subdomain are shown in Figure 3.15. No such differences in the defence response were observed for the *focBr1-*

2 mutant; this also is expected as the mutation lies before the P-loop in the NB-ARC domain and involves a conserved amino acid change.

The experiments described in this chapter support the hypothesis that a gain of function mutation in *BrSNC1* provides a greater level of resistance against an avirulent isolate of *L. maculans* that is effective at 25°C. In the two inoculation assays, carried out at 20°C and 25°C on cotyledons and true leaves of older plants with mutant genotypes, *Brsnc1* plants produced significantly smaller lesions than the wild-type, signifying a strong ETD response. Moreover, *Brsnc1* plants had significantly less pathogen DNA present compared to the wild-type in inoculated leaf tissue. This suggests that *Brsnc1* is an autoactivated mutant, similar to the autoactivated mutations investigated by Tameling et al. (2006). Furthermore, phenotypic responses match those observed in *snc1* gain of function mutant in *A. thaliana*; as opposed to other constitutively resistant mutants, *snc1* does not exhibit spontaneous lesions (Zhang et al., 2003). However, the *PR1* expression of the *Brsnc1* was lower than expected, Li et al. (2010) found *PR1* levels to be significantly higher in *snc1*-1 *A. thaliana* mutants compared to the wild-type.

SNC1 is an important activator of the defence pathway and is generally kept to very low levels of expression, preventing downstream signalling from inducing the resistance response. One of the proteins that negatively regulates *SNC1* is encoded by *BON1*. A loss of function *Atbon1-1* mutant was found to found to activate defence responses and have a dwarf phenotype in *AtSNC1*-containing ecotypes. However, in a loss-of-function double mutant with *snc1-11*, lacking both a functional *SNC1* and *BON1* mutants, the dwarf *bon1* phenotype was supressed and the enhanced resistance was lost (Yang et al., 2004).

Therefore, from the phenotypes described in this study by Yang et al. (2004), it was expected that *focBr1-1* plants, which showed dwarf phenotypes and had a mutation in a potential *SNC1* homolog, would show a similar autoactivated resistant phenotype. However, this was not the case; higher levels of susceptibility were observed in *focBr1-1* mutants at both temperatures. Thus, the mutation in *focBr1-1*, is hypothesised to be a loss-of-function resulting in ADP binding on the NB-ARC domain, effectively maintaining an 'off' state. Downstream pathways inducing the defence response would not be activated resulting in a susceptible response. The dwarf phenotype may be a result of another mutation generated in the TILLING process, highlighting the necessity to carry out further backcrosses on the mutant lines. Alternatively, *FocBr1* may be involved in a different pathway to *SNC1*. These findings add weight to the hypothesis that *BrSNC1* acts as a temperature-sensitive component of the defence response in *B. rapa*. *FocBr1* is also postulated to be involved in a resistance response pathway.

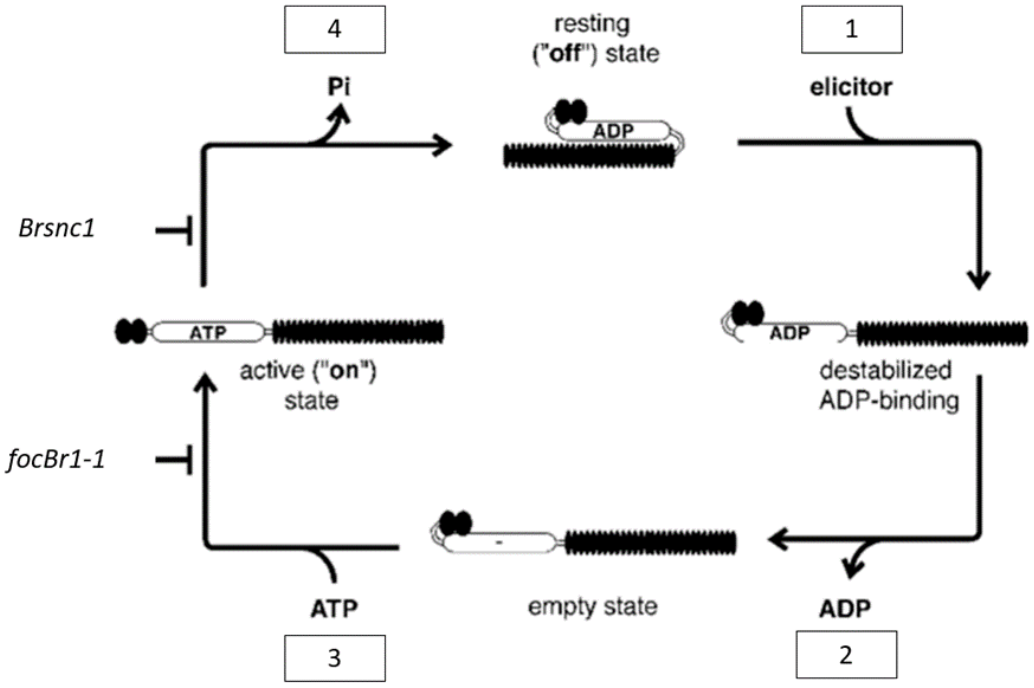


Figure 3.15 Model of the NB-ARC domain functioning as a molecular switch in the regulation of R-protein-mediated signaling and hypothesised effects of *Brsnc1* and *focBr1-1* mutants on its function. In the off state the NB-ARC tightly binds ADP and this interaction is stabilized by the LRR domain. (1) Upon effector recognition, the LRR-NB-ARC interaction is disturbed, reducing its affinity for ADP. (2) This reduced affinity leads to a rapid dissociation of ADP. (3) The empty state binds ATP, triggering a conformational change, this causes a disruption of the TIR-NB-ARC interaction. This on state can now activate signaling, leading to induction of defence responses. (4) Hydrolysis of the bound ATP by intrinsic ATPase activity switches the protein to the off state. The steps hypothesised to be affected in *Brsnc1* (gain of function) and (*focBr1-1*) loss of function mutants in the P-loop of the NB subdomain are indicated.

Schematic adapted from Tameling et al., 2006.

3.4.3. ABA may be part of the mechanism

Both indole-3-aldehyde and 3-indoleacetamide are intermediates in the production of IAA via different pathways (TAA1 and CYP79B) (Sugawara et al. 2009). However, significantly high levels of both these intermediates did not appear to cause any increase in IAA in *Brsnc1* plants. Downstream enzymes could be compensating for higher levels of these intermediates, thus maintaining similar IAA levels to the wild-type.

Alternatively, these intermediates could be precursors for other products. In crucifers, indolic secondary metabolites, made up of indole or indole-related rings with at least one sulphur atom, are known to play an important role in pathogen defence (Bottcher, 2014; Howlett et al., 2001). These phytoalexins are associated with the ETD response, accumulating 1 or 2 days after infection by *L. maculans* (Rouxel et al*.,* 1989; Rouxel et al*.,* 1991).

ABA was significantly reduced in both *Brsnc1* and *focBr1* plants. ABA can play several roles in plants, including protecting against drought stress through closure of stomata. ABA has also been found to have an antagonistic effect on Brassinosteroids (Peleg and Blumwald, 2011). *L. maculans* produces ABA when growing in *B. napus* during the biotrophic stage of infection (Darma et al., 2019). The reason for this is unclear, although it is postulated that it may benefit the fungus by inhibiting the growth of other microorganisms, or through delaying leaf senescence thus allowing the fungus time to grow into the stem. ABA is known to supress *SNC1* expression, thus low levels may activate resistance (Figure 1.1). Future studies of *focBr1-1* and *Brsnc1* genotypes could investigate how the levels of phytohormones differ compared to the wild-type during the resistance response.

4 Gene expression associated with the defence response of temperature-sensitive and -resilient *B. napus R* genes

4.1. Introduction

The changes in gene expression following compatible and incompatible interactions of *B. napus* and *L. maculans* have been studied in both pathogen and host. These investigations have identified and analysed the expression of key genes associated with the defence responses through time following the activation of ETD pathways. Several *R* genes in *B. napus* are known to display temperature-sensitivity. However, the temperature-sensitivity of this resistance response has not been studied. Moreover, the effects of temperature-sensitive breakdown in the resistance response, such as which genes are up- or down-regulated, are unknown.

Temperature has a large impact on plant defence responses, with resistance often breaking down at increased temperatures. The pathways involved in this inhibition at increased temperature are unclear in crop plants, although the temperature-sensitivity pathway in the model plant *A. thaliana* is becoming more understood. Studies in *A. thaliana* have identified intracellular immune receptor NLR genes and SA as being key components in the mechanism behind high temperature inhibition of the defence response (Zhu et al., 2010; Wang et al., 2009).

Therefore, in investigating the temperature sensitivity of the defence response in *B. napus* against *L. maculans*, genes associated with SA are of interest. Expression of *PR1* is SA responsive, thus *PR1* is a commonly used as a marker gene for SA (Sasek et al., 2012). The antifungal group of PR proteins PR1 accumulate to high concentrations locally after pathogen infection (Selitrennikoff, 2001). When inoculated with an avirulent isolate of *L. maculans*, *PR1* was found to be significantly up-regulated from 5 dpi (Sasek et al., 2012).

Three types of cotyledon assays were done to explore the temperature-sensitivity of the resistance-response in *B. napus* against *L. maculans*. The first assay identified temperature-sensitive and -resilient lines from Topas ILs by comparing their resistance phenotypes at 20^{o}C and 25^{o}C. The second study compared defence related gene expression through time between the temperature-sensitive and -resilient lines at 20^{o}C and 25^{o}C with qPCR analysis. The third study used RNA-sequencing to further investigate differences in the defence response of the two lines.

The point method of cotyledon inoculation is often used in defence response studies on the *B. napus* – *L. maculans* pathosystem. A potential issue with this method of inoculation is that the cause of the specificity of the resistance phenotypes has not been tested. Whilst it is hypothesised that the resistance phenotypes result only from an intact *Avr-R* gene specific interaction, it is possible that the observed defence response could also result from other, non-specific molecules produced by the fungus during its culture. This alternative, non-specific induction of the resistance response may be caused by recognition of PAMPs by pattern recognition receptors leading to PTI (de Wit., 2016). As both the *R* genes and *Avr* genes, present in the *B. napus* an *L. maculans* isolates used in these studies are known, the compatibility for each interaction is known prior to inoculation. To investigate, lesion phenotypes resulting from non-specific triggers and those resulting from a specific *Avr-R* gene interaction are compared in this study.

4.1.1. Objectives

1. To test the effect of inoculating *B. napus* cotyledons with *L. maculans* culture filtrate on the phenotypic host response

2. To determine any differences in temperature-sensitivity of responses to *L. maculans* between *LepR3*, *Rlm2*, *Rlm4* and *Rlm7* in *B. napus* single *R* gene ILs at 20°C and 25°C.

3. To compare expression of *PR1* and a putative *FocBo1* homolog, *BnaC07g33990*, between temperature-sensitive and -resilient *B. napus* single *R* gene ILs at 20°C and 25°C.

4. To compare the defence response of temperature-sensitive and -resilient *B. napus* single *R* gene ILs at 20°C and 25°C through time using RNA-Seq analysis.

4.2. Materials and Methods

4.2.1. Response to *L. maculans* culture filtrate cotyledon inoculation in *B. napus*

L. maculans isolate 99-97 (*AvrLm2-4-7*) was grown in Gamborg's B5 medium by inoculating 600 mL of medium with 600 µl of a conidial spore suspension (10^8 spores/mL). A control medium was also prepared without the pathogen (Appendix A.1). The media was incubated for 10 days at 26°C under continuous shaking (130 rpm) (fungal growth in media shown in Appendix C.1). The solution was passed through a sterile 0.22 µm MILLEX GP PES membrane filter unit (MERCK Millipore, Cork, IRL). The single *R* gene IL Topas-*Rlm7* produced by Larkan et al. (2016) and the susceptible double-haploid Topas line, were used. Cotyledons were inoculated with the control medium, *L. maculans* culture filtrate and conidial suspensions, using the method described in 2.2.2, with ten biological replicates per treatment.

4.2.2. Temperature-sensitivity of *R* genes in Topas introgression lines and cultivars

4.2.2.1. Cotyledon inoculation assays at 20°C and 25°C

Cotyledon inoculation assays at 20°C and 25°C were done with *L. maculans* isolates JN3 (*AvrLm1-4-5-6-7-8*) and 99-79 (*AvrLm2-4-7*) to identify temperature-sensitive and resilient *R* genes from a set of *B. napus* ILs, containing *Rlm2, Rlm4, Rlm7* or *LepR3*, produced by Larkan et al. (2016). These *R* genes were each introgressed into a common susceptible doubled-haploid Topas background line through reciprocal back-crossing, resulting in single-*R* gene ILs. *B. napus* cultivars containing the same set of *R* genes were also included to check the responses; Bristol (*Rlm2*), Surpass (*LepR3*), Adriana (*Rlm4*), Excel (*Rlm7*) and Roxet (*Rlm7*). Bristol, Surpass, Adriana and Excel are commonly used as a differential set to determine the *Avr* genes present in *L. maculans* isolates. Cultivar Roxet was the source of the *Rlm7* back-crossed into Topas. The *Rlm7* in Excel comes from the cultivar Caiman and may be a different allele from cultivar Roxet.

Cotyledons were grown and inoculated as described in section 2.2.2 under the same growth conditions. At 24 hours prior to inoculation, plants being treated at an elevated temperature were moved into another CE chamber, set to the same programme with an increased temperature of 25°C. Ten-day old *B. rapa* seedlings were inoculated by the wound-inoculation method described by Huang et al. (2011). Disease assessment was done 12 dpi using a 0-9 scale produced by Larkan et al. (2016) and adapted from that of Koch et al. (1991) (Appendix A.2).

Table 4.1 List of experiments in this chapter with host plant and pathogen material used.

Cotyledon inoculation experiment	***B. napus* material used**	***L. maculans* isolate used**
Response to *L. maculans* culture filtrate	Topas, Topas-*Rlm7*	99-97 (*AvrLm2-4-7*)
Identification of temperature-sensitive and -resilient *R* genes at 20°C and 25°C	Topas, Topas-*Rlm2*, Topas-*Rlm4*, Topas-*Rlm7*, Topas-*LepR3,* Bristol (*Rlm2*), Surpass (*LepR3*), Adriana (*Rlm4*), Excel (*Rlm7*), Roxet (*Rlm7*)	JN3 (*AvrLm1-4-5-6-7-8*) 99-79 (*AvrLm2-4-7*)
PR1 and *BnaC07g33990* expression analysis at 20°C and 25°C	Topas, Topas-*Rlm4*, Topas-*Rlm7*, Topas-*LepR3*	JN3 (*AvrLm1-4-5-6-7-8*)
RNA-Seq analysis at 20°C and 25°C	Topas-*Rlm4*, Topas-*Rlm7*	JN3 (*AvrLm1-4-5-6-7-8*)

a 0-9 scale adapted from that of Koch et al. (1991) (Appendix A.2). ILs were then identified as temperature -sensitive or -resilient based upon this phenotypic assessment.

4.2.2.2. Effect of temperature on growth rate of *L. maculans* isolates

The growth rates of the two isolates used in the assay, JN3 and 99-79, at 20°C and 25°C were compared by measuring the radius of the cultures. Cultures were grown on V8 agar Petri dishes from mycelial disc inoculum. Mycelial plugs of 6 mm diameter were removed from the growing perimeter of isolates grown on V8 agar and placed, with mycelia face down, on the V8 agar surface. These donor isolates were previously grown in darkness at 18°C, produced from 20µl of 10^7 μl^{-1} conidial spore suspensions, revived from storage at -20°C.

4.2.3. Expression analysis of *PR1* and *BnaC07g33990* at 20°C and 25°C

4.2.3.1. Plant growth

Plants were grown and inoculated as described in section 2.2.2. Two leaf discs (8 mm diameter) were taken from the wound sites of infected cotyledons with a Rapid-Core biopsy punch (ProSciTech) at 0, 1, 4 and 7 dpi. Samples were placed in 2 ml skirted screw-cap Eppendorf tubes, along with a zirconium oxide (5 mm) grinding ball (Retsch). Samples were submerged in liquid nitrogen before storage at -80°C. Frozen tissue was ground into a fine powder using a Retsch Mixer Mill set to 25 Hz for 1 min (Retsch).

4.2.3.2. RNA isolation, DNAse treatment and cDNA synthesis

RNA was extracted from cotyledon samples stored at -80°C. Tissue samples were homogenized in TRI Reagent (Merck, Darmstadt, Germany) (1 ml per 50–100 mg of tissue) in a Retsch Mixer Mill (Retsch, UK) in 2 ml sterilised screw-cap tubes. Following complete homogenization, the TRI Reagent® Protocol was followed using the reagents given. The resulting supernatant was dissolved in 20 µl diethylpyrocarbonate (DEPC) water (MERK, UK). A Nanodrop spectrophotometer (Labtech International, UK) was used to quantify the RNA. RNA was stored at -80°C.

RNA samples were diluted with DEPC treated water to produce 8 µl RNA dilutions for each sample containing 1 µg of RNA. A Promega RNase free DNase kit was used to do the DNase

treatment of RNA samples. A DNase digestion reaction was done by adding 1 µl of RQ1 RNase-free DNase 10x reaction buffer and 1 µl of RNase free DNase to each of the RNA samples. The solutions were then incubated at 37°C for 30 min after which the reaction was ended by adding 1 µl of RQ1 DNase stop solution (Promega, Hampshire, UK). Samples were then incubated at 65°C for 10 min to inactivate the DNase. Before reverse transcriptase-PCR, the treated samples were first heated to 70°C for 5 min then cooled on ice for 5 min. Synthesis of cDNA was done using the qPCRBIO cDNA Synthesis Kit, as per the protocol. TE buffer was used to dilute the cDNA samples by 1:20, producing templates for PCR and qPCR. Samples were stored at -20°C.

4.2.3.3. Quantitative PCR

Expression levels of genes of interest were measured using quantitative PCR, with two technical replicates per sample. Specific primers used are given in Table 4.1. 2 µl of sample cDNA was added to 10 µl of SYBR green mix (Brilliant III Ultra-Fast SYBR Green QPCR Master Mix with low ROX, Agilent Technologies), 5 µl primer mix (150 nM) and 3 µl of HPLC water. A quantitative PCR machine (Agilent Mx3000P QPCR System, Cheshire, UK) was set to run the following cycling profile; 1 cycle of denaturation at 95°C for 3 min, 40 amplification cycles of (95°C for 15 sec and 60°C for 1 min) and 1 cycle of (95°C for 1 min, 55°C for 30 sec and 95°C for 30 sec). After each cycle, the amplified product was logged automatically by measuring the fluorescence.

4.2.3.4. Analysis of expression data

Expression of *PR1* and *BnaC07g33990* were assessed from the qPCR result analysis. The expression profiles for each genotype and temperature treatment analysis were calculated from the ΔCt value [ΔCt = (Ct gene - Ct actin)] and -ΔΔCt value [ΔΔCt = (Ct treat. gene - Ct treat. actin) - (Ct control. gene - Ct control. actin)], respectively, obtained by MxPro (Mx3005P, version 4.10, Stratagene, United States) software. The data were statistically analysed using GenStat (19th edition) software. The up-regulated genes were defined as having a fold-change greater than 2 with $P < 0.05$ and a fold change of 0.5 or less was used to define down-regulated genes when $P < 0.05$. A general ANOVA with Fisher's least significant difference (LSD) procedure was then done to determine significant differences between treatments.

Table 4.2 Primers used in this chapter

Gene	Forward primer	Reverse primer	Reference
PR1	TCAGCGCCGTGAACCTTT	TGAAGGCATTCTCCATTGCA	Liu et al. (2007)
BnaC07g33990 (*FocBo1* putative ortholog)	CGACATGGCATTCGATTTTGAGTCT	TCCACACTGTTGTCTGTACTGAGTT	Adapted from Miyaji et al. (2017)
BnActin	CTCACGCTATCCTCCGTCTC	TTCTCCACCGAAGAATGCT	This work

4.2.4. RNA-Seq analysis

Transcriptome analysis by RNA-seq at 0, 1, 4 and 7 dpi of *L. maculans* JN3 inoculated temperature-sensitive and -resilient cotyledons of *B. napus* ILs was done to examine differences in the defence response of the two lines at 20°C and 25°C. Plants were grown in CE chambers set to a 12-hour light/ 12-hour darkness cycle with a constant temperature of 20°C and a relative humidity of 70% for 10 days. At 24 hours prior to inoculation, plants being treated at 25°C were transferred to another CE chamber, set to that temperature with all other conditions kept the same. Ten-day old *B. rapa* seedlings were inoculated as previously described in section 2.2.3. A larger number of plants than needed were grown for each treatment, from this stock, five biological replicates from different cotyledons were taken for each treatment.

Four leaf discs (8 mm diameter) were taken from the cotyledons of each plant (two leaf discs per cotyledon) and prepared following the methodology described previously in section 3.2.7.1. Frozen tissue was ground into a fine powder using a Retsch Mixer Mill (Retsch). From this stage, the E.Z.N.A.® Plant RNA Kit (Omega Bio-tek) was used to extract RNA following the protocol provided. An additional step to remove further DNA was incorporated into the protocol, with the DNase I Digestion Set, (Omega Bio-tek) following the steps provided. Eluted RNA from each sample was divided into three aliquots for quantification, quality checking and RNA-seq.

Five replicates were initially produced for each treatment/ time point. RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Labtech International, UK) and a Qubit 3 fluorometer. The concentration and integrity of the four replicates with the highest quality RNA was also determined on a Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, USA), run on RNA nano chips (Agilent Technologies, USA), at Warwick Genome Facility. The three replicates for each treatment/ time point with the greatest RNA integrity (RIN) values were then submitted to the GENEWIZ New Jersey facility for library preparation and RNA-sequencing (Illumina Hi-Seq).

4.2.4.1. Initial RNA-Seq data analysis

Preliminary RNA-seq data analysis was carried out by David Hughes at Rothamsted Research. The reference sequence for *B. napus* genome was for Darmor bzh and was taken from the EnsemblPlants genome at https://plants.ensembl.org/Brassica_napus/Info/Index, with files downloaded from ftp://ftp.ensemblgenomes.org/pub/plants/release-45/.../brassica_napus.
The fungal reference genome was for the same JN3 isolate that was used in the cotyledon assay and was taken from http://fungi.ensembl.org/Leptosphaeria_maculans/Info/Index, with files

downloaded from ftp://ftp.ensemblgenomes.org/pub/fungi/release-45/.../leptosphaeria_maculans.

The RNA-seq processing pipeline was as follows; Step 1) *Trimmomatic* to clean/trim the raw fastq data files. Step 2) *HISAT2* to map the reads for each RNA-Seq sample to the *B.napus* reference. Step 3) *featureCounts* to estimate transcript abundance. Step 4) BioConductor R package *DESeq2* to analyse the transcript abundance table. Steps 1-3 were done on Rothamsted's Galaxy platform. The initial work done in step 4 was also done on Galaxy, and later work was done locally in R.

4.3 Results

4.3.1. Response to *L. maculans* culture filtrate in *B. napus* cotyledon inoculation

A cotyledon inoculation assay of Topas-*Rlm7* and Topas was done to check if lesion phenotypes produced in cotyledon assays develop in response to a specific interaction between *Rlm7* and *Avrlm4-7*. It was found that these cannot be replicated by secreted molecules produced by *L. maculans* during *in vitro* growth.

Cotyledons in Figure 4.1.A only show some chlorosis following treatment with the medium control. Figure 4.1.B shows no difference between phenotypes on susceptible Topas or resistant Topas-*Rlm7* cotyledons following inoculation with the culture filtrate. In Figure 4.1.C, a strong phenotypic difference can be seen between cotyledons of the susceptible Topas and resistant Topas-*Rlm7* lines following inoculation with conidial suspension. Large grey lesions on the cotyledons of the susceptible Topas line show a clear difference to the small dark lesions, associated with a strong HR resistance response, on Topas-*Rlm7*. The responses to the culture filtrate inoculation (Figure 4.1.B) were lighter in colour had more diffuse borders compared to the resistance responses produced on Topas-*Rlm7* inoculated with the conidial suspension (Figure 4.1.C).

No quantitative differences were found in lesion severity following culture filtrate inoculation on Topas and Topas-*Rm7* (Figure 4.2). Phenotypes produced from conidial suspension were found to be significantly different between Topas and Topas-*Rlm7* ($P < 0.01$). Significant differences were also found between phenotypes produced from culture filtrate and those from conidial suspension, for both Topas and Topas-*Rlm7* ($P < 0.01$).

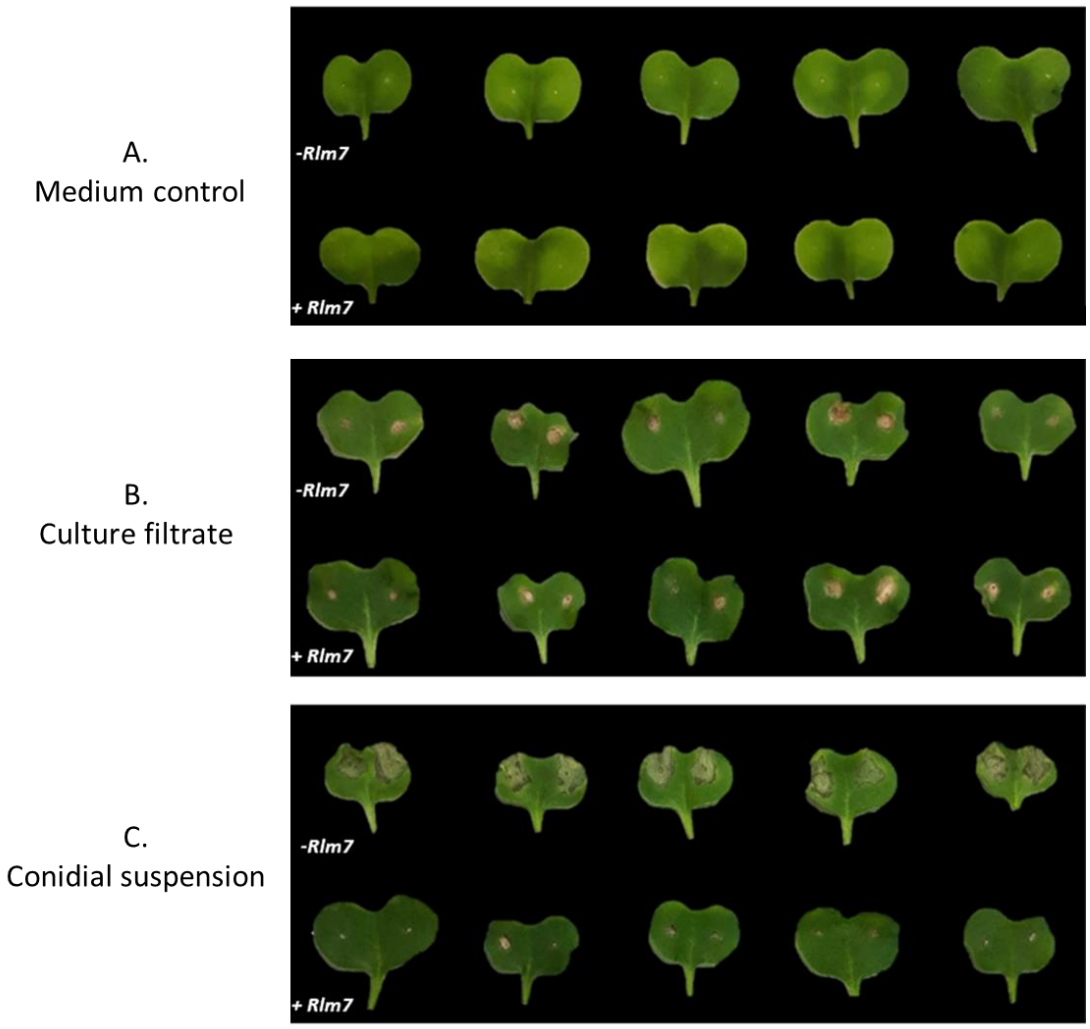


Figure 4.1 Phenotypes of Topas and Topas-*Rlm7* *B. napus* cotyledons inoculated with culture filtrate or conidial suspension of *L. maculans* (*AvrLm4-7*) or a medium control
Cotyledons of *B. napus* susceptible Topas line and introgression line Topas-*Rlm7*, were point inoculated (10 days old) with (A) medium control, (B) culture filtrate and (C) conidial suspension of *L. maculans* isolate 99-79 (*AvrLm2-4-7*). Photographs of resulting phenotypes were taken 11 days following inoculation.

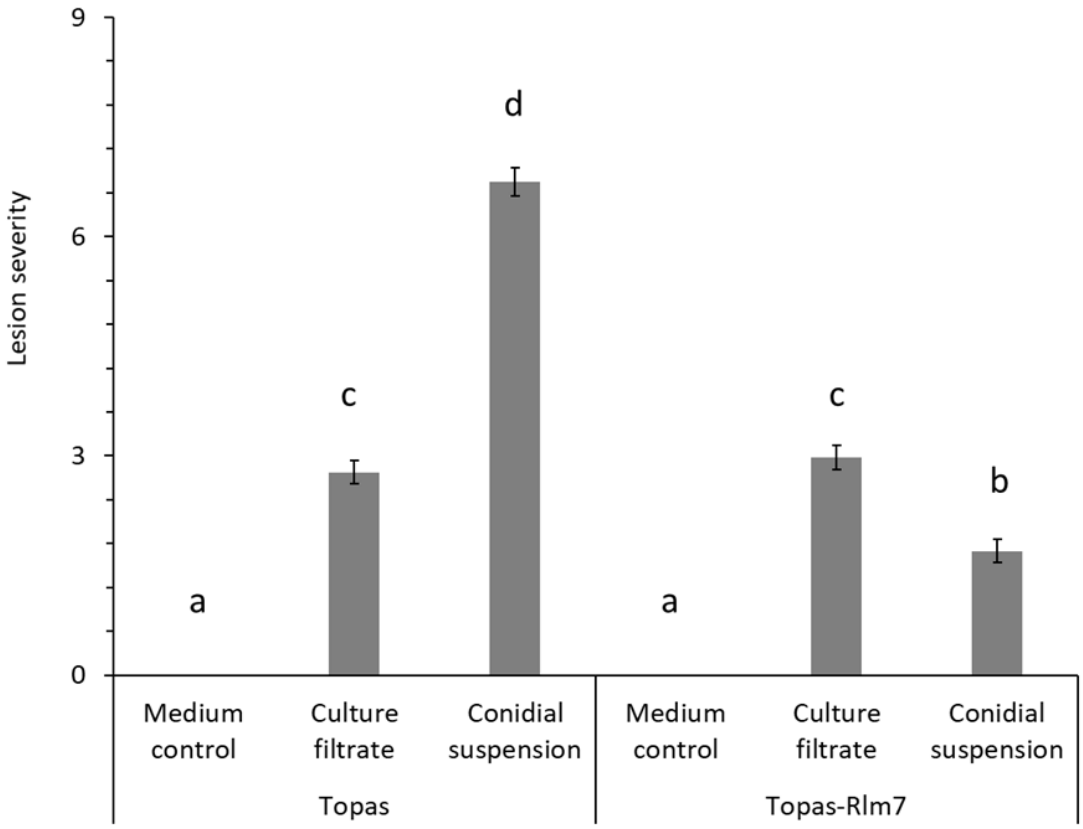


Figure 4.2 Average lesion score (0-9 scale) assessed on cotyledons of *B. napus* introgression lines Topas-*Rlm7* and Topas, following point inoculation with a conidial suspension or culture filtrate of *L. maculans* isolate 99-79 (*AvrLm2-4-7*) and a medium control. Assessment was carried out at 11 days post-inoculation with 10 µl of 10^7 ml^{-1} medium control, culture filtrate or conidial suspension. Ten biological replicates were included per treatment. Error bars indicate the SEM. Average scores sharing the same letter are not significantly different ($P < 0.01$) in a multiple comparison Fishers' unprotected least significant difference test (9 df).

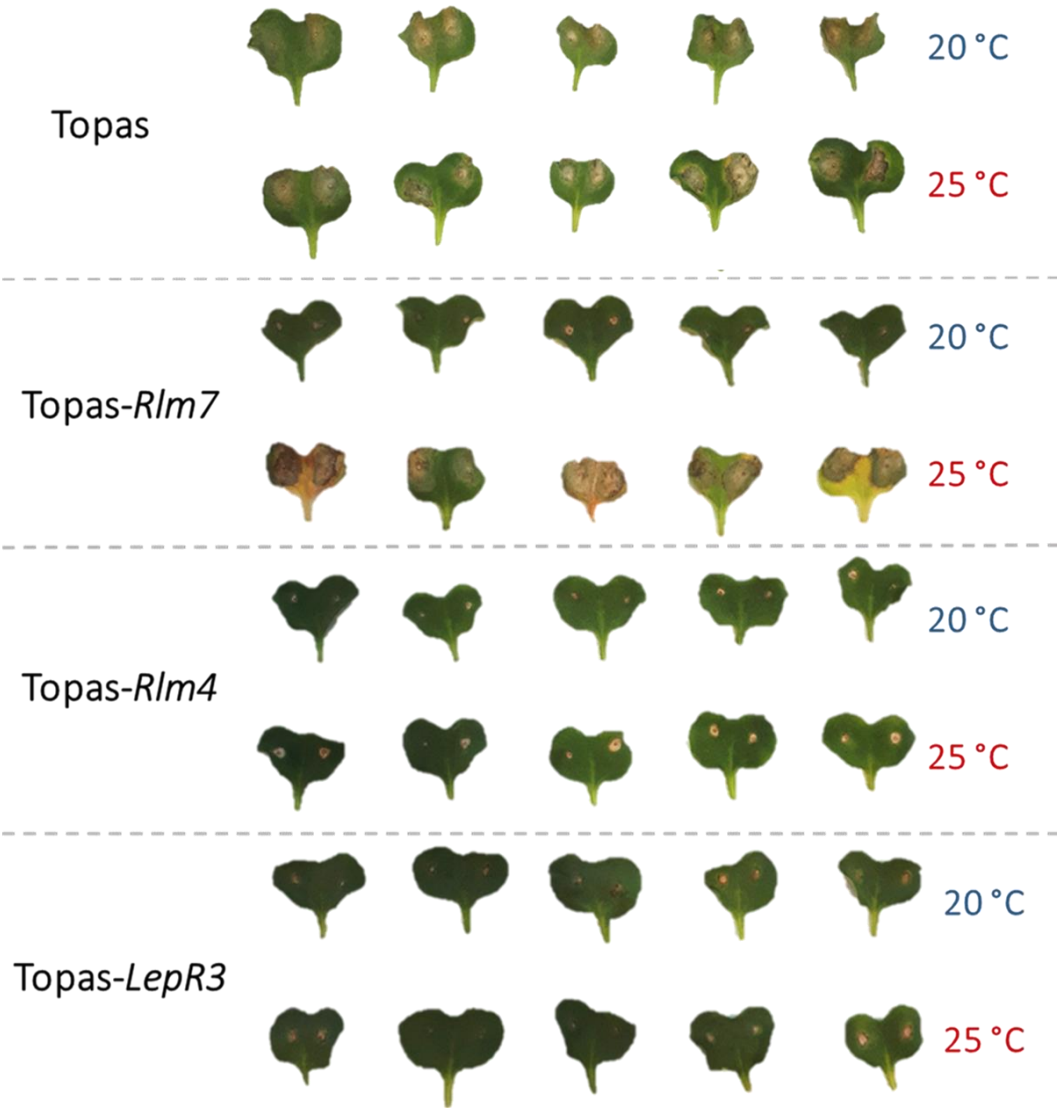


Figure 4.3 Topas-*Rlm7* displays a temperature-sensitive defence response phenotype at 25°C; resistance of Topas-*LepR3* and Topas-*Rlm4* is temperature-resilient. Cotyledons of the susceptible doubled-haploid background Topas line and single *R* gene *B. napus* Topas introgression lines (*Rlm7*, *Rlm4* or *LepR3*) and were point inoculated with 10 µl of 10^7 spores/ml conidial suspensions of *L. maculans* isolate JN3 (*Av1-4-5-6-7-8*). Cotyledons photographed at 12 days post inoculation. Seedlings were grown at 20°C or 25°C for 13 days, prior to which all were grown at 20°C.

4.3.2. Temperature-sensitivity of *R* genes in Topas introgression lines and cultivars

4.3.2.1. Cotyledon inoculation assay at 20°C and 25°C

Cotyledon inoculation assays were done at 20°C and 25°C to determine temperature-sensitive and -resilient *R* genes from the set of *B. napus* Topas ILs and differential cultivars. At 25°C a clear difference in the phenotype of Topas-*Rlm7*, compared to Topas-*Rlm4* and Topas-*LepR3*, following inoculation with an avirulent *L. maculans* isolate was seen; lesions were significantly larger, showing a susceptible phenotype (Figure 4.3). However, at 20°C Topas-*Rlm7* had a resistant response. Topas-*Rlm4* and Topas-*LepR3* showed clear resistance responses at both 20°C and 25°C. As expected, the susceptible control, Topas, showed a susceptible response at both 20°C and 20°C.

Quantitative differences in defence responses between Topas ILs (*LepR3*, *Rlm2*, *Rlm4* and *Rlm7*) and the differential set of cultivars at 20°C and 20°C were assessed to determine temperature-sensitivity of resistance response using *L. maculans* isolate JN3 (*AvrLm1-4-5-6-7-8*) (Figures 4.4) and 99-79 (*AvrLm2-4-7*) (Figure 4.5). *Rlm4* was found to be temperature-resilient, in both Topas-*Rlm4* and in the cultivar Adriana (containing *Rlm4*) when inoculated with both isolates JN3 and 99-79. *LepR3* was also found to be temperature-resilient, in Topas-*LepR3* and in the cultivar Surpass (containing *LepR3*) when inoculated with JN3 (as 99-79 does not have *AvrLm1*, it cannot be used to determine temperature-resilience of *LepR3*, the corresponding *R* gene).

Topas-*Rlm2* and cultivar Bristol (*Rlm2*) both showed a clear susceptible phenotype when inoculated with JN3, this was as expected as the isolate does not contain *AvrLm2* (Figure 4.4). For this reason, isolate 99-79 (*AvrLm2*) was also included in the assay, which produced a strong resistance phenotype in Topas-*Rlm2* (Figure 4.5). However, in cultivar Bristol cotyledons, inoculation with 99-79 showed only an intermediate resistance phenotype (taken as a lesion severity score of 5) at 25°C. Furthermore, this resistance response was temperature-sensitive, as the difference between severity scores at 20°C and 25°C was significant.

Rlm7 was found to display significantly more severe symptoms at 25°C compared to 20°C with both *L. maculans* isolates in Topas-*Rlm7*. Following inoculation with *L. maculans* isolate JN3, the average lesion severity for Topas-*Rlm7* was 3.0 at 20°C, increasing to 6.3 at 25°C. Similarly, average severity scores resulting from inoculation with isolate 99-79 were 3.6 at 20°C and 6.7 at 25°C. Cultivar Roxet (containing *Rlm7*) also showed temperature-sensitivity when it was inoculated with JN3.

However, in cultivar Excel, *Rlm7* behaved in a temperature-resilient manner when inoculated with both isolates, displaying a clear resistance response at both temperatures with no significant difference between severity scores at 20°C and 25°C. Average lesion severity scores at 20°C were 1.2 and 1.1 when inoculated with isolates JN3 and 99-79, respectively. This only marginally increased at 25°C, with severity scores 1.8 and 1.3, respectively.

Thus, *Rlm7* was determined to be temperature-sensitive in the Topas-*Rlm7* IL and in cultivar Roxet (from which the *Rlm7* was introgressed for the Topas-*Rlm7* line (Larkan et al., 2016) and temperature-resilient in cultivar Excel.

4.3.2.2. Effect of temperature on growth rate of *L. maculans* isolates

Growth of *L. maculans* was assessed at temperatures 20°C and 25°C to ensure differences in phenotype were not just related to differences in pathogen growth rate. Both isolates used showed no significant differences in their growth rate between the two temperatures (Figure 4.6).

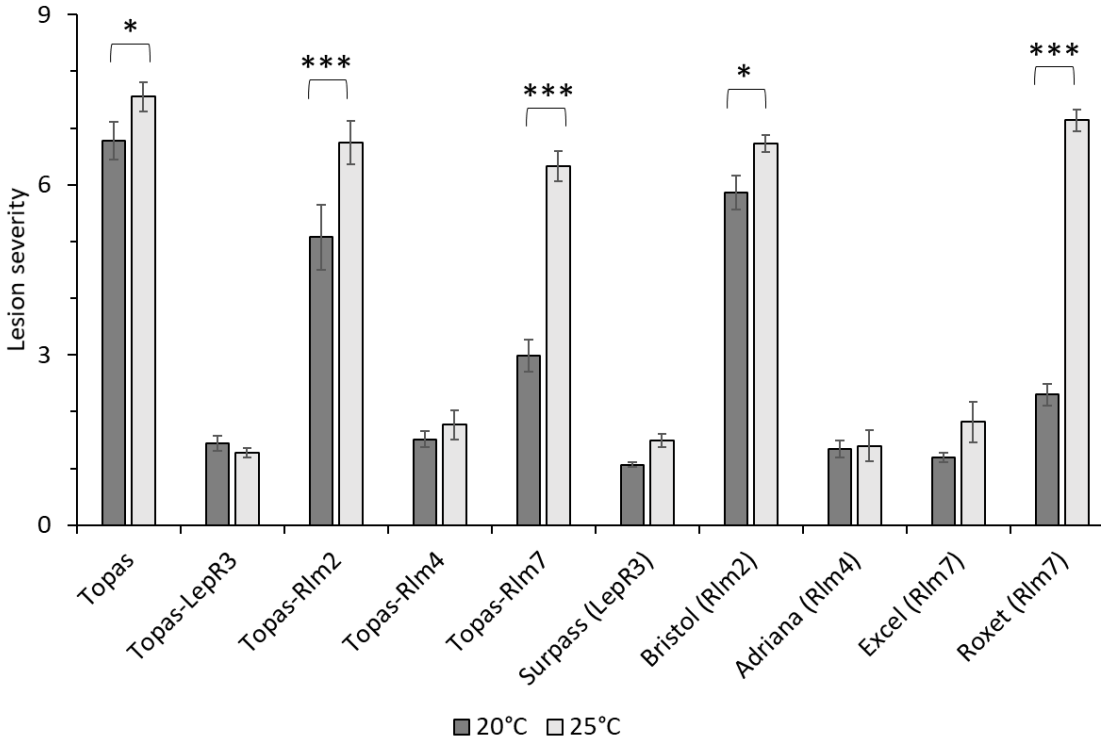


Figure 4.4 Average lesion score (0-9 scale) assessed on cotyledons of *B. napus* Topas introgression lines, containing *LepR3*, *Rlm2*, *Rlm4* and *Rlm7*, and a differential set of cultivars containing each of these *R* genes, following wound inoculation with 10 µl of 10^7 ml^{-1} conidial suspension of *L. maculans* isolate JN3 (*Av1-4-5-6-7-8*) at 13 dpi. Four wound sites were assessed per plant. Eight biological replicates were included for each of the introgression lines and six biological replicates were included for each the differential set of cultivars for each assay and each assay which was done twice. Bars represent mean lesion score and error bars indicate the standard error of the mean. (* $P < 0.05$, *** $P < 0.001$)

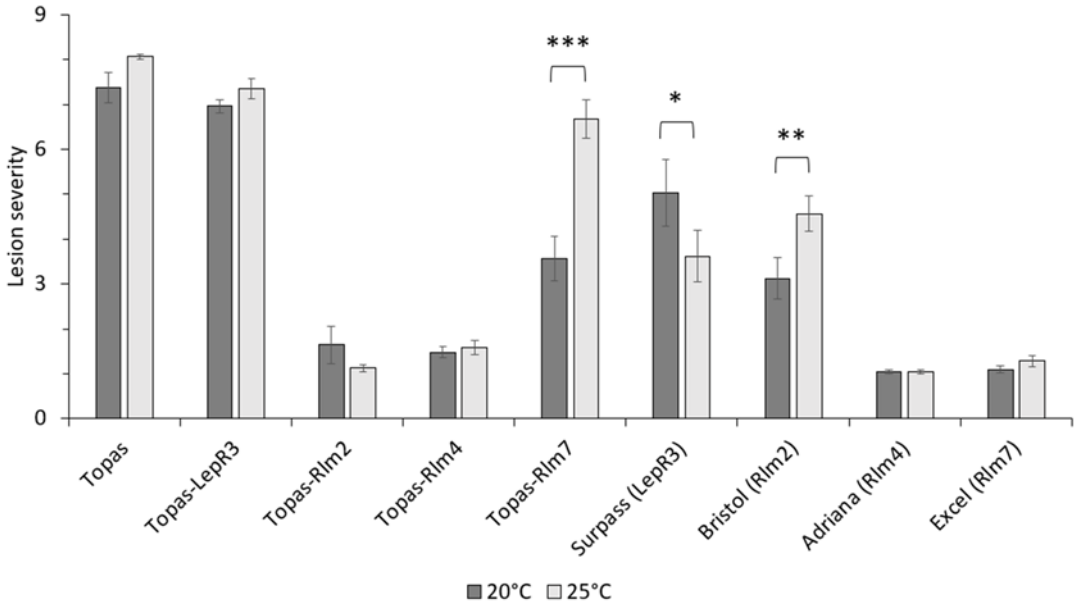


Figure 4.5 Average lesion score (0-9 scale) assessed on cotyledons of *B. napus* Topas introgression lines, containing *LepR3*, *Rlm2*, *Rlm4* and *Rlm7*, and a differential set of cultivars containing each of these *R* genes, following wound inoculation with 10 µl of 10^7 ml^{-1} conidial suspension of *L. maculans* isolate 99-79 (*AvrLm2-4-7*) at 13 dpi. Four wound sites were assessed per plant. Eight biological replicates were included for each of the introgression lines and six biological replicates were included for each the differential set of cultivars for each assay, and each assay which was done twice. Bars represent mean lesion score and error bars indicate the standard error of the mean. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

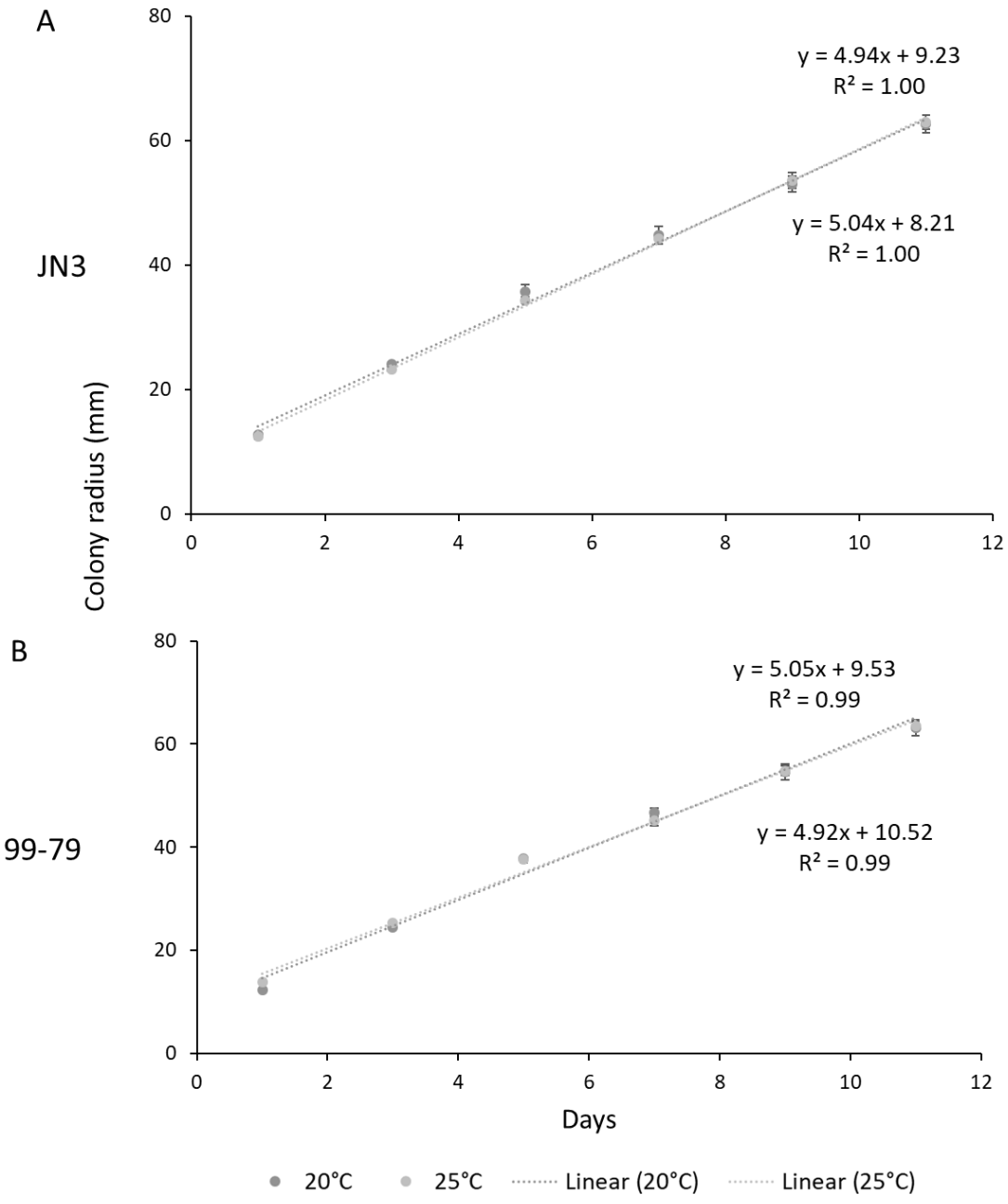


Figure 4.6 Increased temperature from 20°C to 25°C had no effect on the growth of *L. maculans* isolates (A) JN3 (*P* = 0.26) or (B) 99-79 (*P* < 0.66). Mycelial discs were transferred from fungal colonies onto V8 media Petri dishes and placed in controlled environment cabinets set to a constant temperature of 20°C or 25°C. Colony radius was determined by measuring the diameter of fungal growth.

4.3.3. Expression analysis of *PR1* and *BnaC07g33990*

The expression of *PR1* and *BnaC07g33990,* (previously identified as a partial putative homolog of *FocBo1* and *FocBr1*) were measured in temperature-sensitive (Topas-*Rlm7*) and -resilient (Topas-*Rlm4* and Topas-*LepR3*) *B. napus* single *R* gene ILs at 20°C and 25°C at 1, 4 and 7 dpi following incompatible interactions. A contrast analysis was done on ΔCt values for the susceptible Topas IL treated with a water mock or with the JN3 isolate of *L. maculans*. No significant difference was found between the two treatments for both *PR1* and *BnaC07g33990* expression (Appendix C.2 and C.3). Therefore, Topas mock samples were not included in graphs.

4.3.3.1. *PR1* expression analysis

There was a significant influence of genotype and time (dpi) on *PR1* expression as well as significant temperature-by-time, genotype-by-time and temperature-by-genotype-by-time interactions (Table 4.3). The greatest difference in expression per time in *PR1* for all genotypes occurred between 1 and 4 dpi at 20°C (Figure 4.7). The strongest expression of *PR1* occurred in Topas-*LepR3*, which reached a peak at 4 dpi with a 2086698 fold change increase at 20°C and a 767571 increase at 25°C. Topas-*Rlm7* had a 126263-fold change at 20°C and 1295 at 25°C. *PR1* expression at 1, 4 and 7 dpi was analysed; at 1 and 7 dpi no significant differences were found between genotypes, but differences were found at 4 dpi. At 4 dpi, no significant difference in *PR1* expression between 20°C and 25°C was found for Topas-*Rlm4* and Topas-*LepR3*, both of which displayed a temperature-resilient phenotype. A significant reduction in *PR1* expression at 25°C, compared to 20°C, was found for Topas-*Rlm7*, which also has a temperature-sensitive defence phenotype (Figure 4.8).

4.3.3.2. *BnaC07g33990* expression analysis

There was a significant influence of genotype and time (dpi) on relative *BnaC07g33990* expression, as well as significant temperature-by-time interaction (Table 4.4). The greatest differences in *BnaC07g33990* expression between lines were observed at 4 dpi (Figure 4.9). At 25°C, at 4 dpi, *BnaC07g33990* was significantly down regulated in Topas-*Rlm4* and Topas-*Rlm7* (Figure 4.10). The susceptible Topas line showed a very similar level of *BnaC07g33990* expression at 20°C and 25°C. Topas-*LepR3* was found to have the greatest expression levels of *BnaC07g33990* at 4 dpi 20°C, with average expression fold changes of 74.

Table 4.3 ANOVA test to evaluate the effect of temperature (20°C or 25°C), genotype (*Rlm7*, *Rlm4*, *LepR3* and susceptible Topas introgression lines) and time (1, 4, or 7 dpi) on relative *PR1* expression. *df* Degrees of freedom

Source	*df*	Mean square	*F*-value	*P*-value
Temperature	1	1.62	0.35	0.557
Genotype	3	64.10	13.83	<.001
Time	2	1913.22	412.92	<.001
Temperature × Genotype	3	2.26	0.49	0.693
Temperature × Time	2	65.66	14.17	<.001
Genotype × Time	6	19.78	4.27	0.001
Temperature × Genotype × Time	6	14.38	3.1	0.011
Error	53			

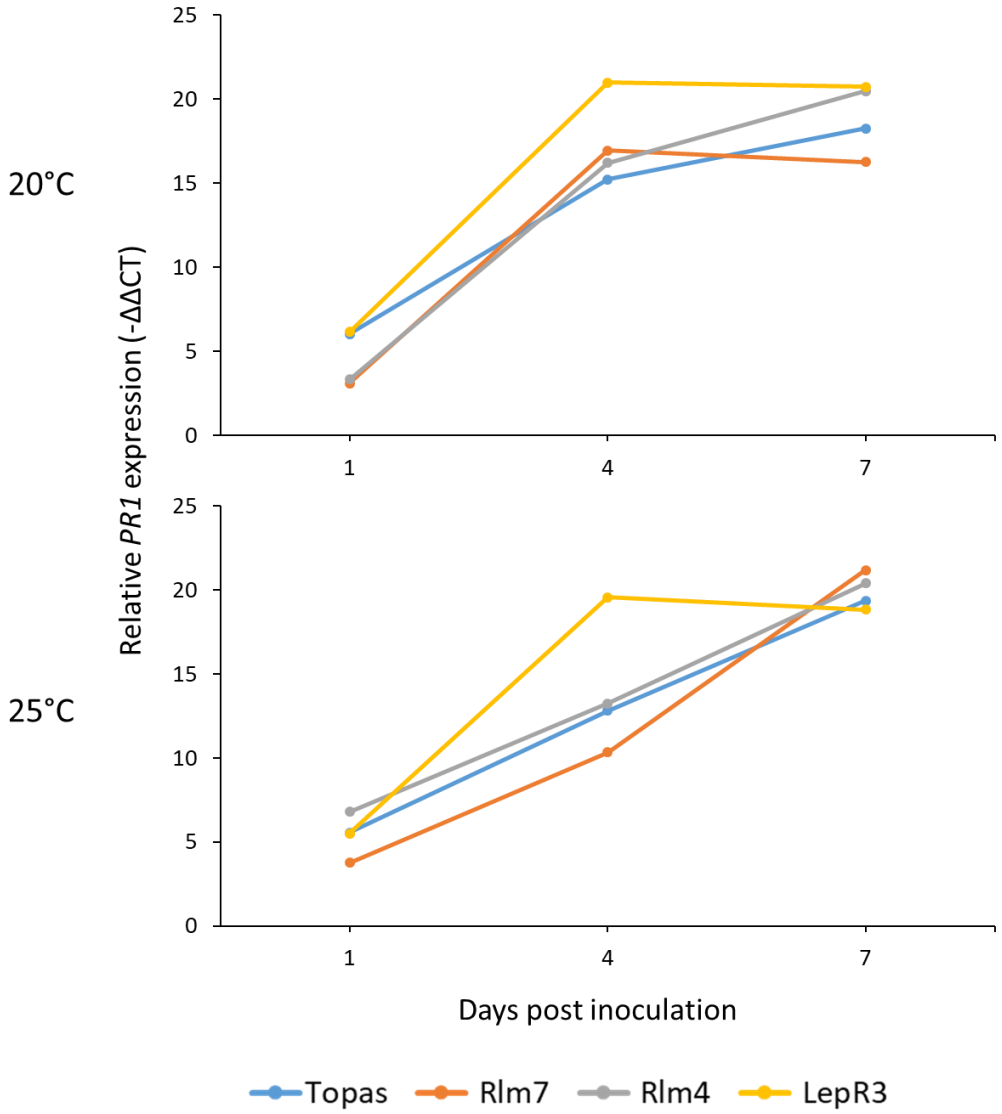


Figure 4.7 *PR1* expression of single *R* gene *B. napus* Topas introgression lines *Rlm7*, *Rlm4* and *LepR3* and the susceptible Topas line at 1, 4- and 7-days post inoculation with *L. maculans* isolate JN3 (*AvrLm1-4-5-6-7-8*) relative to an uninoculated control. Inoculation was done by point inoculation, with 10µl of 10^7 spores/ml conidial suspension. Seedlings were grown at (a) 20°C or (b) 25°C (constant) for 13 days, prior to which all were grown at 20°C. The expression values of *PR1* were assessed upon the qPCR result analysis of -ΔΔCt value (ΔΔCt = (Ct *PR1* - Ct *Actin*) treatment - (Ct *PR1*- Ct *Actin*) control). Analysis of differences at 4 dpi shown if Figure 4.8.

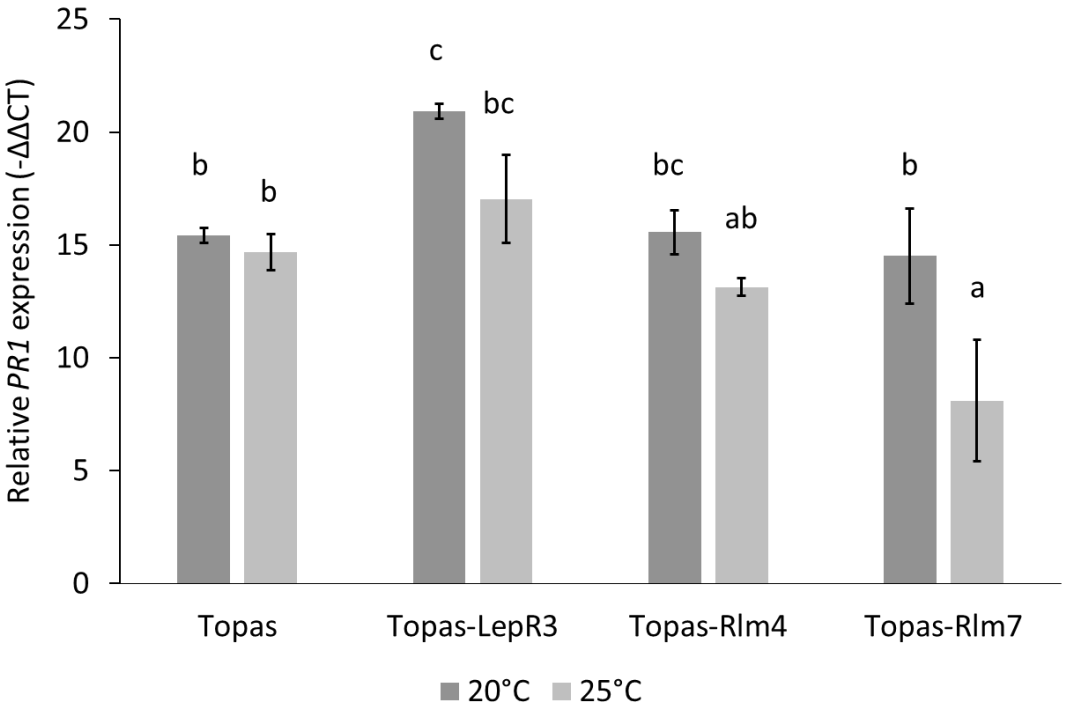


Figure 4.8 Relative *PR1* expression is less at 25°C compared to 20°C at 4dpi for temperature-sensitive Topas-*Rlm7* following inoculation with an avirulent isolate of *L. maculans*. Plants were grown at 20°C for 10 days, before half were exposed to an elevated temperature of 25°C. After an initial period of 24 hr to allow plants to adapt to the new temperature, all plants were inoculated by point inoculation, with 10µl of 10^7 spores ml^{-1} conidial suspension of isolate JN3 (*AvrLm1-4-5-6-7-8*). Bars represent mean expression relative to an uninoculated control calculated from at least three biological replicates -ΔΔCt value (ΔΔCt = (Ct *PR1* - Ct *Actin*) treatment - (Ct *PR1*- Ct *Actin*) control). Error bars indicate standard error of the mean. For each gene, different lowercase letters indicate significant differences between mean values (general ANOVA with Fisher's least significant test; $P < 0.01$).

Table 4.4 ANOVA test to evaluate the effect of temperature (20°C or 25°C), genotype (*Rlm7*, *Rlm4*, *LepR3* and susceptible Topas introgression lines) and time (1, 4, or 7 dpi) on relative *BnaC07g33990* expression. *df* Degrees of freedom

Source	*df*	Mean square	*F*-value	*P*-value
Temperature	1	2.22	0.69	0.409
Genotype	3	44.15	13.81	<.001
Time	2	37.70	11.8	<.001
Temperature × Genotype	3	3.67	1.15	0.34
Temperature × Time	2	35.75	11.18	<.001
Genotype × Time	6	5.88	1.84	0.111
Temperature × Genotype × Time	6	2.91	0.91	0.496
Error	48			

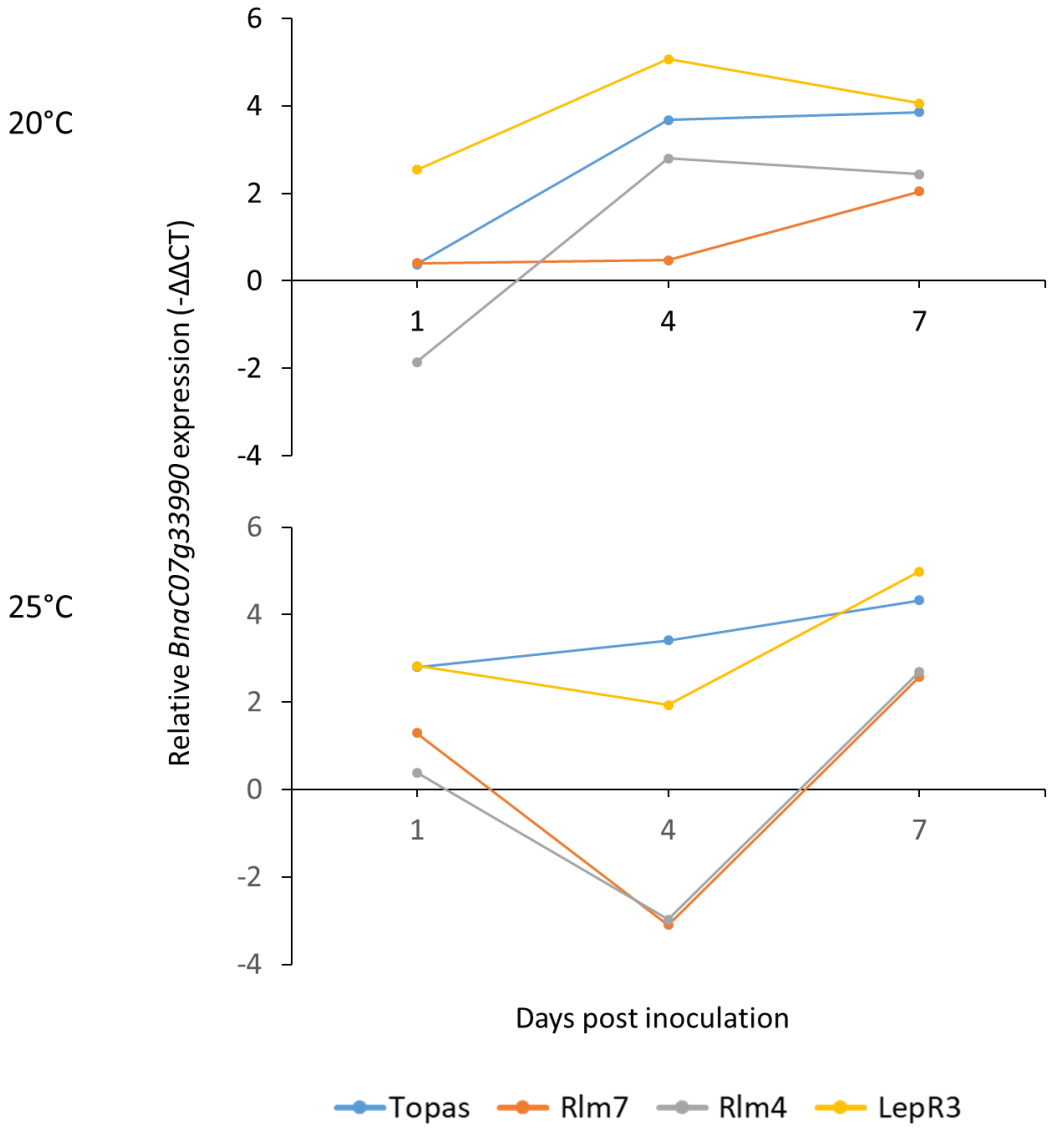


Figure 4.9 *BnaC07g33990* expression of single *R* gene *B. napus* Topas introgression lines *Rlm7*, *Rlm4* and *LepR3* and the susceptible Topas line at 1, 4 and 7 days post inoculation with *L. maculans* isolate JN3 (*Av1-4-5-6-7-8*). Inoculation was done by point inoculation, with 10 µl of 10^7 spores ml^{-1} conidial suspension. Seedlings were grown at (a) 20°C or (b) 25°C (constant) for 13 days, prior to which all were grown at 20°C. The expression values of *BnaC07g33990* were determined from the qPCR result analysis of -ΔΔCt value (ΔΔCt = (Ct *BnaC07g33990* - Ct *Actin*) treatment - (Ct *BnaC07g33990*- Ct *Actin*) control). Analysis of differences at 4 dpi shown if Figure 4.10.

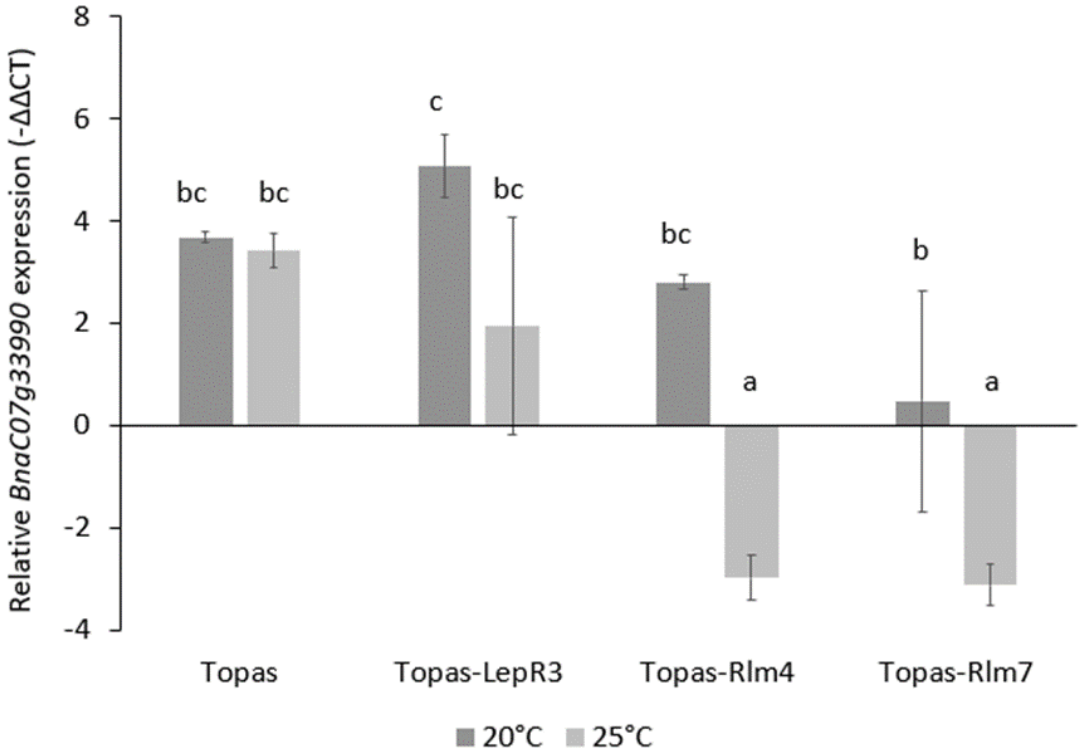


Figure 4.10 Relative *BnaC07g33990* expression in *B. napus* single *R* gene Topas introgression lines *Rlm7*, *Rlm4* and *LepR3* and the susceptible Topas line at 4 days post inoculation with *L. maculans* isolate JN3 (*AvrLm1-4-5-6-7-8*).. Plants were grown at 20°C for 10 days, before half were exposed to an elevated temperature of 25°C. After an initial period of 24 hr to allow plants to adapt to the new temperature, all plants were inoculated by point inoculation, with 10µl of 10^7 spores/ml conidial suspension of *L. maculans* isolate JN3 (*AvrLm1-4-5-6-7-8*). Bars represent mean expression relative to an uninoculated control, calculated from at least three biological replicates (ΔΔCt = (Ct *BnaC07g3399*0 - Ct *Actin*) treatment - (Ct *BnaC07g33990* - Ct *Actin*) control). Error bars indicate standard error of the mean. For each gene, different lowercase letters indicate significant differences between mean values (general ANOVA with Fisher's least significant test; $P < 0.05$).

4.3.4. RNA-Seq analysis

RNA-seq was done to investigate differences in defence responses of *B. napus* plants with temperature-sensitive and -resilient *R* genes Topas-*Rlm7* and Topas-*Rlm4*, respectively. Samples were taken at four timepoints, 0, 1, 4 and 7 dpi, after inoculation with the *L. maculans* isolate JN3. An initial data quality assessment found one sample (a 0-dpi control) displayed 'over-represented sequences' which appear to be ribosomal RNA. It did not cluster with any other samples in the subsequent analysis and was therefore excluded from this preliminary analysis. Mapping the sequences found overall alignment rates were about 80%.

4.3.4.1. Sample separation and clustering

Ideally, a Principal Coordinates Analysis (PCoA) plot of the samples should show separation between different treatments and clustering of replicates. The plot in Figure 4.11 shows clear separation between 0 dpi (D0) and 1 dpi (D1), and between 1 dpi (D1) and 4/7 dpi (D4/D7). A PCoA plot for just the 4 and 7 dpi (D4/D7) samples (Figure 4.12) shows separation between *Rlm4* (L4) and *Rlm7* (L7), and some separation between 4 dpi (D4) and 7 dpi (D7).

4.3.4.2. Expression of selected genes

Figure 4.13 shows the log-fitted expression of six *B. napus* genes of interest, across the 16 treatments. *PR1* was selected as a marker for SA and two homologs of *PDF1.2* were used as markers for JA-ET signaling. *PR1* and one of the *PDF1.2* homologs (*BnaC02g23620D*) showed similar trends in their expression levels across treatments; from 4 dpi they were upregulated in both Topas-*Rlm7* and Topas-*Rlm4* at 20°C and 25°C. Lower levels of *PR1* and *PDF1.2* expression was observed in Topas-*Rlm7* lines compared to Topas-*Rlm4* at 25°C. The other *PDF1.2* gene (*BnaA07g32130D*) showed a different expression pattern across samples; upregulation occurred earlier at 1 dpi. *FocBn1* (*BnaC07g33990D*), a partial putative homolog of *FocBo1*, *BnaC07g33980*D and *BnaC07g34000D* had lower levels of expression and appear to be more constant across the treatments.

4.3.4.2. Significant genes

As expected, large numbers of genes show a change in expression with time following inoculation (Table 4.5). A smaller yet significant number showed a change in expression between *Rlm4* and *Rlm7* and between 20°C and 25°C.

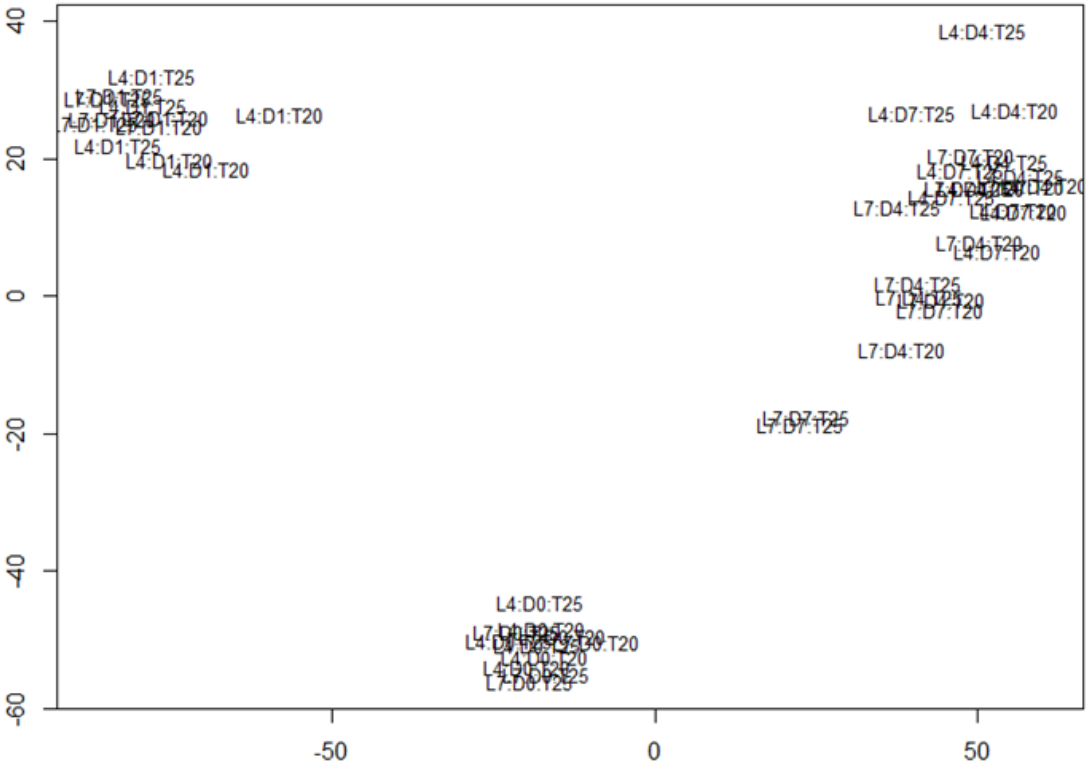


Figure 4.11 PCoA plot of the samples showing clear separation between 0dpi (D0) and 1 dpi (D1), and between 1 dpi (D1) and 4/7 dpi (D4/D7). Note that day 0 controls cluster separately.

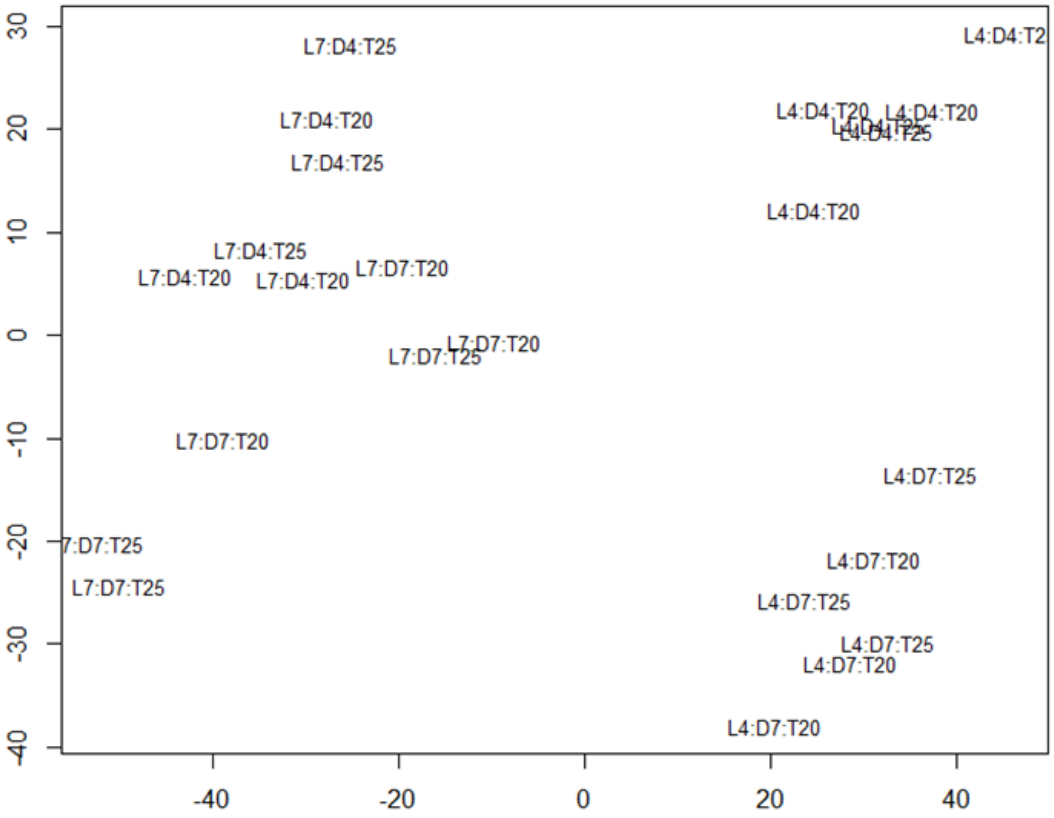


Figure 4.12 PCoA plot for just the 4/7dpi (D4/D7) samples, showing separation between *Rlm4* (L4) and *Rlm7* (L7), and some separation between 4 dpi (D4) and 7 dpi (D7).

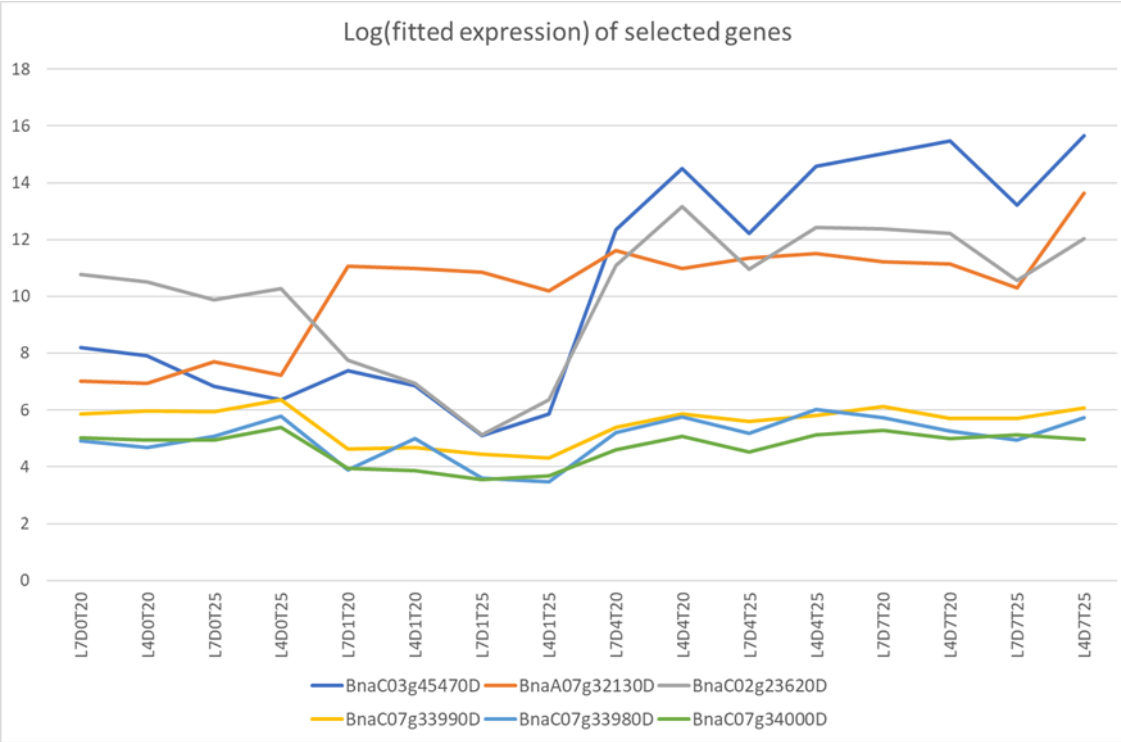


Figure 4.13 The log-fitted expression (or abundance) of six *B. napus* genes of interest, across the 16 treatments. The genes of interest included in this analysis were *PR1* (*BnaC03g45470D*), *PDF1.2* gene family (*BnaA07g32130D* and *BnaC02g23620D*), the putative partial homolog of *FocBo1* in *B. napus* (*BnaC07g33990D*) and *SNC1* "pseudogenes" (*BnaC07g33980D* and *BnaC07g34000D*).

Table 4.5 The numbers of "significant genes" for the five main effects in this experiment.

Effect	p-value	Up regulated	Down regulated
1 dpi vs 0 dpi	10^-10	3137	3857
4 dpi vs 0 dpi	10^-10	2029	1710
7 dpi vs 0 dpi	10^-10	1412	1405
Rlm7 vs *Rlm4*	0.05	939	1001
25°C vs 20°C	0.05	324	611

4.4. Discussion

4.4.1. Response to *L. maculans* culture filtrate in *B. napus* cotyledons

Results from the cotyledon inoculation assay comparing defence phenotypes following culture filtrate and pathogen inoculation demonstrate that an intact host-pathogen interaction is required in order to produce *Avr-R* gene specific resistance phenotypes. Whilst inoculation with *L. maculans* culture filtrate caused small, pale lesions with diffuse edges to form on both susceptible and resistant Topas lines, the phenotype was clearly different to that produced following a compatible reaction with pathogen isolates. This phenotype cannot be mimicked by using culture filtrates of the avirulent pathogen, confirming that effectors produced during infection cause a differential response on susceptible and resistant genotypes. This in agreement with de Wit's (2016) findings that effectors are responsible for differential resistance and susceptibility phenotypes, and these effectors are only present in pathogen-infected plants and are not produced when the pathogen is grown *in vitro*.

4.4.2. Phenotypic assay; temperature-sensitivity of *LepR3*, *Rlm2*, *Rlm4* and *Rlm7*

Rlm7 was found to behave differently in different cultivars. In the Topas IL and the cultivar Roxet (from which it was introgressed to the Topas IL), *Rlm7* showed a clear temperature-sensitive phenotype, breaking down at 25°C in cotyledon assays. In contrast, *Rlm7* in cultivar Excel (whose *Rlm7* originated from the cultivar Caiman) showed strong temperature-insensitivity at 25°C, leading to the suggestion that there are two separate alleles. It has been previously suggested that differences in temperature-sensitivity and virulence may be due to background effects of the cultivar (Huang et al., 2014). To confirm if there are two different alleles, cloning of *Rlm7* from both genetic sources should be done and genetic sequences compared. This information would be useful to breeders in increasing the understanding of effective *R* genes

4.4.3. Responses of temperature-sensitive and -resilient *R* genes following an incompatible reaction with *L. maculans* at 20°C or 25°C

Findings from the qRCR *PR1* expression study support the interpretation of the cotyledon inoculation assay at 20°C and 25°C. Taken together, Topas-*Rlm7* can clearly be identified as having a temperature-sensitive defence response. In contrast, Topas-*Rlm4* and Topas-*LepR3* can both be described as temperature-resilient at 25°C. Topas-*LepR3* has a stronger *PR1* expression, even compared to the temperature-resilient *Rlm4*. It is postulated that this could be because

LepR3, which codes for an RLP, belongs to a different class of *R* genes to *Rlm4* and *Rlm7* which are likely wall-associated kinase-like WAKLS (Larkan et al., 2019). Therefore, Topas-*Rlm4* was selected as the temperature-resilient candidate to be included in the RNA-seq assay.

RNA-seq analysis also revealed lower expression levels of *PR1* and *PDF1.2* (SA and ET hormone markers respectively) in Topas-*Rlm7* lines compared to Topas-*Rlm4* at 25°C. Even though ET and JA pathways are known to act antagonistically to the SA pathway, this increase in *PR1* and *PDF1.2* expression at 4 dpi, following the recognition of *L. maculans AvrLm4-7*, is supported by previous studies (Becker et al., 2017; Šašek et al., 2012). One possible explanation for this result could be an additional wound response caused by the inoculation process. In investigating the defence responses in *B. napus* against *L. maculans*, Haddadi et al. (2016) and Becker et al. (2017) used the point inoculation method, whereas Šašek et al. (2012) chose to infiltrate cotyledons in order to induce the defence response simultaneously in a greater number of plant cells. Whilst both point inoculation and infiltration inoculation are commonly used in studying the defence responses, both methods involve wounding the plant, thus initiating the wound response alongside the defence response. The wound response involves increases in ET, JA and ABA to induce wound healing at the wounding site (Stotz et al., 2000). This may lead to some potential confusion in gene expression studies, as these JA and ET pathways may cause cross-talk confliction with the SA defence response pathway. However, by including mock controls, this issue is reduced, and results can be analysed.

Sasek et al (2012) found a simultaneous induction of SA and ET pathways following the recognition of *AvrLm1* in *B. napus* plants; and suggested it could relate to the pathogens contrasting infection stages, transitioning from an asymptomatic infection stage to lesion formation. Becker et al (2017) also observed high levels of *PR1* and *PDF1.2* expression at 7 dpi following incompatible interaction. By using laser microdissection and quantitative PCR, they were able to identify spatial gradients of gene activity in response to *L. maculans* around the site of infection. Through measuring the distribution of *PR1* and *PDF1.2*, they discovered an antagonistic spatial relationship between SA and JA-ET signaling pathways in resistant host cotyledons.

Indolic glucosinolates have been shown to be essential for defence against pathogens as well as promoting callose formation (Buxdorf et al., 2013; Clay et al., 2009). Becker et al (2017) found a marker for indole glucosinolates, CYP79B2, was most highly expressed in tissue next to the infection site with high combined SA and JA-ET signaling. Frerigmann and Gigolashvili (2014) also found that joint application of SA and JA produced the greatest expression of a key transcription

factor for indolic glucosinolates. From these studies and preliminary analysis of RNA-seq data, it is hypothesised that the accumulation of phytoalexins derived from antifungal indolic glucosinolates are higher in the temperature-resilient Topas-*Rlm4* lines than in the temperature-sensitive Topas-*Rlm7* line.

Future analysis of this RNA-seq data will explore and compare the gene expression profiles over time in temperature-resilient Topas-*Rlm4* and temperature-sensitive Topas-*Rlm7* at 20°C and 25°C. Genes whose log fitted expression correlate well with that of candidate genes such as *PR1* and *PDF1.2* will be identified. Genes differentially expressed between 20°C and 25°C and the transcriptional changes between temperature-sensitive and -resilient ILs will be explored.

4.4.4. PCoA plots show changes in pathogen life stage

The RNA-seq PCoA plots display a clear separation between 0 dpi, 1 dpi and 4/7 dpi, this could reflect the change of life stages the pathogen goes through following inoculation. *L. maculans* starts off as a biotroph, however by 4/7 dpi the pathogen appears to have switched to its necrotrophic stage. Haddadi et al, (2017) found genes associated with SA and JA production to be upregulated at the initial and late stages of infection, respectively, as *L. maculans* transitioned from biotrophic to necrotrophic during the infection of *B. napus* cotyledons. However, this was observed slightly later, at 6 – 8 dpi. Ma et al., (2018) found expression of *AvrLm1* by *L. maculans* lead to enhanced induction of cell death, acting as a virulence mechanism to support this transition from biotrophy to necrotrophy.

4.4.5. Expression of *BnaC07g33990* is temperature-dependent in Topas-*Rlm4* and Topas-*Rlm7*

In *B. napus*, *BnaC07g33990* was identified as a putative partial homolog of *FocBo1* and *FocBr1*. However, this gene was split in two in Darmor bzh. Following an incompatible *Avr-R* gene interaction at 25°C, relative expression levels of *BnaC07g33990* were found to decrease at 4 dpi in Topas-*Rlm7* and Topas-*Rlm4* but not in Topas-*LepR3*. This difference in expression could be because *LepR3* belongs to a different class of *R* gene to *Rlm4* and *Rlm7*; *LepR3* was found to code for an RLP whereas *Rlm4* and *Rlm7* map closely to *Rlm9* which was recently found to be a WAKL gene (Larkan et al., 2016; Stotz et al., 2018; Larkan et al., 2019). RLP and WAKLs could possibly have different defence response pathways. Future studies could use plants with CRISPR-Cas9 mutations in *BnaC07g33990* to investigate its role in the temperature-dependence of the defence response in lines containing WAKLs and RLPs.

5 General Discussion

This thesis aimed to explore some of the factors affecting the ability of *Brassica* plants to defend against *L. maculans* at increased temperatures. With climate change, maximum summer temperatures and frequency of extreme weather events are likely to increase. This chapter will discuss the main findings from this project and propose future research. Results from the field study pointed to high maximum June temperatures to be linked to more severe phoma stem canker, as we see more extreme weather due to climate change this could increase the severity of the disease. To effectively prepare winter oilseed cultivars against the threat of future disease in a warming climate it is necessary to understand the temperature-sensitivity of the resistance response and use this information to improve the defence responses of crops. How the results of this study may help in developing more temperature-resilient crops will also be discussed.

5.1. Increased temperature effects the efficacy of both *R* gene mediated and quantitative resistance against *L. maculans* in the stems of *B. napus* plants

This study suggests that *R* genes also operate in the stems of adult winter oilseed rape plants, providing some protection against the phoma stem canker pathogen (Chapter 2). This protection appeared to be reduced in plants grown at a constantly elevated temperature of 25°C (Figure 2.9). CE experiments were supported by field experiment results, which found maximum June temperature to be correlated to phoma severity (Figure 2.5). This finding highlights the importance of understanding the mechanism of temperature-sensitivity. Although several *R* genes in *B. napus* are known to display temperature-sensitivity, these genes are currently described as only operating throughout autumn when, in the UK, temperatures are not generally high enough to affect their efficacy. Future experiments should be done with near-isogenic material, with or without *R* genes, to confirm this hypothesis. For this assay, it is suggested that the Topas ILs produced by Larkan et al. (2016) could be used. In this way, different *R* genes could be assessed for their efficacy against *L. maculans* in the stems of adult plants. Inoculation should be done following the method of inoculating the stems directly to circumvent the effects of any *R* genes operating in the leaves.

Previous studies have investigated the effect of increased temperature on quantitative resistance, reaching different conclusions. The results from this study could help explain these

differences in findings. Both experiments done by Huang et al. (2006; 2009) used constant temperature regimes in CE settings, as was the CE experiment described here in Chapter 2 (section 2.2.3.2). Findings from these CE experiments are generally in agreement with the hypothesis that quantitative resistance provides a mechanism to reduce the effect of elevated temperatures (Huang et al., 2006; Huang et al., 2009). Hubbard and Peng (2018) set the temperature conditions to mimic the heat waves that occur in Canadian Prairies; gradually increasing temperature from a seven-hour night-time period at 18°C to reach a seven hour day time of 32°C. Their findings support the field experiment data in this study, showing no correlation between canker severity and maximum June temperature for cultivars with only quantitative resistance. Therefore, it is suggested that quantitative resistance maintains its efficacy when increased temperature is not sustained for long periods of time. This may be an effect of a circadian clock regulation of plant immunity, in which some defense related genes and closure of stomata are modulated in a circadian manner (Hua, 2013; Roden and Ingle, 2009).

Alternatively, the differences seen in the results of these studies on QR could be due to the cultivars containing different forms of QR. Hubbard and Peng (2018) suggested that the cultivars included in their study may contain genes responsible for QR against *L. maculans* that are separate from those associated with physiological responses to temperature. The genes behind the QR in the cultivars Darmor and ES Astrid, both of which were weakened in efficacy at 25°C, could be linked with physiological responses to temperature. This could be determined in a future experiment using CE chambers set to different elevated temperature regimes. From these CE and field experiments, it is concluded that combining temperature-resilient *R* genes and quantitative resistance provides the best protection against *L. maculans*, the phoma stem canker pathogen at increased temperature.

5.2. *BrSNC1* appears to play a role in the temperature-sensitivity of *B. rapa* resistance against *L. maculans*.

Candidate orthologs of the temperature-sensitive *AtSNC1* and temperature-resilient *FocBo1* in *B. rapa* were investigated for their involvement in the temperature-sensitivity of the resistance response against *L. maculans*. Mutants of these *BrSNC1* and *FocBr1* genes were assessed for temperature-sensitivity of the defence response at 20°C and 25°C (Chapter 3). Mutations in candidate *FocBo1* and *SNC1* orthologs are hypothesised to have opposing effects in *B. rapa* resistance to *L. maculans*. A temperature-resilient autoimmune phenotype was observed in a putative gain of function *Brsnc1* mutant, while a more susceptible phenotype was observed for

the loss of function *focBr1-1* mutant (Figures 3.7 to 3.10). These results shed some light on the mechanism behind the temperature-sensitivity of resistance in *B. rapa*; with *BrSNC1* indicated to be involved in the temperature-dependence of the defence response.

The hypothesised pathways, following activation of the defence response, for *BrSNC1*, *Brsnc1* and *focBr1-1* at 20°C and 25°C are given in Figure 5.1. In the wild-type R-o-18 line, the resistance response is temperature-sensitive. At an increased temperature of 25°C, resistance is lost; this could be due to multiple components of the temperature-sensitive pathway, such as the thermoregulatory switch from immunity to growth, controlled by *PIF4* as described by (Gangpappa et al., 2017). In the *Brsnc1* mutant, this resistance remains effective at 25°C, it is hypothesised that increased temperature does not inhibit Brsnc1 protein expression as it is autoactivated (Figure 3.11) due to a mutation in the P-loop region of the NB-ARC domain of *Brsnc1*. The downstream pathway following an incompatible R-Avr protein interaction in *Focbr1-1* mutants is hypothesised to not be activated due to the mutation in the P-loop region of the NB-ARC domain of *focBr1-1*, causing it to be functionally switched off from activating the downstream pathway. Alternatively, *FocBr1* may operate in a different pathway.

Most autoimmune mutants are associated with dwarfism and/ or spontaneous cell death (van Wersch et al., 2016). While *Brsnc1* plants showed an auto-activated autoimmune resistance response when inoculated with an avirulent *L. maculans* isolate at 20°C and 25°C, this was not constitutively expressed, in a manner seen in both overexpressing and point-mutation *A. thaliana SNC1* dwarf mutants (Zhang et al., 2003). *Brsnc1* plants also show the same growth phenotype as the R-o-18 wild-type. Therefore, there are no apparent fitness pay-offs for this heightened resistance response. However, the mutant lines were grown in CE cabinets so a reduction in fitness may not be apparent.

The next steps in this study will be to carry out further back-crosses (to confirm whether the phenotypes are due to the selected mutations) and segregation tests for *Brsnc1* and *focBr1-1*. Hormones and expression of defence response related genes should be measured following an incompatible reaction in wild-type, *Brsnc1* and *focBr1-1* genotypes. This would confirm the phenotypic responses described in this study (sections 3.3.4.1 and 3.3.4.2) and help characterise the pathway of temperature-resilient defence response. It would also be beneficial to determine if the wild-type R-o-18 line has resistance to *F. oxysporum*. If so, *focBr1-1* would be hypothesised to also have increased susceptibility to *F. oxysporum*.

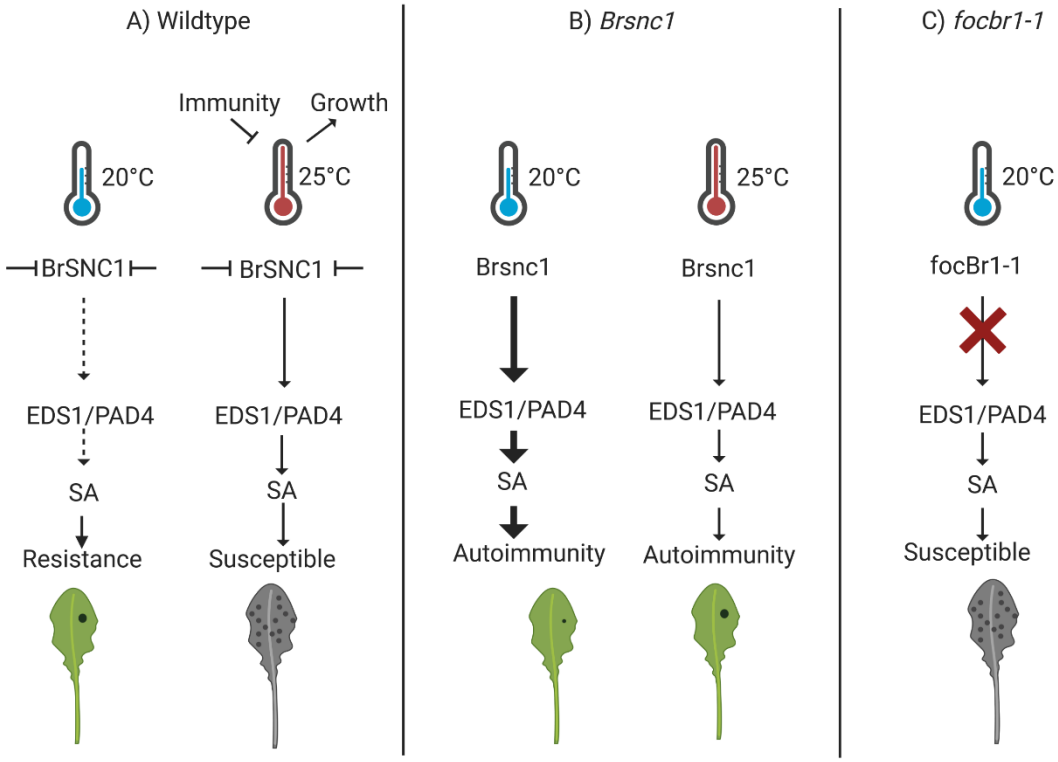


Figure 5.1 Proposed pathways following activation of the defence response for *BrSNC1*, *Brsnc1* and *focBr1-1* at 20°C and 25°C. (A) Wild-type situation; in analogy to *A. thaliana*, it is hypothesized that EDS1/PAD4 act downstream of BrSNC1 and activate SA biosynthesis leading to resistance. The *R* gene in R-o-18 is unknown and therefore the pathway to resistance is unknown (dashed lines). The defence response is supressed at 25°C by multiple components of the temperature-sensitive resistance response (B) In the *Brsnc1* mutant this resistance remains effective at 25°C, it is hypothesised increased temperature does not inhibit *Brsnc1* expression as it is autoactivated due to a mutation in the P-loop region of the NB-ARC domain of *Brsnc1*. (C) In the *Focbr1-1* mutant, *Focbr1* is hypothesised to be inactivated due to the mutation in the P-loop region of the NB-ARC domain of *focBr1-1*, causing it to be functionally switched off from activating the downstream pathway. The blue, red or black arrows indicate pathways that occur at 20°C, 25°C, or which are independent of temperature, respectively. Arrow thickness indicate the amount of protein activity. Figure created with BioRender.com.

5.3. Temperature-sensitive and -resilient *B. napus* plants show differences in defence related gene expression following incompatible *L. maculans* interaction.

Differences in the resistance response at 20°C and 25°C of temperature-resilient Topas-*LepR3* and Topas-*Rlm4* compared to the temperature-sensitive Topas-*Rlm7* were characterised both phenotypically and in the expression of the SA marker *PR1* (Chapter 4). RNA-seq was also done on temperature-resilient Topas-*Rlm4* and Topas-*Rlm7* with findings from preliminary analysis supporting the *PR1* expression analysis; *PR1* is upregulated from 4 dpi. RNA-seq analysis also revealed lower levels of *PR1* and *PDF1.2* expression in Topas-*Rlm7* lines compared to Topas-*Rlm4* at 25°C.

The next step for interpreting this RNA-seq data will be to identify genes that are differentially expressed in temperature-sensitive and -resilient Topas IL resistance response at 20°C and 25°C. Differentially expressed genes associated with the SA-mediated signaling pathway, ET-mediated signaling pathway and JA-mediated signaling pathway will be of particular interest and will allow us to create expression profiles over time.

5.4. Applications for developing oilseed cultivars with temperature-resilient resistance to phoma stem canker

Findings of this study point towards multiple factors that can influence a *B. napus* plant's ability to defend against *L. maculans* at elevated temperatures which may be utilised to improve the resilience the crop to this disease. The results in Chapter 2 highlight that temperature-resilient elements are present in existing cultivars, in the forms of quantitative resistance and temperature-resilient *R* genes, such as *Rlm4* and *LepR3*. The observation that high maximum June temperatures increase the severity of phoma stem cankers could be used to identify cultivars with increased temperature resilience. Furthermore, breeders may be able to use this to increase the temperature resilience of future cultivars through crossing selections and field assessments.

BrSNC1 is hypothesised to play a key role in temperature-sensitivity of the defence response in *B. rapa*. The next step would be to identify a homologous gene in *B. napus* and then generate a mutant that would hypothetically display a similar temperature-resilient autoimmune phenotype. Although efforts to engineer disease resistance crops have so far been met with issues relating to associated fitness cost, new studies are exploring strategies with a more targeted approach. One such application has been designed to express defence proteins with

minimal adverse effects on plant growth and development (Xu et al., 2017). Based on the knowledge that translation of key immune regulators, such as TBF13, are rapidly and transiently induced upon pathogen recognition, a "TBF1-cassette" consisting of both an immune-inducible promoter and two pathogen-responsive upstream open reading frames (uORFs) of the TBF1 gene was produced. Applying this TBF1-cassette leads to the uORFsTBF1-mediated translational control over the production of *snc1-1* in *A. thaliana* allows broad-spectrum disease resistance to be engineered without reducing plant fitness.

Additional components that contribute to temperature-sensitivity in *B. napus* may yet be discovered; RNA-seq analysis results should help in discovery of more genes associated with temperature-resilience that may be selected for in breeding programmes for future cultivars or used to engineer temperature-resilient crops. Differentially expressed genes in temperature-resilient Topas-*Rlm4* (compared to temperature-sensitive Topas-*Rlm7*) could be useful markers for selecting the most suitable genotypes when developing oilseed cultivars with temperature-resilient resistance to *L. maculans*.

5.5. Conclusions

To summarise, the results of this study increase our understanding of how the resistance response of oilseed plants against *L. maculans* are affected by temperature and may aid in developing more temperature-resilient crops for the future. High maximum June temperatures appear to increase the severity of phoma stem cankers but cultivars with higher levels of quantitative resistance and more temperature-resilient *R* genes may be better able to defend against the disease particularly as we experience hotter summers. As well as ensuring future cultivars contain these quantitate and major *R* gene resistance traits already in use, breeders may also look towards marker selection for temperature resilience once these are known. Greater understanding of the *BrSNC1* gene could also provide potential pathways towards crops that are better able to defend against pathogens as climate change brings about increases in summer temperatures. In this way, the future production of oilseed in the UK and Europe may be made more sustainable in the face of a changing climate.

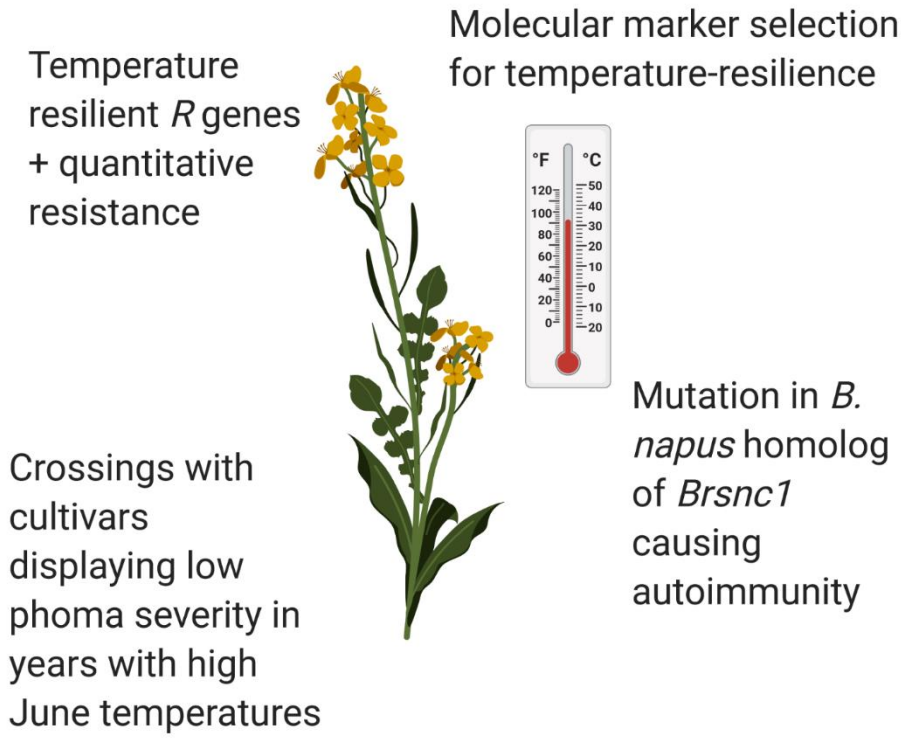


Figure 5.2 Applications for developing oilseed cultivars with temperature-resilient resistance to phoma stem canker based on the outcomes of this study. Figure created with BioRender.com

Appendix

A.1. Pathogen culture media

V8 agar media was prepared in 500 ml Duran bottles. Once autoclaved, the solution was left to cool to 50°C, at which point 20 units/ml penicillin and 40 units streptomycin was added to reduce bacterial contamination. Agar was then poured in 9 cm Petri dishes.

V8 Agar (400 ml)

8 g Agar

0.8 g $CaCO_2$

80 ml V8 juice

320 ml Distilled de-ionised water

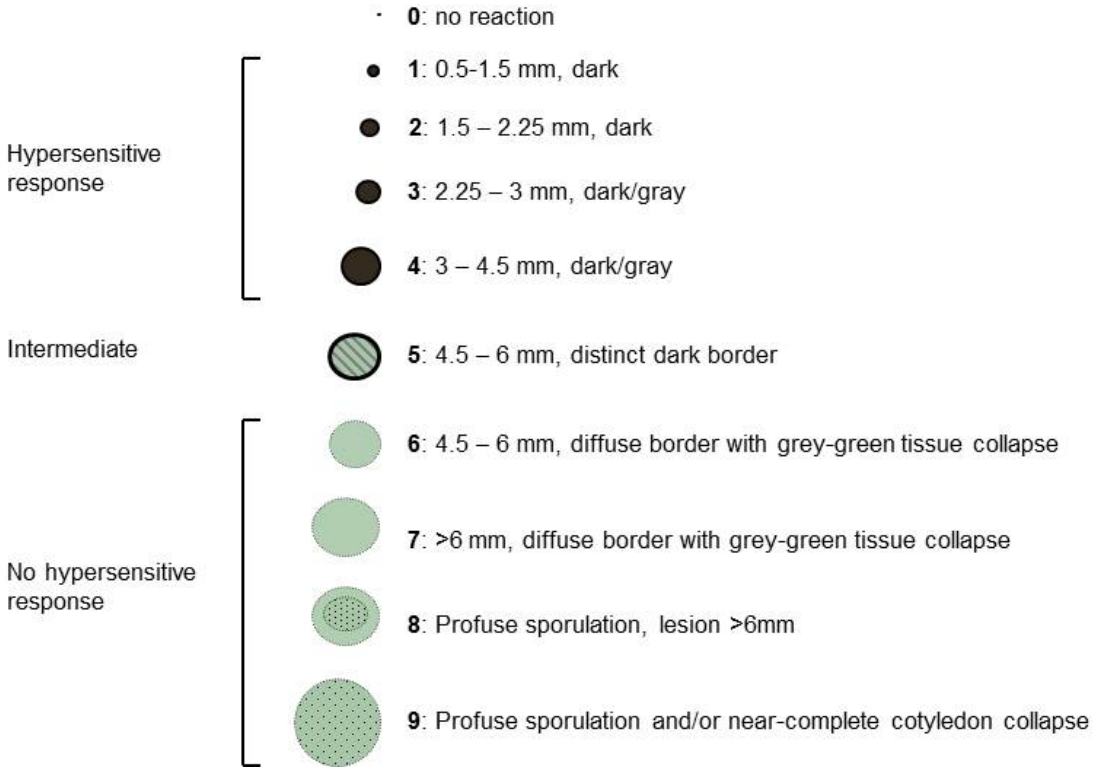
Gamborg's B5 media (1000 ml)

3.16 g Gamborg's B5 with vitamins (Duchefa)

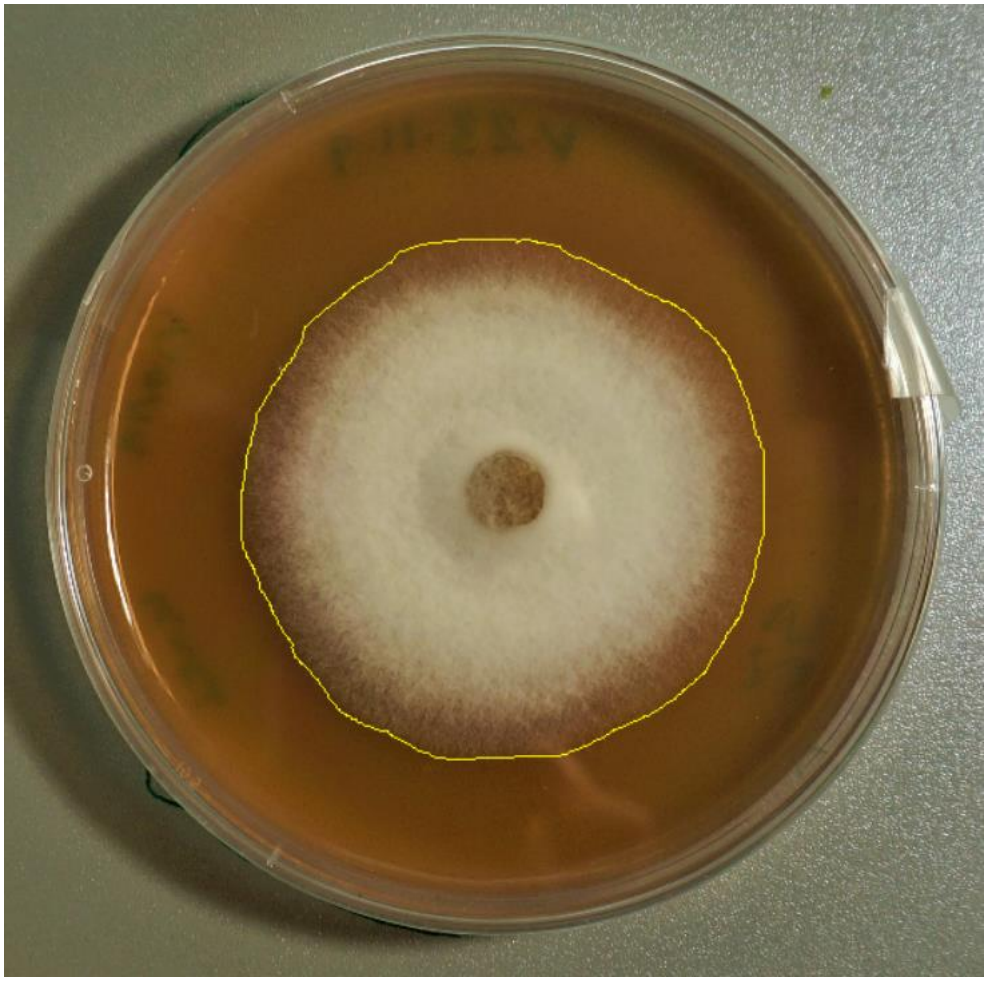
30 g of saccharose

10 mM MES buffer, pH 6.8

A.2. Phenotypic scoring guide for cotyledon assessment of *B. napus* defence response against *L. maculans* using the scoring method adapted from Koch et al., (1991), using a schematic from Larkan et al. (2016)



A.3. *L. maculans* colony growing on V8 media, with the outline of mycelial growth traced freehand on Image J, to measure the area of growth to determine the radius.



A.4. Correlation analysis for canker severity score and autumn monthly Rainfall

Month	Canker correlation coefficient
September rainfall	0.3285
October rainfall	0.4878
November rainfall	0.4860
December rainfall	0.2871

A.5. Correlation analysis for canker severity score and mean maximum temperature for each month of the growing season.

Month	**Canker correlation coefficient**
September temperature	0.2521
October temperature	0.1754
November temperature	0.1888
May temperature	0.2701
June temperature	0.3329
July temperature	0.2608

A.6. Estimated parameters for the simple linear regression equation y = a + b.*Tmax*, where a is the intercept and b is the slope.

Group	a	b
1	-2.23	0.1322
2	-2.68	0.1534
3	-0.53	0.0809
4	3.54	-0.0337
5	-0.62	0.0673
6	-2.06	0.1056
7	1.12	0.0238
8	7.82	-0.1563

R gene	a	b
LepR3	-1.1	0.0801
None	4.47	-0.0663
Rlm4	0.35	0.0575
Rlm7	-2.38	0.1393

A.7. Oilseed rape growth stages (Sylvester-Bradley and Makepeace 1985)

0 - Germination and emergence	**5** - Pod development
1 - Leaf production	**5,3** - 30% potential pods
1,0 - Both cotyledons unfolded	**5,5** - 50% potential pods
1,1 - First true leaf	**5,7** - 70% potential pods
1,2 - Second true leaf	**5,9** - All potential pods
1,5 - Fifth true leaf	**6** - Seed development
1,10 - About tenth true leaf	**6,1** - Seeds expanding
1,15 - About fifteenth true leaf	**6,2** - Most seeds translucent but full size
2 - Stem extension	**6,3** - Most seeds green
2,0 - No internodes ('rosette')	**6,4** - Most seeds green-brown mottled
2,5 - About 5 internodes	**6,5** - Most seeds brown
3 - Flower bud development	**6,6** - Most seeds dark brown
3,0 - Only leaf buds present	**6,7** - Most seeds black but soft
3,1 - Flower buds enclosed by leaves	**6,8** - Most seeds black and hard
3,3 - Flower buds visible from above	**6,9** - All seeds black and hard
3,5 - Flower buds raised above leaves	**7**- Leaf senescence
3,6 - Flower stalks extending	**8** - Stem senescence
3,7 - First flower buds yellow ('yellowbud')	**8,1** - Most stem green
4 - Flowering	**8,5** - Half stem green
4,0 - First flower opened	**8,9** - Little stem green
4,1 - 10% all buds opened	**9** - Pod senescence
4,3 - 30% all buds opened	**9,1** - Most pods green
4,5 - 50% all buds opened	**9,5** - Half pods green
4,7 - 70% all buds opened	**9,9** - Few pods green
4,9 - 90 % all buds opened	
5 - Pod development	

B.1. DNA sequence alignment of the three *SNC1* alleles in *A. thaliana*, *B. rapa* and *B. napus*) and three *FoBr1* alleles (in *B. oleracea*, *B.rapa* and *B. napus*). Colours indicate translational coding for amino acids

B2. Phenotypic observations of *B. rapa* R-o-18 TILLING mutants

TILLING line (direct RevGen)	Phenotype
R-o-18	Healthy plant
focBr1-2	Healthy plant
focBr1-1	Dwarfed phenotype Faceted stem Late flowering Rounded leaves
Brsnc1	Healthy plant

B3. ANOVA test to evaluate the effect of genotype (*Brsnc1*, *focBr1-1*, *focBr1-2, FocBr1-2* and *FocBr1-2*/*focBr1-2*) on relative *PR1* expression compared to R-o-18 wild-type. df Degrees of freedom

Source	*df*	Mean square	*F*-value	*P*-value
Genotype	5	3.051	4.22	0.007
Error	23	0.724		

B4. ANOVA test to evaluate the effect of genotype (*Brsnc1*, *focBr1-1*, *focBr1-2, FocBr1-2* and *FocBr1-2*/*focBr1-2*) on relative *BrSNC1* expression compared to R-o-18 wild-type. df Degrees of freedom

Source	*df*	Mean square	*F*-value	*P*-value
Genotype	5	0.458	0.74	0.6
Error	23	0.617		

B5. ANOVA test to evaluate the effect of genotype (*Brsnc1*, *focBr1-1*, *focBr1-2, FocBr1-2* and *FocBr1-2*/*focBr1-2*) on relative *FocBr1* expression compared to R-o-18 wild-type. df Degrees of freedom

Source	*df*	Mean square	*F*-value	*P*-value
Genotype	5	5.8061	566.77	<.001
Residual	23	0.01024		
Total	28			

C.1. Gamborg's B5 media inoculated with *L. maculans* (left) and the control without fungus (right)

C.2. Contrast performed for mock and *L. maculans* inoculated Topas lines for *PR1* expression analysis

Source	***df***	s.s.	m.s.	**F-value**	***P*-value**
treatment	1	48.86	48.86	1.18	0.284
Residual	46	1911.39	41.55		
Total	47	1960.26			

C.3. Contrast performed for mock and *L. maculans* inoculated Topas lines for *BnaC07g33990* expression analysis

Source	***df***	**s.s.**	**m.s.**	**F-value**	***P*-value**
Treatment	1	6.31	6.31	1.17	0.286
Residual	36	193.388	5.372		
Total	37	199.699	5.397		

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