July 2018

Student Report No. 51

Exploitation of resistance genes from oilseed rape for control of light leaf spot

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This is the final report of a PhD project (214-0010) that ran from August 2014 to July 2018. The work was funded by the University of Hertfordshire, the GEN Foundation and a contract for £54,000 from AHDB.

CONTENTS

1. Abstract

The aim of this project was to use novel genetic and genomic information to improve understanding of resistance against *Pyrenopeziza brassicae* (light leaf spot) in oilseed rape.

A major gene locus for resistance on *Brassica napus* chrA1 was further studied using flanking marker information. Approximate physical location of this marker at the bottom of chrA1 was confirmed by using sequence homology and the synteny between *B. napus* and *B. rapa* genomes. KASP markers were developed for the single nucleotide polymorphisms (SNPs) identified between the flanking marker and the telomere of chrA1. Genotyping and linkage mapping of newly developed KASP markers led to the identification of markers linked to the south of the resistance locus, providing a defined chromosomal region. Six candidate resistance genes were identified, based on the functional annotations for the gene content in the corresponding chromosomal region available from the Darmor-*bzh* and Z11 genome sequences of *B. napus* and the *Brassica* pan-transcriptome. These included four receptor-like kinases (RLKs) and two nucleotide-binding site leucine rich repeat genes (NLRs).

Segregation for resistance against *P. brassicae* has been identified in a doubled haploid *B. napus* population (Q DH) developed by incorporating genetic diversity from ancestral *Brassica* species, *B. rapa oleifera* and *B. oleracea atlantica*. Resistance against *P. brassicae* segregating in this population was studied with controlled environment, glasshouse and winter oilseed rape field experiments. Composite interval mapping analysis had identified 17 QTL across 10 chromosomes, using data for the percentage leaf area covered with *P. brassicae* sporulation and five QTL (with LOD ≥ 3.2) across five chromosomes for the *P. brassicae* DNA data. There were some QTL hotspots on chromosomes C1, C3, C6 and C9, where QTL from different experiments and/or traits were co-located.

This work also reports the identification of specific interactions between *B. napus* and *P. brassicae* using controlled environment and glasshouse experiments. There were two main phenotypes of resistance, formation of black necrotic flecking and limitation of *P. brassicae* asexual sporulation (acervuli), which often appeared to be correlated. Interestingly, there appeared to be a quantitative nature to the black flecking phenotype. Considering various effects of resistance or susceptibility to *P. brassicae* (i.e. leaf deformations, necrotic flecking, different amounts of pathogen colonisation and asexual sporulation), it can be suggested that recognition of *P. brassicae* by resistant lines/cultivars occurs at a late stage of *P. brassicae* colonisation, possibly during the phase of asexual sporulation.

2. Introduction

Light leaf spot, caused by *Pyrenopeziza brassicae,* is an economically damaging disease of brassicas. A number of severe disease epidemics have been reported in winter oilseed rape (*Brassica napus*) in the UK since a major epidemic was recorded in 1974 (Simons & Skidmore 1988). Severe disease epidemics have also been reported in some regions in continental Europe. The disease was first reported from France in 1978 and it has persisted at different levels, with severe epidemics in the 1980s and 2000s (Karolewski *et al.* 2006; Pilet *et al.* 1998). Light leaf spot is considered to be less damaging in Germany (Karolewski *et al.* 2006), despite its widespread occurrence in the late 1980s (Pilet *et al.* 1998). However, increased incidence of the disease on oilseed rape has been recorded in recent years in Germany. Light leaf spot also occurs in Poland, with severe damage in mild winters (Karolewski *et al.* 1999; Koike *et al.* 2007). New Zealand experienced a severe outbreak of light leaf spot in vegetable brassicas in 1978 that led to a record of the teleomoph, *P. brassicae,* under natural conditions (Cheah *et al.* 1980). Widespread occurrence of the disease has been reported in some other regions with wet, cool climates (Staunton and Kavanagh 1966; Koike *et al.* 2007). Recent studies have reported the occurrence of light leaf spot on *Brassica* species in Oregon, USA (PNW plant disease handbook 2016).

Since the major outbreak of light leaf spot disease in the UK, disease severity has varied greatly between different cropping seasons and different regions (Simons & Skidmore 1988; Fitt *et al.* 1998). Previously, severe epidemics have been recorded in Scotland and northern England, where the weather conditions are favourable for disease development (Figueroa *et al.* 1995). However, according to recent disease survey data from Defra-funded winter oilseed rape pest and disease survey (CropMonitor 2016), the severity of epidemics has increased progressively across the UK, thereby causing increased yield losses (Figure 1). This frequent, widespread occurrence of light leaf spot has made it a high priority for many oilseed rape growing areas in the UK.

2.1. *Pyrenopeziza brassicae*

Pyrenopeziza brassicae, causative organism of light leaf spot disease in oilseed rape, is a discomycete fungus, which has been taxonomically accommodated within the family Dermateaceae of the order Helotiales within the phylum Ascomycota. Their vegetative structures consist of septate mycelia that are haploid in chromosome numbers (Webster & Weber 2007). According to the most recent classification of ascomycete mating type loci, the two mating types are designated as *MAT-1* and *MAT-2*. The family Dermateaceae is characterized by the formation of darkly-pigmented (i.e. grey, black or brown coloured) apothecia that emerge directly on the substrate without producing stromata (Webster & Weber 2007).

However, this pathogen was also known by its anamorph (imperfect stage), *Cylindrosporium concentricum*, for many years; this was initially described by Greville (1823) considering the

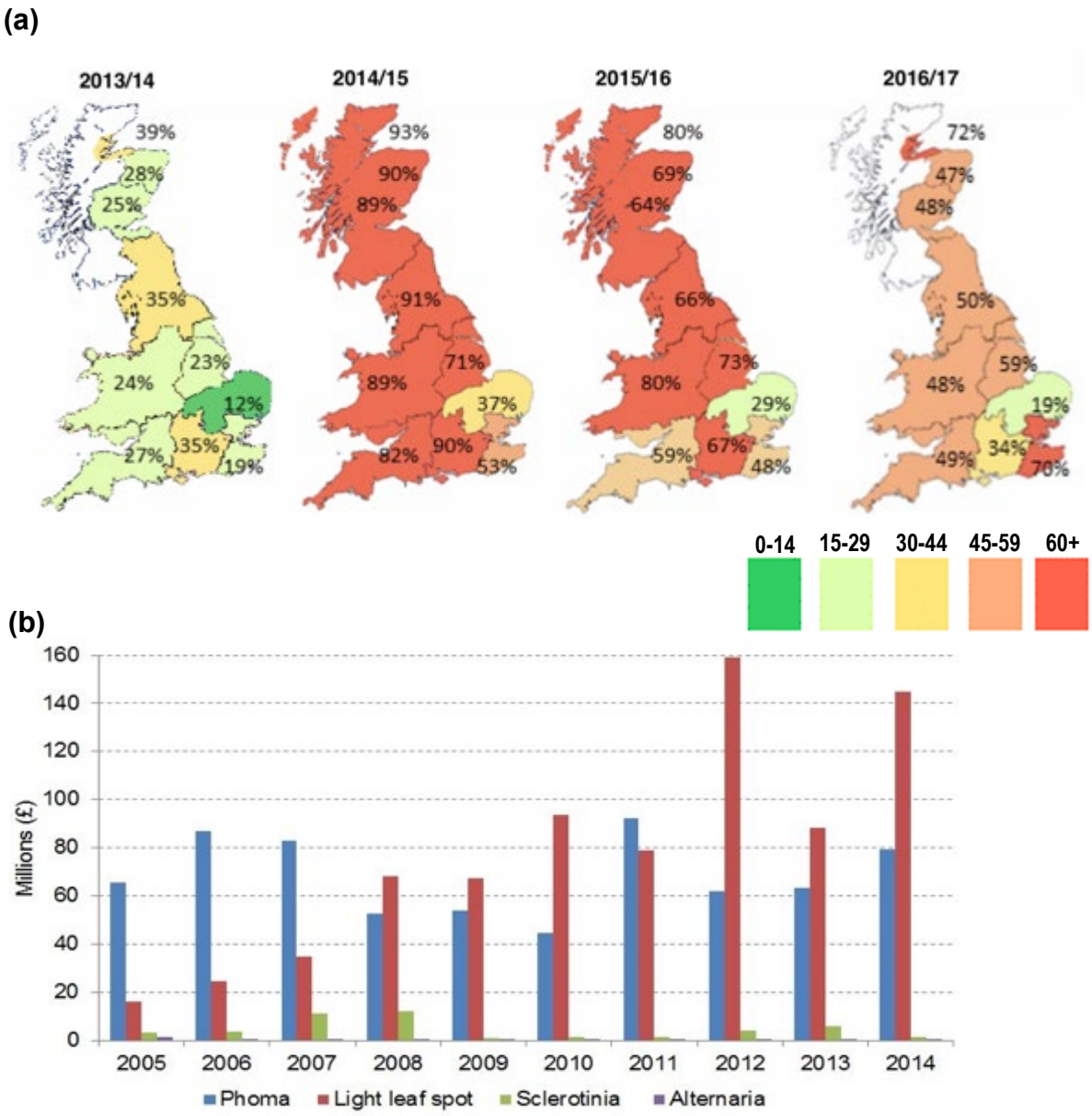

Figure 1: Comparison of forecasted light leaf spot epidemics in different regions in the UK between four cropping seasons and yield losses caused by the disease since 2005 in England.

(a) According to the regional light leaf spot forecasts, the percentage of crops predicted to have more than 25% affected plants has increased over the past three recent cropping seasons. The percentages on the map indicate % crops with >25% affected plants. (b) Yield losses caused by four major oilseed rape pathogens in England. Since 2008, light leaf spot has become the most damaging disease in winter oilseed rape in England. (Images from Rothamsted Research light leaf spot forecast (Accessed 10 May 2018) and CropMonitor 2016, respectively).

concentric ring-like pattern of the asexual sporulation. Ascomata (sexual sporulating structures) of *P. brassicae* were first observed on culture media by Thomson (1936) and Cabral (1940), and later described as immature apothecia by Hickman *et al.* (1955). The occurrence of *P. brassicae* apothecia under natural conditions was first reported by Staunton and Kavanagh in 1966, based on their observations in diseased vegetable brassica crops in Ireland. The precise identification of *P. brassicae* was made by Rawlinson *et al.* (1978a), who described it as the teleomorph of *Cylindrosporium concentricum* (Rawlinson *et al.* 1978a; Cheah *et al.* 1980).

2.2. Light leaf spot disease cycle

Light leaf spot is a polycyclic disease with epidemics that are initiated in autumn by wind-dispersed ascospores (Gilles *et al.* 2001). Infected crop debris remaining in the field after harvest provides the primary inoculum for the disease development in winter oilseed rape crops. Ascospores are produced within apothecia via sexual reproduction of the pathogen at this stage (Rawlinson *et al.* 1978a). Germinating ascospores on host surfaces enter into the host by direct penetration through the cuticle. Involvement of cutinases in *P. brassicae* infection has been demonstrated and cutinolytic activity of the pathogen has been suggested to assist the penetration (Davies *et al.* 2000; Li *et al.* 2003).

After infection, the pathogen enters into a long asymptomatic growth phase where it proliferates within the sub-cuticular space between the cuticle and the epidermis of the host leaves. During winter, the first symptom of light leaf spot is onset of asexual sporulation, which involves development of white acervuli on leaf surfaces. This indicates the end of the asymptomatic growth phase of the pathogen (Rawlinson *et al.* 1978a; Boys *et al.* 2007). *P. brassicae* acervuli consist of numerous conidiophores that produce splash-dispersed conidia, which are known to cause secondary infections (Evans *et al.* 2003; Karolewski *et al.* 2004). In addition, ascospores produced on senescent *P. brassicae*-infected leaves can also contribute to secondary disease spread in spring (Evans *et al.* 2003).

P. brassicae infections induce the formation of leaf and stem lesions on host plants (Fitt *et al.* 1998; Rawlinson *et al.* 1978a), causing reduced photosynthetic area and thereby reduced yield at harvest. Early infections during autumn and winter are able to kill seedlings or decrease plant vigour. Secondary infections that occur late in the cropping season affect floral parts, leading to pod disease. This results in premature ripening and pod shattering, causing further yield loss. Also, this pathogen interferes with the hormonal balance of the host plant, resulting in leaf distortion and stunting of plants (Ashby 1997; Gilles *et al.* 2000; Rawlinson *et al.* 1978a).

2.3. Management of light leaf spot disease

Currently, light leaf spot disease control in the UK includes the use of fungicides (Fitt *et al*. 1998). Because of the long asymptomatic growth phase of the pathogen, there is a considerable time between the initial infection and the symptom appearance (incubation period). Hence, timing of the first fungicide application is a key factor to successful disease control. Considerable variations in disease severity between different regions and different cropping seasons also need to be considered. Most often the first fungicide application has to be made before symptom development. This can lead to unnecessary applications of fungicides that cause economic losses to farmers and also increase risk of fungicide-insensitivity development within pathogen populations (Boys *et al*. 2007; Fitt *et al*. 1998; Gilles *et al*. 2000). Reduced sensitivity to azole fungicides has been reported

within *P. brassicae* populations in the UK (Carter *et al*. 2013). Moreover, recent changes in the EU legislation to withdraw fungicides with certain active substances will limit the available chemical control measures.

Deployment of cultivar resistance against *P. brassicae* can be used as a successful alternative to the chemical control methods. However, there is a limited understanding about the genetic resistance operating in different commercial oilseed rape cultivars. According to the AHDB recommended list (RL) disease resistance rating for light leaf spot (2015/16), oilseed rape cultivars with good resistance (rating 6 and 7, where 9 is most resistant and 1 is very susceptible) are available for the North region, whereas moderately resistant cultivars (rating 5 and 6) are available for the East and West regions (AHDB Cereals and Oilseed 2018). Resistance breakdown of some cultivars with good resistance ratings has been reported, with recent light leaf spot epidemics resulting. Also, there is increasing concern among oilseed rape breeders and growers about the need for more comprehensive field assessment criteria, which consider all the aspects of the disease. Inadequate information about cultivar resistance among vegetable brassica species has limited the deployment of cultivar resistance in vegetable brassicas.

Moreover, cultural practices can also be incorporated into disease management programmes. Removal/reduction of the amount of initial inoculum by cultural practices enhances the disease control. Cheah and Hartill (1985) demonstrated that ploughing of vegetable brassica crop debris caused rapid degradation of *P. brassicae* apothecia and the loss of viability of ascospores. Crop rotation is also effective in controlling the initial inoculum. Field experiments at Rothamsted in two cropping seasons were reported to have a greater disease incidence earlier in the cropping season as well as a greater rate of disease increase in crops when they followed oilseed rape rather than cereals crops (Figueroa *et al*. 1994).

2.4. Resistance in oilseed rape against *P. brassicae*

2.4.1. Operation of host resistance during the pathogen life cycle

Analysis of infection and disease development stages of pathogen life cycles is a useful tool to identify possible resistance mechanisms operating in the host against that particular pathogen. In the case of *P. brassicae*, the life cycle can be divided into spore adhesion and germination, cuticular penetration, colonisation, asexual sporulation and spore dispersal stages. Pre-existing structural host defence mechanisms, such as thickness and composition of epicuticular waxes and the surface topology, can act against the adhesion and germination of *P. brassicae* ascospores and conidia. Application of the herbicide Dalapon (2,2- dichloropropionoc acid) on oilseed rape caused increased susceptibility to *P. brassicae* due to its effect on altering epicuticular waxes (Rawlinson *et al*. 1978b). Some surfactants and mechanical damage have also been recorded to result in decreased amounts

of wax and an increased hydrophilic nature on leaf surfaces, facilitating the adherence, germination and penetration of the pathogen.

Penetration of the pathogen is known to occur through the activity of cutinases (Davies *et al*. 2000). Therefore, cutinase inhibitory actions or substrate alterations can be proposed as possible host defence mechanisms. After the penetration, the asymptomatic growth phase of the pathogen starts with the formation of a hypomycelium followed by the proliferation of fungal hyphae to produce mycelial plates within the sub-cuticular space (Rawlinson *et al*. 1978a). At this stage, two-way communication occurs between pathogen and host plant in which the pathogen attempts to utilise the host metabolism for its growth and reproduction, whereas the host strives against the pathogen following the recognition of pathogen signals. Therefore, both major and minor gene-mediated resistance can operate to prevent the pathogen proliferation (Boys *et al*. 2007).

Involvement of extracellular cutinases (*Pbc1*) (Li *et al*. 2003), extracellular proteases (*Psp1*) (Batish *et al*. 2003) and cytokinins (Ashby 1997) has been identified during the sub-cuticular growth phase of the pathogen and they are considered as the key pathogenicity determinants of *P. brassicae*. Moreover, intercellular growth of hyphae in the upper mesophyll during the onset of asexual sporulation has been observed, indicating another possible point for the operation of major gene-mediated resistance. Delayed leaf senescence, attributed either to the genetic composition of the crop or to environmental factors, can provide resistance against the sexual sporulation of the pathogen, resulting in reduced amounts of secondary inoculum (Boys *et al*. 2007).

2.4.2. Evidence of host resistance against *P. brassicae*

There have been several studies on the operation of host resistance against *P. brassicae* in different brassica species. Pilet *et al*. (1998) reported 10 (six environmentally stable) QTL segregating in a *Brassica napus* doubled haploid (DH) population derived from a cross between two oilseed rape cultivars, Darmor-*bzh* and Yudal. Bradburne *et al*. (1999) published the first report about qualitative resistance in *B. napus* against *P. brassicae*. Two different resistance phenotypes derived from two ancestral species, *B. rapa* and *B. oleracea atlantica*, were recognized: no obvious symptoms or asexual sporulation; development of dark flecks. The genetic locus for ‘no asexual sporulation’ (*PBR1*) was located on linkage group A1 and the corresponding locus for ‘dark flecking’ (*PBR2*) was placed on linkage group C16 (Bradburne *et al*. 1999). In addition, a major gene for resistance against *P. brassicae* has been characterized and mapped to the bottom end of the chromosome A1 on *B. napus* (Boys *et al.* 2012) using a doubled haploid (DH) mapping population developed by introgressing resistance in cultivar Imola derived from resistant lines studied by Bradbourne *et al*. (1999). The phenotype of this resistance is characterised by the presence of black necrotic flecking with no asexual sporulation of *P. brassicae*. Furthermore, there have been several studies on various aspects of resistance against *P. brassicae* in oilseed rape and vegetable brassicas. Involvement of gene-for-gene interactions in the interaction between *P. brassicae* and *B. oleracea* was reported by

Simons and Skidmore (1988). Effectiveness of resistance elicitors, consisting of acibenzola-S-methyl, *cis*-jasmonate and β-aminobutyric acid, was compared with that of triazole fungicides for controlling light leaf spot disease on winter oilseed rape. It has been reported that resistance elicitors gave better control of the disease at some stages of the crop growth than fungicide treatments (Oxley & Walters 2012).

2.4.3. Exploitation of host resistance against *P. brassicae*

It is clearly evident that light leaf spot disease causes substantial economic losses to oilseed rape and vegetable brassica production in the UK and can be an emerging disease threat to other oilseed rape growing areas. It has been difficult to achieve effective management of light leaf spot with currently available cultivars and identification of durable resistance against *P. brassicae* remains a challenge. Understanding of the molecular genetic mechanisms underpinning the *B. napus* – *P. brassicae* interactions is essential for developing effective, durable disease-management strategies. Compared to the understanding of light leaf epidemiology, substantial gaps remain in understanding of the operation of brassica resistance and *P. brassicae* pathogenicity. Improved understanding of mechanisms of resistance operating against *P. brassicae* can provide useful tools for breeding for disease resistance. Even though there have been few sources of resistance identified, the potential for use of these sources in development of sustainable resistance against *P. brassicae* has increased substantially in the light of recent advancements in molecular techniques and genomic resources. Genome sequence information is available for *B. napus* (Chalhoub *et al*. 2014), together with its diploid ancestral species, *B. rapa* (Wang *et al*. 2011) and *B. oleracea* (Liu *et al*. 2014) and for *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000). Recently, the genomes of allotetraploid *B. juncea* and its B genome progenitor *B. nigra* were sequenced (Yang *et al.* 2016). With this information, resistance genes against *P. brassicae* mapped in previous studies can be further examined to characterise the genetic basis of resistance. Sequence information can also be used to increase the marker density of linkage maps to assist in breeding programmes.

2.5. Aim and objectives

The aim of this PhD project is to provide a better understanding of the operation of resistance against the light leaf spot pathogen *P. brassicae* in *B. napus*. The specific objectives are,

1. To study specific host-pathogen interactions in the *Brassica napus-Pyrenopeziza brassicae* pathosystem
2. To phenotypically analyse the Q doubled haploid population for resistance against *P. brassicae* and map resistance QTL
3. To fine map a major resistance locus against *P. brassicae* at the bottom of *B. napus* chromosome A1 and to identify candidate resistance genes

3. Materials and methods

3.1. Specific host-pathogen interactions in the *Brassica napus-Pyrenopeziza brassicae* pathosystem

There were three experiments done under controlled environment or glasshouse conditions, which included several oilseed rape cultivars and breeding lines, to study specific host-pathogen interactions and meristematic infection by *P. brassicae*. Table 1 provides a summary of these experiments.

3.1.1. Investigation of the phenotype/s of resistance

Three oilseed rape cultivars/lines that produced a characteristic black flecking phenotype in response to *P. brassicae* infection (cv. Imola, Q83 and Q88) and cv. Bristol (considered to contain a major gene for resistance against *P. brassicae* that has been rendered ineffective) were selected. Single conidial isolates of *P. brassicae* were prepared from acervuli taken from different oilseed rape cultivars/lines. *P. brassicae* inoculum (conidial suspensions of 10^5 spores/ml) was prepared from four single conidial isolates (Table 2) sub-cultured on MEA media.

Plants were grown in controlled environment conditions until they reached growth stage 1,4-1,5. Plants were arranged with 16 pots in each tray in a split-plot design generated using Experiment Design Generator and Randomiser (EDGAR) (Brown 2005). The four isolates were randomly assigned to main plots (trays) and four cultivar/line treatments were randomly assigned to the sub-plots (pots) within each of the main plots. For each cultivar/line, four replicate plants were tested for each of the isolates. Plants were spray-inoculated with conidial suspensions. At 24 dpi (days post inoculation), plants were harvested and scored for the presence/absence of a necrotic response. After scoring, plants were individually placed in polyethylene bags and incubated at 4 °C for 5 days to induce *P. brassicae* sporulation. Disease assessment was done using the 1-6 scale (Appendix 1) and also by estimating the percentage leaf area (of the fully expanded leaves) covered with *P. brassicae* sporulation. The number of deformed leaves (leaf curling, distortion, etc.) was also recorded.

3.1.2. Investigation of the effect of resistance on spread of *P. brassicae* to upper leaves through growth of meristematic tissues

Oilseed rape cultivars Imola (resistant to *P. brassicae*), Tapidor (susceptible) and three Q DH lines; Q70, Q62, Q79 (with early flowering time and susceptible) were included in this experiment. Diseased leaves from oilseed rape cultivars Marathon, Bristol, Tapidor and susceptible Q DH lines were collected from glasshouse-grown plants and incubated at 4 °C for five days to induce sporulation. Pathogen inoculum (conidial suspensions of 10^5 spores/ml) consisting of *P. brassicae* populations was prepared from incubated leaves.

Table 1: Summary of the controlled environment and glasshouse experiments used to investigate *Brassica napus* – *Pyrenopeziza brassicae* interactions

Description of the experiment	Cultivars/ breeding lines used	*P. brassicae* inoculum	Method of disease assessment
1. Comparison of the resistance response to *P. brassicae* of cultivar Imola and Q DH lines that produce a black flecking phenotype[+]	Cvs Imola, Bristol and two Q DH lines (Q83 & Q88)	Conidial suspensions prepared from single spore isolates of *P. brassicae*	Presence of a necrotic response; 1-6 scale*, visual assessment of the % leaf area covered with *P. brassicae* sporulation
2. Investigation of the persistence of meristematic infection[+]	Cvs Imola, Tapidor and four Q DH lines with early flowering	Conidial suspensions prepared from *P. brassicae* populations[#]	% leaf area covered with *P. brassicae* for individual leaves; quantification of *P. brassicae* DNA using qPCR
3. Study of the specific interactions between different oilseed rape cultivars/ lines with single spore isolates of *P. brassicae*[†]	Seven commercial oilseed rape cultivars, cv. Imola and two DH lines from the Q DH population (Q69 & Q83)	Conidial suspensions prepared from single spore isolates of *P. brassicae*	1-6 scale*, visual assessment of the % leaf area covered with *P. brassicae* sporulation; presence of a necrotic response

[+] Controlled environment experiment

* See section Appendix 1 for description of the 1-6 scale for light leaf spot assessment

[#] Conidial suspensions were prepared from leaf samples collected from a glasshouse experiment set up to study the segregation of resistance in the Q DH population

[†] Glasshouse (temperature-regulated) experiment

Table 2: Origin of *Pyrenopeziza brassicae* isolates used to investigate the production of the black necrotic flecking phenotype of resistance

Four isolates that originated from different oilseed rape cultivars/lines with a black necrotic phenotype were selected to study the isolate specificity of the black necrotic flecking phenotype observed in cv. Imola and two DH lines.

Isolate name[‡]	Type	Origin		Year	Mating type
		Cultivar/line[†]	Location*		
17WOSRQ107-SA1	Single acervulus isolate	Q107	Glasshouse experiment	2017	*MAT-1*
17WOSRQ88-SS3	Single conidial isolate	Q88	Glasshouse experiment	2017	*MAT-1*
17WOSRQ89-SS4	Single conidial isolate	Q89	Glasshouse experiment	2017	*MAT-1*
17WOSR-I6	Single conidial isolate	Imola	Glasshouse experiment	2017	*MAT-2*

[‡] Isolate names consist of the year, crop species, specific line/cultivar followed by coding for isolate type (SA – single acervulus, SS – single spore).

[†] All four isolates were derived from oilseed rape cultivars/lines known to produce a black necrotic phenotype after inoculation.

* Isolates originated from diseased leaf samples taken from a glasshouse experiment set up to study the segregation of resistance in the Q DH population.

Plants were grown in a controlled environment and arranged in a randomised complete block design with five pots in each tray (block) with six replicate blocks. Each cultivar/line had at least four replicate plants. Plants at growth stage 1,3-1,4 were spray-inoculated (particularly on the shoot tips) with *P. brassicae* conidia (10^5 spores/ml + 0.005% Tween 80). Disease assessment was done at 28 dpi. Individual leaves were scored for the percentage leaf area covered with *P. brassicae* asexual sporulation (acervuli) and individual plants were scored using the 1-6 scale (Appendix 1). Presence of other symptoms such as plant/leaf deformations (leaf curling, distortion, etc.) was also recorded. After disease assessment, individual leaves including the internode were removed from each plant and placed separately in labelled 15 ml or 50 ml Falcon tubes. The shoot apex was also removed from each plant and placed separately in labelled 2 ml tubes. Samples were freeze-dried and ground to a fine powder. DNA was extracted and diluted to 20ng/μl. Amount of *P. brassicae* DNA was quantified using qPCR (Boys *et al.* 2012).

3.1.3. Identification of differential interactions between *B. napus* cultivars/lines and single-spore isolates of *P. brassicae*

Seven commercial oilseed rape cultivars (acquired from commercial sources), cv. Imola (provided by Mark Nightingale, Elsoms Seeds Ltd.) and two DH lines from the Q DH population (provided by Dr. Rachel Wells, John Innes Centre, Norwich) were used in this experiment (Table 3). Eight single spore isolates of *P. brassicae* were selected (Table 4). Isolates were sub-cultured onto malt extract agar (MEA) media for spore production. *P. brassicae* inoculum was prepared by adding 10 ml of sterilised distilled water onto cultures, mixing with mycelia and filtering with a Miracloth (Calbiochem, USA). The spore numbers were counted with a Bright-Line haemocytometer (Sigma Aldrich, UK) and suspensions were diluted to give a concentration of 10^5 spores/ml.

Oilseed rape cultivars/lines were grown in 9 cm pots containing a 1:1 mixture of all-purpose compost (Miracle Gro, UK) and John Innes No.3 compost (LBS Horticulture, Lancashire, UK) in glasshouse conditions until they reached growth stage 1,4-1,5. Plants were arranged in a split-plot design generated using Experiment Design Generator and Randomiser (EDGAR) (Brown 2005). The eight isolates were randomly assigned to main plots (blocks) and ten cultivar/line treatments were randomly assigned to the sub-plots (pots) within each of the main plots. For each cultivar/line, four replicate plants were tested for each of the isolates.

Plants at growth stage 1,3-1,4 were spray-inoculated with *P. brassicae* conidial suspensions (10^5 spores/ml incorporated with 0.005% Tween 80 just before inoculation). Spray inoculation was done using a 50 ml Travellers' sprayer (Boots, UK) until leaves were fully covered with fine droplets of conidial suspensions. The average spray volume per plant was 1.0-1.5 ml. Main plots (blocks) were covered individually with polyethylene covers for 48 h after inoculation to maintain high humidity to facilitate spore germination and infection. Glasshouse conditions were set at a 12 h daylength and 16 °C/14 °C day/night temperatures, respectively. At 24 dpi, plants were harvested and placed in

Table 3: Characteristics of the oilseed rape cultivars and breeding lines selected to study specific interactions with *Pyrenopeziza brassicae*

Differential host-pathogen interactions were studied using seven commercial oilseed rape cultivars (with varying resistance ratings from the AHDB Cereals and Oilseeds recommended list trials), cv. Imola and two DH lines known to have resistance against *P. brassicae*.

Cultivar/ line	Type	Origin	RL rating for light leaf spot*	Description
Bristol	Conventional[#]	Cargill, France	2 (1996/97)	Suggested to carry a major gene for resistance against *P. brassicae*, which was rendered ineffective, increasing the susceptibility to the pathogen
Marathon	Restored hybrid	DSV, Germany	5 (2015/16)	Listed as medium resistant against *P. brassicae* and having poor resistance against *L. maculans*
Cuillin	Restored hybrid	KWS, Germany	8 (2014/15)	Recommended for north region with good resistance against *P. brassicae*
Cracker	Restored hybrid	LS Plant Breeding, UK	7 (2015/16)	Known to have good resistance rating against *P. brassicae* and poor resistance against *L. maculans*
Temple	Conventional[#]	Elsoms Seeds, UK	7 (2014/15)	Recommended for north region with good resistance against *P. brassicae*
Trinity	Conventional[#]	Lantmannen SW Seed, Sweden	6 (2016/17)	Recommended for east/west region with medium resistance against *P. brassicae*
Excel	Restored hybrid	DEKALB, UK	5 (2011/12)	Known to have good resistance rating against *L. maculans*
Imola	Conventional[#]	KWS, Germany	NA[‡]	Has a major gene for resistance against *P. brassicae*, which produces a characteristic black flecking phenotype
Q69	DH breeding line	Smooker *et al.* 2011	NA[+]	DH breeding line developed by crossing a synthetic *B. napus* with oilseed rape cultivar Tapidor; appeared to have little or no sporulation when inoculated with *P. brassicae* populations
Q83	DH breeding line	Smooker *et al.* 2011	NA[+]	DH breeding line developed by crossing a synthetic *B. napus* with oilseed rape cultivar Tapidor; appeared to have little or no sporulation when inoculated with *P. brassicae* populations and gives a necrotic response

* AHDB Cereals and Oilseeds recommended list (RL) rating for light leaf spot on 1-9 scale, where 9 is most resistant (http://cereals.ahdb.org.uk/varieties/running-the-recommended-lists.aspx). RL rating for each cultivar has been taken from the most recent record available (given in brackets next to the RL rating) in RL trials, except for cv. Bristol where the value has been taken from Karolewski *et al.* (2006).

[#] Open pollinated cultivar

[‡] Cultivar is not commercially available

[+] Pre-breeding material

Table 4: Origin of *Pyrenopeziza brassicae* isolates used to investigate the differential interactions with *Brassica napus*

Single conidial isolates of *P. brassicae* were prepared according to the method described in section 2.3.1. Eight isolates that originated from different oilseed rape cultivars were used in a controlled environment experiment to study the specific interactions with different oilseed rape cultivars/lines.

Isolate name‡	Type	Origin		Year	Mating type
		Cultivar	Location		
15WOSR64-SS1	Single conidial isolate	Bristol	Hereford*	2015	*MAT-1*
15WOSR81-SS1	Single conidial isolate	Temple	Hereford*	2015	*MAT-2*
17WOSR-I1	Single conidial isolate	Imola	Glasshouse experiment†	2017	*MAT-2*
15WOSR76-SS2	Single conidial isolate	Cracker	Hereford*	2015	*MAT-2*
15WOSR78-SS1	Single conidial isolate	Anastasia	Hereford*	2015	*MAT-2*
17WOSR-I4	Single conidial isolate	Imola	Glasshouse experiment†	2017	*MAT-2*
15WOSR5.2-SS2	Single conidial isolate	Catana	Boxworth*	2015	*MAT-2*
17WOSR-CUI	Single conidial isolate	Cuillin	Glasshouse experiment†	2017	*MAT-2*

‡ Isolate names consist of the year, crop species, specific line/cultivar followed by coding for isolate type (SA – single acervulus, SS – single spore).
* Isolates originated from diseased leaf samples collected from winter oilseed rape field experiments at Hereford, Herefordshire or Boxworth, Cambridgeshire in the 2014/15 cropping season.
† Isolates originated from diseased leaf samples taken from a glasshouse experiment set up to study the segregation of resistance in the Q DH population.

polyethylene bags individually and incubated at 4 °C for 5 days to induce *P. brassicae* sporulation. After incubation, disease assessment was done using a 1-6 scale and also by estimating the percentage leaf area (of the fully expanded leaves) covered with *P. brassicae* sporulation. The number of deformed leaves (leaf curling, distortion, etc.) and the presence of a necrotic resistance response were also recorded.

3.2. Phenotypic analysis of the Q doubled haploid population for resistance against *Pyrenopeziza brassicae* and mapping of resistance QTL

Phenotype data for the Q DH population were obtained from a series of experiments in field and controlled environment conditions using visual and molecular techniques. The mapping of resistance QTL segregating in the Q DH population was done using phenotype data obtained in these experiments and the linkage map described by Smooker *et al*. (2011).

3.2.1. Phenotyping of resistance against *P. brassicae* in the Q DH population in a winter oilseed rape field experiment

In the 2015/16 cropping season, a field experiment was established at the Limagrain UK Ltd field site at Rothwell, Lincolnshire. On 29 August 2015, 62 lines from the Q DH population, *B. rapa oleifera* '29' (one of the parental lines of the Q DH population), and oilseed rape cultivars Cuillin and Marathon were sown in two row mini plots (1.2 m long, 22 cm wide) using a Hege drill. Field plots were inoculated by spreading infected oilseed rape crop debris from the previous cropping season and the plots received no fungicide treatment. Light leaf spot assessment was done on a 1-6 scale (Appendix 1). In addition, leaves were sampled on three different occasions, on 19 November 2015, 19 February 2016 and 04 April 2016, for DNA extraction and analysis using qPCR by randomly selecting 6-10 plants from Q DH lines and control cultivars. Leaves sampled on 04 April 2016 were incubated at 4 °C for 5 days and light leaf spot severity was assessed by visual estimation of the % leaf area covered with *P. brassicae* sporulation. Leaf samples collected at each time point were placed individually in 15 ml or 50 ml Falcon tubes and stored at -20 °C until use for DNA extraction. Frozen leaf samples were each freeze-dried and ground separately with a mortar and pestle to a fine powder. A sub-sample of 20 mg was taken from each ground leaf sample and DNA extraction of individual samples was done. Samples were adjusted to a final DNA concentration of 20 ng/µl. The amount of *P. brassicae* DNA in 50 ng of total extracted DNA was measured by quantitative PCR using *P. brassicae* diagnostic primers.

3.2.2. Phenotyping of resistance against *P. brassicae* in the Q DH population in controlled environment/glasshouse experiments

- *Controlled environment experiment described in this section was done by Katherine Cools (née Downs) at Rothamsted Research, Harpenden, UK.*

Controlled environment experiment

This experiment included 89 lines from the Q DH population and oilseed rape cultivar Canberra (AHDB RL rating (2007/08) 7, resistant) was included as a control. The conidial suspension (10^4 conidia/ml) was prepared from diseased oilseed rape leaves collected from oilseed rape field experiments at Rothamsted, Harpenden. Plants were grown in 7 cm diameter pots and maintained in a glasshouse at 20 °C for three weeks. Plants were spray-inoculated using an aerosol sprayer (Chrom Atomiser, Camlab; Cambridge, UK) until drops ran off the leaves. Inoculated plants were maintained in a controlled environment (CE) cabinet (RES simple cabinet) at 16 °C with 12 h photoperiod (light intensity - 190 E/m^2s^1) and 80% relative humidity. Plants were individually covered with polyethylene bags (26 cm x 38 cm) for 48 h after inoculation to maintain high humidity. Light leaf spot severity on each plant was assessed at 23 days post inoculation by visually estimating the percentage areas of third and fourth leaves covered with *P. brassicae* acervuli.

Glasshouse experiment

This experiment included 84 lines from the Q DH population, two of parental lines (*B. rapa oleifera* '29' & cv. Tapidor), cvs Imola (with a characteristic black flecking resistance phenotype against *P. brassicae* infection), Cuillin (RL resistance rating 7), Marathon (RL resistance rating 4) and Bristol (RL resistance rating 2). Pathogen inoculum (10^5 conidia/ml) was prepared from leaves with light leaf spot collected from an oilseed rape field trial at Morley (Norfolk) in the 2015/2016 cropping season. The glasshouse experiment was arranged in an alpha design generated using an alpha design generator from the Indian Agricultural Statistics Research Institute (IASRI) (Parsad *et al.* 2007). All the lines/cultivars were divided into four batches and assessed in a series of four experiments over a 5-month period. Each batch consisted of 27 cultivars/lines, with control cultivars and some Q DH lines repeated in each of the four experiments. In each experiment, plants were grown in a temperature-regulated glasshouse and spray-inoculated with *P. brassicae* conidial suspensions at growth stage 1,4-1,5. Plants were covered with a polyethylene cover for 48 h after inoculation to maintain 100% humidity to facilitate spore germination and infection. Glasshouse conditions were set up at a 12 h daylength and 16 °C/14 °C day/night temperatures, respectively.

In the glasshouse experiment, plants were harvested at 24 days post inoculation and placed individually in polyethylene bags and incubated at 4 °C for 5 days to induce sporulation. Disease assessment was done using a 1-6 scale (Appendix 1) and visually estimating the percentage leaf area covered with *P. brassicae* acervuli. Presence of a necrotic response and the number of deformed leaves (leaf curling, leaf distortions, etc.) were also recorded. The fourth true leaf from each plant was removed and placed in a 50 ml Falcon tube and stored at -20 °C for qPCR analysis. Frozen samples were freeze-dried, ground to a fine powder and DNA extraction was done using the DNAMITE plant kit following the manufacturer's protocol. DNA samples were diluted to 20ng/μl and the amount of *P. brassicae* DNA was quantified using qPCR.

3.2.3. Genetic mapping of the resistance QTL segregating in the Q DH population

Phenotyping data for the Q DH lines were obtained from a winter oilseed rape field experiment, controlled environment experiment and the glasshouse experiments. Mean % leaf area covered with *P. brassicae* sporulation (arcsine-transformed) and qPCR (log_{10}-transformed) data were treated as quantitative phenotype data in the QTL analysis. Qualitative phenotype data for the presence/absence of a necrotic response were treated as a single locus for the purpose of mapping by linkage analysis. A published genetic linkage map for the Q DH population, which comprised of 357 SSR markers over 19 linkage groups with a total genetic distance of 1,381 (Smooker *et al.* 2011) was used for the QTL analysis.

QTL mapping within individual experiments was implemented with QTL cartographer version 2.5 (Wang *et al.* 2012). Prior to advanced QTL detection methods, single-marker analysis was done to

screen the whole genome for marker-trait associations to identify possible QTL. The results obtained from single marker analysis were further refined by doing interval mapping (IM) followed by composite interval mapping (CIM). The QTL threshold was determined by permutation analysis using 1000 iterations at a genome wide significance level of 0.1 and all the QTL exceeding the LOD threshold were recorded. A chromosome walking speed of 1 cM was used for both IM and CIM analysis. The support interval for each of the QTL was determined based on the decrease in LOD 1.5 on either side of the LOD maximum, as suggested by Silva *et al.* (2012). Qualitative phenotype data (black necrotic flecking) were treated as molecular marker data and analysed using MSTmap on-line software (Wu *et al.* 2008). QTL detected by the CIM model were visualised on the linkage map of the Q DH population using MapChart (version 2.32) software (Voorrips 2002) with manual editing.

3.3. Fine mapping of a major gene locus for resistance against *P. brassicae* and identification of candidate resistance genes

3.3.1. Physical localisation of the closest flanking marker (Na14F11) of the resistance locus on the bottom of chromosome A1 (chrA1)

DNA samples from the two parental lines of the N26 DH population; cv. Imola (resistant) and line 218-11 (susceptible), were PCR amplified using primers of the microsatellite (SSR) marker, Na14F11 (Lowe *et al*. 2004). Once the expected DNA band had been identified, PCR reactions were prepared for a total volume of 100 µl for DNA samples from Imola and line 218-11, scaling up volumes of each component of the PCR reaction and the same cycling parameters were used. PCR products of the bulked-up PCR reaction were run on a 2% agarose gel with preparative wells loaded with 100 µl sample volumes. Two separate gels were prepared for two samples of DNA. After staining with EtBr, the gel was placed on a UV table. The gel was exposed to UV light for 45 sec during which the agarose blocks containing the required DNA band were cut out using a scalpel. DNA was isolated from excised gel blocks using a gel extraction kit (MinElute gel extraction kit, QIAGEN Ltd., UK) followed by cloning into a vector using pGEM-T easy vector system (Promega Corporation, USA). Six recombinant bacterial colonies were selected for each of the transformation reactions and plasmid DNA was extracted from bacterial cell pellets using the QIAprep Spin Miniprep Kit (QIAGEN UK Ltd.). The manufacturer's protocols were followed throughout the procedure. Extracted plasmid DNA was restriction digested with NotI and 7 µl of the reaction mixture with 3 µl of gel loading dye was run on a 2% agarose gel and visualized under UV light to check for the presence of the correct insert. Once confirmed, selected recombinant DNA samples (six samples for each of the two parental lines) were sequenced using Sanger sequencing technology (GATC-Biotech, Germany).

Na14F11 marker sequence was aligned to the *B. napus* Darmor bzh genome (Chalhoub *et al.* 2014) using BLAT (BLAST-like alignment tool) available in GENOSCOPE – *Brassica napus* genome

browser (thttp://www.genoscope.cns.fr/blat-server/cgi-bin/colza/webBlat). Additionally, BLAST search of EnsemblPlants genome archive (http://plants.ensemble.org/index.html) was done with the marker sequence to identify sequence homology in the published *B. rapa* genome. Nucleotide sequences of *B. napus* chrA1 and *B. rapa* chrA1 were obtained and sequence output files were imported into Geneious R9.1.8 (Biomatters Limited, New Zealand; Kearse *et al*. 2012). Synteny of the corresponding chromosomal region between the *B. napus* and *B. rapa* chrA1 was analysed using Mauve alignment (multiple genome alignment) (Darling *et al.* 2010) in Geneious R9.1.8 to estimate the physical location of the marker locus. Information on homologous gene sets among *B. rapa*, *B. oleracea*, A and C subgenomes of *B. napus* and their putative orthologs in *A. thaliana* (available in Chalhoub *et al*. (2014) supplementary Table s19) was also used as a guide.

3.3.2. Fine mapping of the major gene locus for resistance against *P. brassicae*

Genome-wide SNP data for *B. napus* (Schmutzer *et al*. 2015) was retrieved from the e!DAL-electronic data archive library (https://doi.ipk-gatersleben.de/DOI/61c4eb77-1d00-48ff-8f1c-37cf9ddcc58a/b46e79d7-80f0-460a-b3b8-2e0429f25a18/2) and used as a source for the identification of marker polymorphism in this study. The corresponding genomic region on chrA1 was selected using genome coordinates of the flanking marker locus identified in section 3.3.1 and the telomere. Information on SNPs present in this genomic region was extracted from files retrieved from e!DAL-electronic data archive library.

A custom filtering pipeline was developed using Galaxy web platform (https://usegalaxy.org/) to identify uniquely mapping SNP loci in the corresponding chromosomal region. This was commenced by creating new genomic coordinates for each identified SNP by adding 100 bp upstream and downstream of each SNP locus to obtain a 200 bp flanking region. *B. napus* Darmor-*bzh* genome sequence was imported into Galaxy and a 200 bp flanking sequence for each SNP was extracted from the genome sequence using a Galaxy workflow. In order to identify SNP loci with unique flanking regions on *B. napus* chrA1 by discriminating homologous regions mainly between *B. napus* A and C genomes and in other genomic regions, the list of 200 bp flanking sequences was batch-BLAST against the *B. napus* genome sequence. Batch-BLAST output for each 200 bp sequence was analysed and SNP loci that give highly homologous matches in other genomic regions were filtered out. The remaining SNP loci were visualised in Geneious R9.1.8, again checked manually and selected for their specificity on chrA1 to be considered as putative SNPs. Only SNPs with fairly uniquely mapped loci were retained. From the list of putative SNPs, 56 SNPs were selected for the development of kompetitive allele specific PCR (KASP™) markers. Flanking sequences of the selected SNPs (100 bp flanking sequence for each SNP) were used to develop KASP primer sets using proprietary Kraken™ software system (LGC Genomics, Hoddesdon, UK) and KASP markers were obtained.

Initially, the two parental lines of the N26 DH population were genotyped with a sub-set of KASP markers. This was done in a Mx3005 qPCR system (Agilent Technologies, UK) in 96-well format following protocols for the preparation and running of KASP reactions, and PCR conditions given in the KASP manual (http://www.kbioscience.co.uk/). Assays were set up as 10 µl reaction systems (wet DNA method) containing 5 µl low rox KASP master mix (LGC Genomics, Hoddesdon, UK), 0.14 µl of primer (KASP assay) mix and 5 µL of 5 ng/µl genomic DNA. PCR cycling conditions for KASP marker assay were set up as 94°C for 15 min, followed by 10 cycles of touch-down PCR from 61°C to 55°C with 0.6°C decrease per cycle, then followed by 26 cycles of 94°C for 20 s and 55°C for 1 min. Three additional cycles of 94°C for 20 s and 57°C for 1 min were done to obtain clear allele discrimination between the samples. End-point fluorescent readings were obtained using the Mx3005 qPCR system.

KASP genotyping assays for the N26 DH population (267 DH lines and the two parental lines) was done at John Innes Centre (Norwich, UK) in two batches of 14 and 32 markers. Genotyping data from the first batch of markers subjected to preliminary linkage mapping (section 5.2.2.6) to identify markers linked to the resistance locus and the second batch of markers were selected from the new genomic interval flanking resistance locus. All the markers were tested in 384-well format and set up as 5 µl reaction systems (dry DNA method) containing 1.8 µl of 5 ng/µl DNA (dispensed into PCR plates and dried in an oven at 65°C for about 30 min), 2.4 µl of KASP master mix and 0.07 µl of primer mix. Thermal cycling was done using the following protocol: hot start at 95 °C for 15 min, followed by ten touchdown cycles from 63°C to 57°C with 0.6°C decrease per cycle, then followed by 35 cycles of 94°C for 20 s and 57°C for 1 min. Fluorescence signals were visualised in a BMG PHERAstar plate reader (BMG LABTECH, Offenburg, Germany) to identify KASP marker polymorphisms.

KASP assay data were clustered and visualised to identify allelic variations in each of the DNA samples at each SNP locus. For each KASP marker, scanned image calls of sample wells were checked manually to identify if any errors had occurred during clustering analysis. Polymorphic markers were scored as ‘a’ for maternal (Imola) allele and ‘b’ for paternal (Line 218-11) allele. Data that were ambiguous (non-parental genotypes) or missing were denoted as ‘u’. The marker genotyping method and the DH lines were validated using a bi-filtering analysis of monomorphic marker data following the method described by Cai *et al.* (2015). Polymorphic markers were used for linkage mapping after excluding the DH lines with a high percentage of non-parental genotypes. Mapping data for the linkage group A1 (chrA1) containing simple sequence repeat (SSR) markers and the phenotype (scored as a qualitative trait and mapped as a single locus) were retrieved from Boys (2009) and combined with the KASP marker genotyping data generated in the present study. Linkage analysis was done using the MSTMap online software (Wu *et al,* 2008, available at http://www.mstmap.org) with maximum distance between markers at 15.0 cM and single LG as the grouping LOD criteria. The Kosambi mapping function was used to estimate map distances.

3.3.3. Identification of candidate resistance genes

Genomic coordinates of the flanking markers, Na14F11 (determined by sequencing of the PCR product amplified from primers for Na14F11) and SNP marker locus, were used to locate the corresponding chromosomal region on the physical map of *B. napus* chrA1. Gene content of this region in the published *B. napus* genome sequence (Chalhoub *et al.* 2014) was analysed with additional information from the genome sequence of *B. napus* cv. DH12075 (unpublished) (provided by Dr. Isobel Parkin, AAFC Saskatoon). *Brassica* pan transcriptome data (He *et al.* 2015, available at http://yorknowledgebase.info) were also used to supplement the gene content due to the presence of unannotated regions of chrA1 in the published *B. napus* genome.

3.4. Statistical analysis of data

Statistical analysis was done with Genstat® (Payne *et al.* 2008) and graphs were plotted using Microsoft® Excel (2010). Analysis of variance (ANOVA) was used to test the effects of cultivar/line and isolate and of their interaction on the different traits measured. Light leaf spot severity (% leaf area covered with *P. brassicae* sporulation) and % leaves deformed were transformed taking the arcsine of the square root of the proportion value. If the *F*-test showed significant effects of any factor, the standard error of the difference (SED) and the least significant difference (LSD) were calculated and presented at a probability level of 5% (i.e. $P \leq 0.05$). The relationships between different measures were analysed using simple linear regression. Differences between different cultivar/lines or different isolates were analysed using comparative analyses of position and parallelism of regression lines.

4. Results

4.1. Specific host-pathogen interactions in the *Brassica napus-Pyrenopeziza brassicae* pathosystem

4.1.1. Investigation of the phenotype/s of resistance

There was no rapid cell death/HR (hypersensitive response) after *P. brassicae* infection. The first visible sign of infection of plants was the leaf deformations that were observed as early as 7 dpi. In the first experiment, the black flecking phenotype was observed on cv. Imola and on the DH lines Q83 and Q88 at 10-14 dpi. Cultivar Imola and the two Q DH lines produced black necrotic flecking mainly along the petioles and leaf veins but also on the leaf lamina. The black flecking on cv. Imola appeared to be different from that on the Q DH lines; cv. Imola produced more intense flecking compared to the DH lines that had less intense brown coloration along the petioles. *P. brassicae* asexual sporulation (acervuli) was first observed at 16-18 dpi and occasionally there were pale-green to yellowish or grey coloured patches observed on the leaf lamina. However, acervuli were mostly observed with no lesions associated with them and appeared in concentric ring-like patterns. The

black flecking phenotype on cv. Imola and the two Q DH lines was often associated with little or no *P. brassicae* sporulation.

In the first experiment, light leaf spot assessment done using the 1-6 scale and by estimating the % leaf area covered with acervuli indicated that there were significant differences ($P \leq 0.05$) between cultivars/lines, between isolates and significant cultivar/line-isolate interactions (Figure 2). There was a significant positive correlation between the two light leaf spot severity assessment criteria (sample correlation coefficient = 0.95, $P < 0.01$, n = 14). The relationships between the % leaves deformed and the light leaf spot severity (light leaf spot score on the 1-6 scale and the % leaf area with *P. brassicae* acervuli) (data obtained from experiments 1 and 3) were analysed. According to the results from the first experiment, there was a positive correlation between the % leaves deformed and light leaf spot severity. However, analysis of data obtained from the third experiment showed no significant correlation between % leaves deformed and light leaf spot severity for any of the isolates tested. This suggests that incorporation of leaf/plant deformation into light leaf spot assessment criteria may not improve the selection for cultivar resistance. However, leaf/plant deformations are the initial signs of infection/disease and therefore, these need to be taken into consideration.

4.1.2. Investigation of the effect of resistance on spread of *P. brassicae* to upper leaves through growth of meristematic tissues

In the second experiment, leaf deformations started to appear at 7 dpi and became severe as the disease progressed. Considering the leaf deformations, younger leaves appeared to be more affected than the older leaves. There was a negative effect on the overall growth of all the plants in terms of the formation of new leaves, stem extension and flowering. At 28 dpi, no flowering was observed in the DH lines Q62, Q70 or Q79 that are known to have early flowering times. Of the five oilseed rape cultivars/lines included in this experiment, cv. Imola and the DH line Q70 (known to give a black necrotic phenotype against *P. brassicae* infections) showed necrotic flecking mainly along the leaf veins, midribs and along the petioles. However, the intensity of the black flecking phenotype was less than that had been observed in other experiments, especially for cv. Imola. Cultivar Imola developed a considerable number of P. *brassicae* acervuli on the leaf lamina, for the first time (Figure 3). For each line/cultivar, there were significant differences in the % leaf area with *P. brassicae* acervuli between different leaves (Figure 4). The amount of *P. brassicae* DNA showed a gradual decrease from the lower canopy leaves to the uppermost leaves.

4.1.3. Identification of differential interactions between *B. napus* cultivars/lines and single-spore isolates of *P. brassicae*

There was a significant positive correlation between the two light leaf spot severity assessment criteria (sample correlation coefficient = 0.98, $P < 0.01$, n = 79). Results obtained from these two

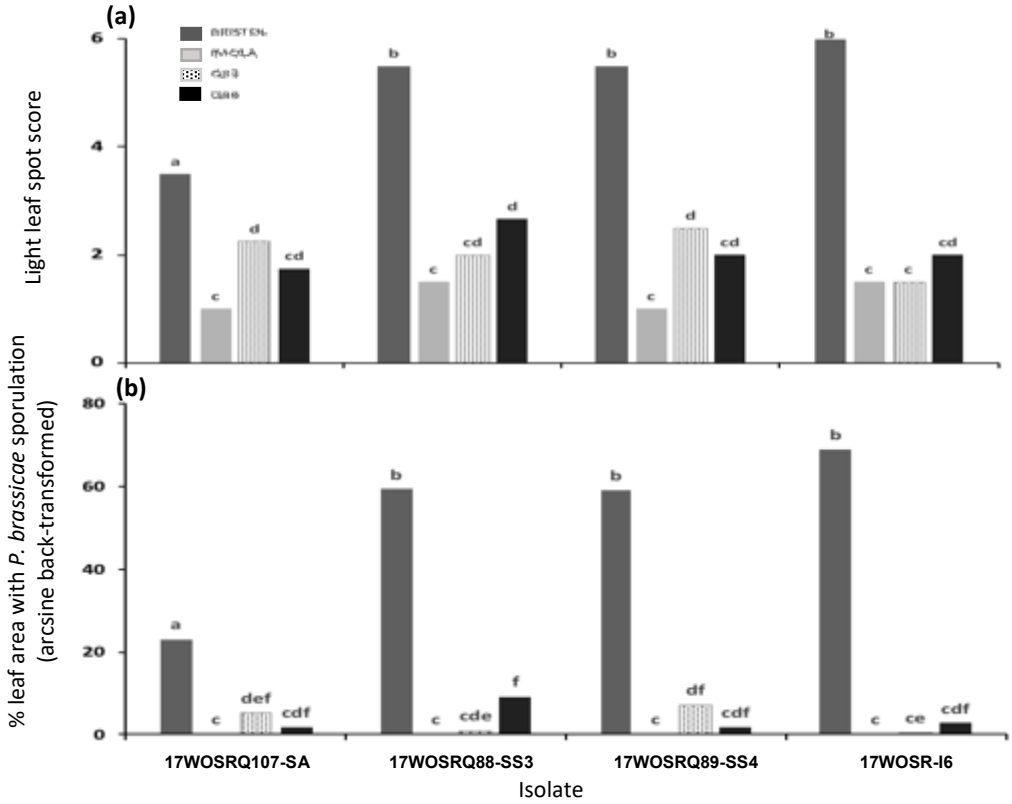

Figure 2: Comparison of resistance against *P. brassicae* in oilseed rape cultivar Imola and Q DH lines with a black flecking phenotype

Experiment 1: In a controlled environment experiment, oilseed rape cv. Imola, two Q DH lines (Q83, Q88, known to produce a black flecking phenotype as a resistance response against *P. brassicae*) and cv. Bristol (susceptible control) were spray-inoculated with single spore isolates of *P. brassicae*. (a) light leaf spot severity measured using a 1-6 scale (1 is resistant) (see Appendix 1 for light leaf spot assessment key). (b) % leaf area with *P. brassicae* asexual sporulation. Bars labelled with the same letter do not differ at a significance level of $P \leq 0.05$ (LSD).

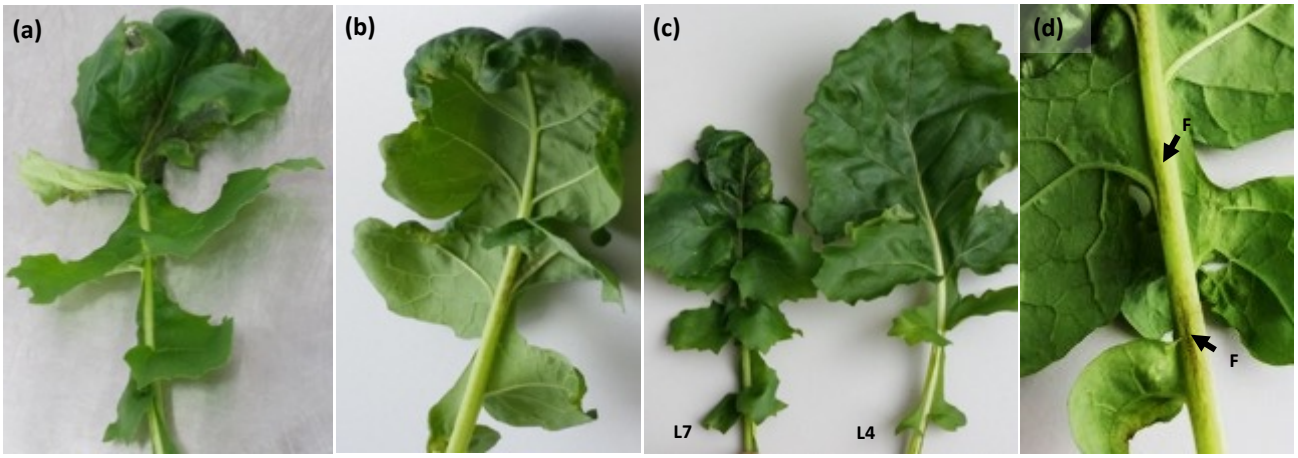

Figure 3: Light leaf spot symptoms on different oilseed rape cultivars/lines inoculated on the meristem with a *P. brassicae* population

Experiment 2: Severe leaf deformations were observed, starting from 7 dpi, in all the lines/cultivar regardless of their level of resistance against *P. brassicae*. (a) & (b) show deformed leaves of the DH line Q62 (susceptible to *P. brassicae*) and cv. Imola (resistant against *P. brassicae*), respectively. Considering the leaf deformations, younger leaves appeared to be more affected than the older leaves. (c) Deformations on a younger leaf (L7) and an older leaf (L4). Cultivar Imola and the DH line Q70 (known to produce a black necrotic phenotype against *P. brassicae* infection) showed necrotic flecking mainly along the leaf veins, midribs and along the petioles, which appeared less intense than that seen in a typical resistance response (d).

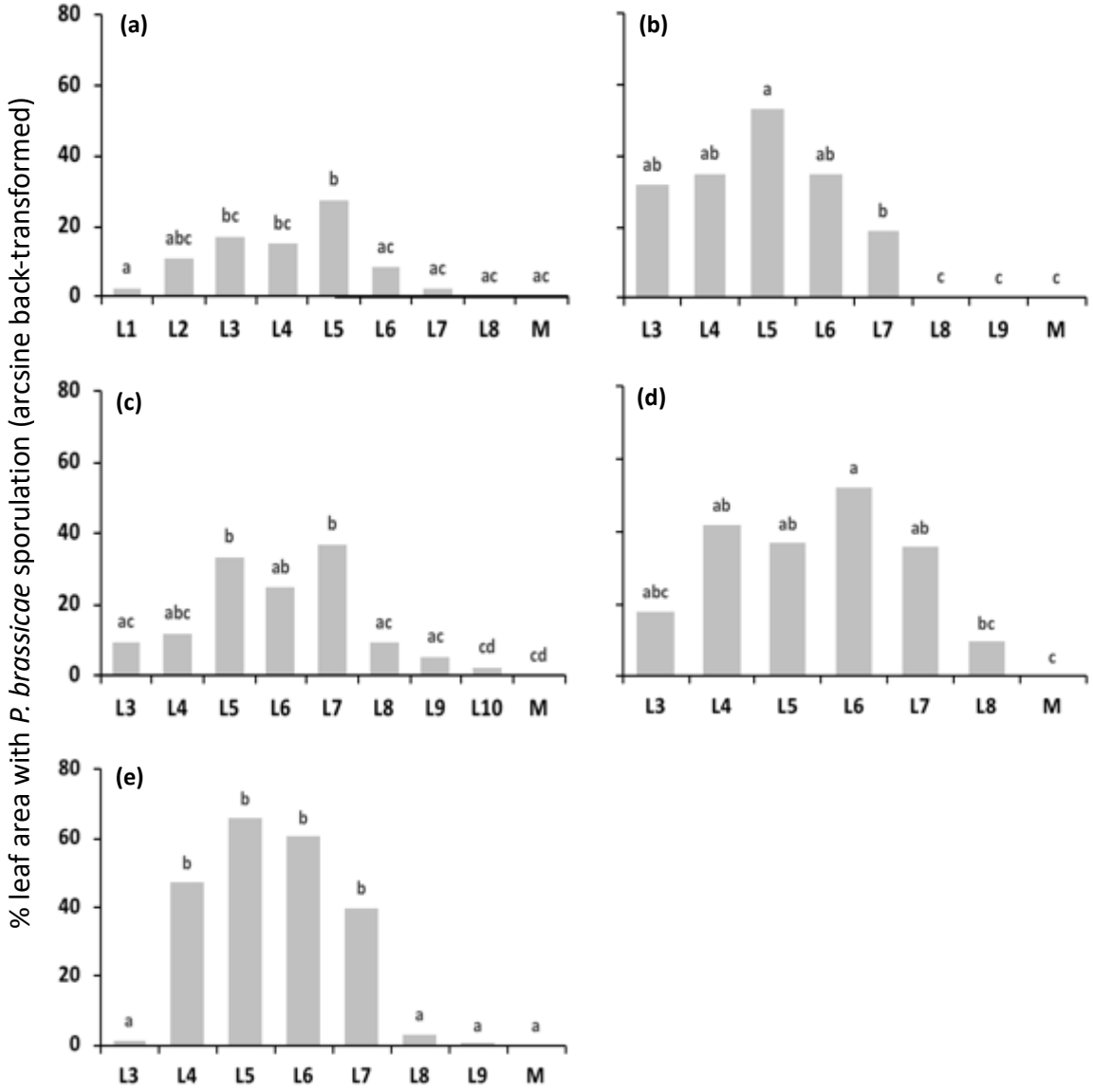

Figure 4: Effect of resistance on light leaf spot severity on different leaves (L1-L10) and the shoot tips (M)

Experiment 2. In a controlled environment experiment, five oilseed rape cultivar/lines were spray-inoculated (particularly on the shoot tips) at growth stage 1,3-1,4. Disease assessment was done at 28 dpi by estimating % leaf area covered with *P. brassicae* asexual sporulation in individual leaves. Graphs show the amount of disease on (a) cv. Imola, (b) Q62, (c) Q70, (d) cv. Tapidor and (e) Q79. Cultivar Imola and Q70, which produced a black flecking phenotype as the resistance response, appeared to have less sporulation on all the leaves compared to the susceptible cv. Tapidor and Q79. Bars labelled with the same letter do not differ at a significance level of $P \leq 0.05$ (LSD) within each graph. Different cultivars/lines appeared to have slightly different growth rates indicated by different numbers of leaves included in the analysis.

Table 5: Light leaf spot severity on different oilseed rape cultivars or lines scored on a 1-6 scale

Experiment 3: In a glasshouse experiment, several commercial oilseed rape cultivars, cv. Imola and two DH lines were spray-inoculated with different *P. brassicae* isolates. At 24 dpi, light leaf spot assessment was done using a 1-6 scale where 1 is most resistant (Appendix 1).

Cultivar/ Line	Light leaf spot score*								Cultivar mean‡
	Isolate	15WOSR 64-SS1	15WOSR 81-SS1	17WOSR -I1	15WOSR 76-SS2	15WOSR 78-SS1	17WOSR -I4	15WOSR 5.2-SS2	17WOSR -CUI	
Bristol		3.0	2.3	3.7	1.5	5.0	4.7	5.5	6.0	3.9[a]
Cracker		2.3	1.0	2.7	3.3	4.0	5.0	5.3	4.7	3.5[b]
Cuillin		2.3	1.5	2.0	2.7	3.3	4.0	4.0	4.7	3.1[c]
Excel		3.0	3.0	4.5	4.0	6.0	5.7	5.0	6.0	4.7[d]
Marathon		3.0	1.8	4.0	2.0	4.0	3.7	4.0	5.5	3.5[ab]
Temple		2.8	1.5	2.0	2.5	4.5	5.5	4.5	5.0	3.5[a]
Trinity		2.5	1.7	3.7	3.0	3.5	5.7	4.3	4.3	3.6[a]
Q69		2.8	2.3	2.5	1.5	2.5	2.0	1.7	2.7	2.3[e]
Q83		2.0	2.7	2.5	2.7	3.0	3.7	2.7	2.7	2.8[f]
Imola		1.0	1.0	1.3	1.0	1.5	1.0	1.7	1.7	1.3[g]
Isolate mean†		2.4[a]	1.9[b]	2.9[c]	2.4[a]	3.7[d]	4.2[ef]	3.9[de]	4.4[f]	3.2

* Light leaf spot severity score for different isolates. For comparison of the means between the cultivar-isolate combinations, the least significant difference (LSD) at 5% significance level was 1.23.
† Values followed by the same letter do not differ at a significance level of $P \leq 0.05$ (LSD=0.37).
‡ Values followed by the same letter do not differ at a significance level of $P \leq 0.05$ (LSD=0.44).

methods indicated that there were significant differences ($P \leq 0.05$) between cultivars/lines, between isolates and significant cultivar/line-isolate interactions (Table 5). Cultivar Imola showed the greatest level of resistance against *P. brassicae* (with no *P. brassicae* sporulation or < 1% of leaf area covered with acervuli and average light leaf spot score ranging from 1.0 to 1.7) for all the isolates tested. The two Q DH lines, Q83 (average leaf area covered with acervuli ranged from 2% - 14.7% and average light leaf spot score ranged from 2.0 to 3.7) and Q69 (average leaf area covered with acervuli ranged from 0.1% - 7.6% and average light leaf spot score ranged from 1.5 to 2.8) showed greater resistance against *P. brassicae* than the commercial oilseed rape cultivars included in this experiment. Cultivar Excel showed the greatest susceptibility to *P. brassicae* (average leaf area covered with acervuli ranged from 10.2% to 76.4% and average light leaf spot score ranged from 3.0 to 6.0) for all the isolates. Considering the different isolates, resistance against isolate 15WOSR81-SS1 was the most common among different lines/cultivars. Resistance against the isolates 15WOSR64-SS1, 17WOSR-I1 and 15WOSR76-SS2 was also observed to be common among most of the lines/cultivars. *P. brassicae* isolates 15WOSR5.2-SS2 and 17WOSR-CUI caused severe disease on all the commercial oilseed rape cultivars, but less disease on cv. Imola, Q83 and Q69. In addition, a range of symptoms was observed in these lines/cultivars inoculated with single spore isolates of P. *brassicae* (Figure 5). Cultivar Imola and the DH line Q83 showed a black flecking phenotype against all the isolates and cvs Bristol, Trinity and the DH line Q69 showed a black flecking against at least one of the isolates.

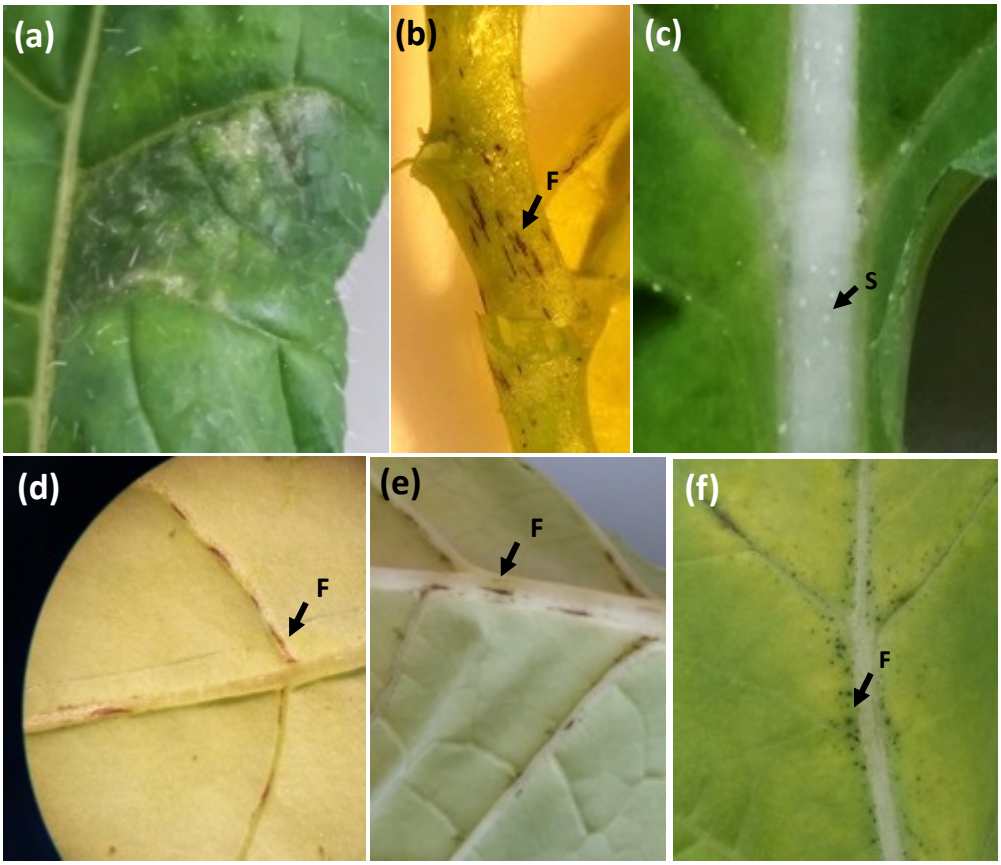

Figure 5: Range of light leaf spot symptoms observed on different oilseed rape cultivars/lines inoculated with single spore isolates of *P. brassicae*

A range of symptoms was observed in different oilseed rape cvs/lines inoculated with single spore isolates of *P. brassicae*. The amount of asexual sporulation (acervuli) (S) observed on different cultivars ranged from no sporulation to *c*. 76% leaf area covered with sporulation. In less susceptible cultivars/lines, sporulation was observed mostly along the leaf veins. (a) leaf lesion on the DH line Q83. (b) Black flecking (F) observed on the stem of the DH line Q69. (c) *P. brassicae* acervuli on the mid rib of cv. Cuillin. (d) Black flecking observed on cv. Bristol. (e) Black flecking observed on cv. Imola. (f) Black flecking observed on cv. Trinity.

4.2. Phenotypic analysis of the Q doubled haploid population for resistance against *Pyrenopeziza brassicae* and mapping of resistance QTL

4.2.1. Phenotyping of resistance against *P. brassicae* in the Q DH population

Of the 62 Q DH lines included in the field experiment, 33 lines were scored for light leaf spot in the final assessment. The Q DH population also segregates for flowering time and vernalisation, which caused the loss of some lines during the winter, and some lines had poor seed germination. Observation of light leaf symptoms on the Q DH population in April indicated that the population was segregating for resistance against *P. brassicae*. Based on the 1-6 scale (1 is most resistant), 17 out of 33 Q DH lines showed no *P. brassicae* acervuli and scored resistant (score 1) against *P. brassicae*. This agreed with a 1:1 ratio of resistant:susceptible lines ($X^2 = 0.27$, $P = 0.60$) (Figure 6). There were significant effects of line/cultivar ($P < 0.01$) on the light leaf spot severity, estimated by severity score as well as by the % leaf area with *P. brassicae* asexual sporulation (Figure 7 (a) and

(b)). Analysis of *P. brassicae* DNA in leaf samples collected in April 2016 showed significant differences between different lines/cultivars (Figure 7 (c)).

In the first controlled environment experiment, presence of *P. brassicae* asexual sporulation was taken as the key measure of susceptibility to *P. brassicae*. Of the 89 Q DH lines included in this experiment, data from a total of 85 Q DH lines and the resistant control cultivar Canberra were included in the data analysis. Visual estimation of the % leaf area covered with acervuli in the controlled environment also showed segregation for resistance against *P. brassicae* in the Q DH population (Figure 8). Average % leaf area covered with acervuli ranged from 0 to 46% and interestingly, the resistant control cultivar Canberra had an average of 20% leaf area covered with acervuli. Of the 85 Q DH lines, 17 lines had no *P. brassicae* acervuli production.

Four main parameters were used in the glasshouse experiment to assess the resistance/susceptibility to *P. brassicae* in the Q DH population: 1-6 scale of light leaf spot severity, visual estimation of the % leaf area covered with *P. brassicae* asexual sporulation (acervuli), presence of a necrotic response and estimation of the amount of *P. brassicae* DNA (pg) in 50 ng of total DNA. An estimation of the leaf deformations in each line/cultivar was also taken as a separate measure. Data analysis and the results presented for this work were for 77 Q DH lines out of 84 Q DH lines included in this experiment and the control cultivars (six cultivars) after removing seven lines with less than three replicates. Of the 77 Q DH lines included in the data analysis, 32 lines had light leaf spot scores of 1 or 2 and had no disease or slight traces of disease, respectively. According to visual estimates of % leaf area covered with acervuli, a total of 39 lines were recorded to have less than 10% leaf area covered with acervuli (Figure 9). Analysis of qPCR data showed a significant effect of line/cultivar on the amount of *P. brassicae* DNA in leaf samples.

4.2.2. Genetic mapping of the resistance QTL segregating in the Q DH population

Composite interval mapping (CIM) analysis for the two quantitative traits measured in three experiments (field, glasshouse and CE) suggested that there were 22 QTL distributed across 13 linkage groups (17 QTL identified with the light leaf spot severity data and five QTL identified with *P. brassicae* DNA data). Considering the resistance QTL identified with light leaf spot severity data, four QTL detected in the controlled environment experiment accounted for 60.5% of overall phenotypic variation (ranging from 10.5 to 18.6%). In contrast, seven QTL were identified in the glasshouse experiment and six QTL were identified in the winter oilseed rape field experiment with phenotypic variation explained ranging from 15.1 to 33.1% and 16.0 to 29.2%, respectively. For *P. brassicae* DNA data (only available for glasshouse and winter oilseed rape field experiments), there was one QTL identified in the glasshouse experiment (accounting for 26.4% of the variation) and four QTL identified in the winter oilseed rape

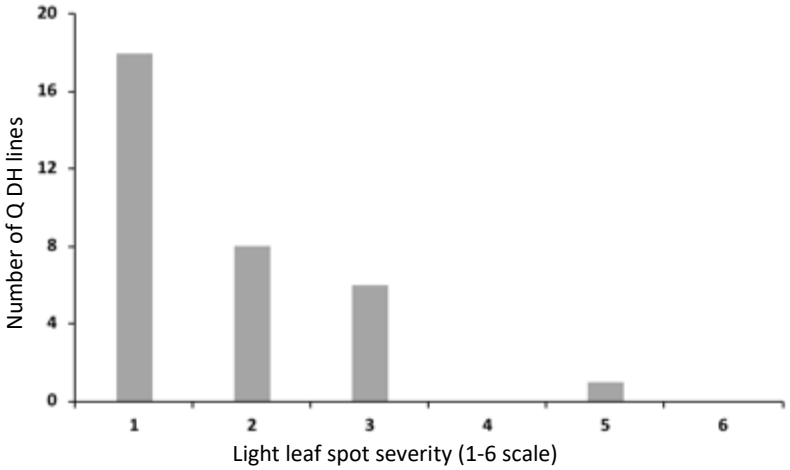

Figure 6: Frequency of Q doubled haploid lines in each category of a 1-6 light leaf spot severity scale in a winter oilseed rape field experiment

The frequency distribution of the Q DH lines (number of DH lines out of the Q DH population) that scored in each category of a 1-6 light leaf spot severity scale (Appendix 1) in a 2015/16 winter oilseed rape field experiment in Lincolnshire. A total of 33 Q DH lines were scored in the assessment.

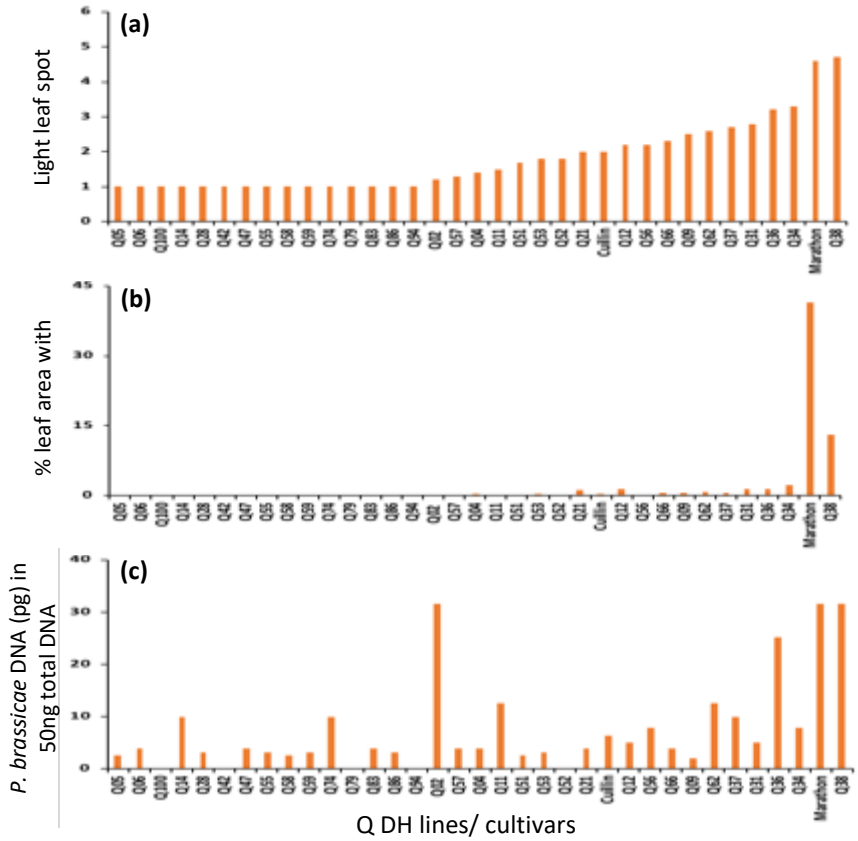

Figure 7: Light leaf spot severity and the amount of *P. brassicae* DNA in different Q DH lines and commercial cultivars grown in a winter oilseed rape field experiment

In the 2015/16 cropping season, a winter oilseed rape field experiment was established in Lincolnshire with a sub-set of the Q DH population and two commercial oilseed rape cultivars, Marathon and Cuillin as susceptible and resistant controls, respectively. Disease severity was measured using (a) a 1-6 scale (1 most resistant) (section 2.5.1); (b) percentage leaf area of different DH lines and cultivars covered with *P. brassicae* sporulation; (c) amount of *P. brassicae* DNA (pg) in leaves taken from different lines and cultivars. Graphs (a), (b) and (c) have been ranked from smallest to greatest, based on the light leaf spot score on the 1-6 scale.

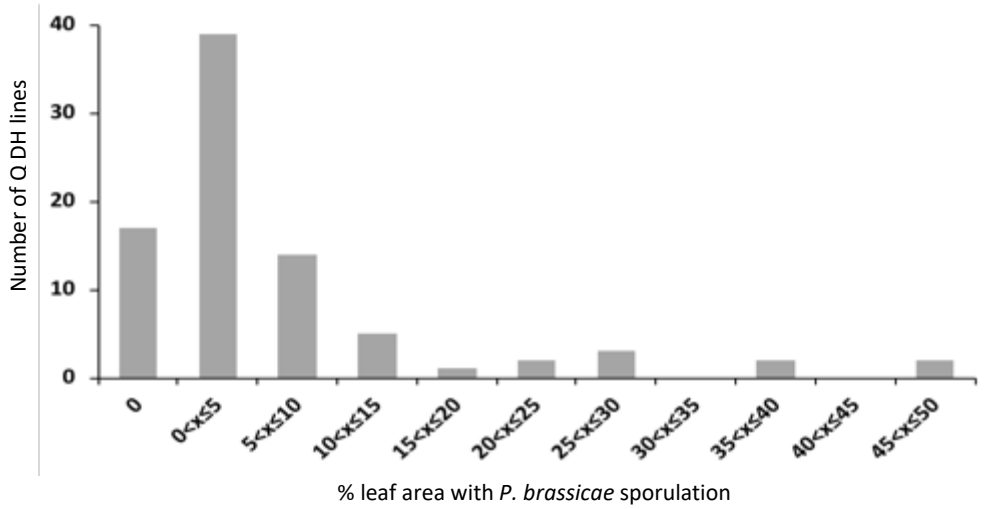

Figure 8: Frequency of Q doubled haploid lines with different amounts of *P. brassicae* sporulation in a controlled environment experiment

In a controlled environment experiment, 89 lines from the Q DH population and the resistant control cv. Canberra were inoculated with a *P. brassicae* population and disease severity was measured by visually estimating the % leaf area covered with *P. brassicae* sporulation. Of these lines, a total of 85 lines were included in the data analysis after excluding four lines with insufficient data. Of these lines, 17 lines were observed to have no *P. brassicae* sporulation. The remaining 72 lines were observed to have varying amounts of *P. brassicae* sporulation.

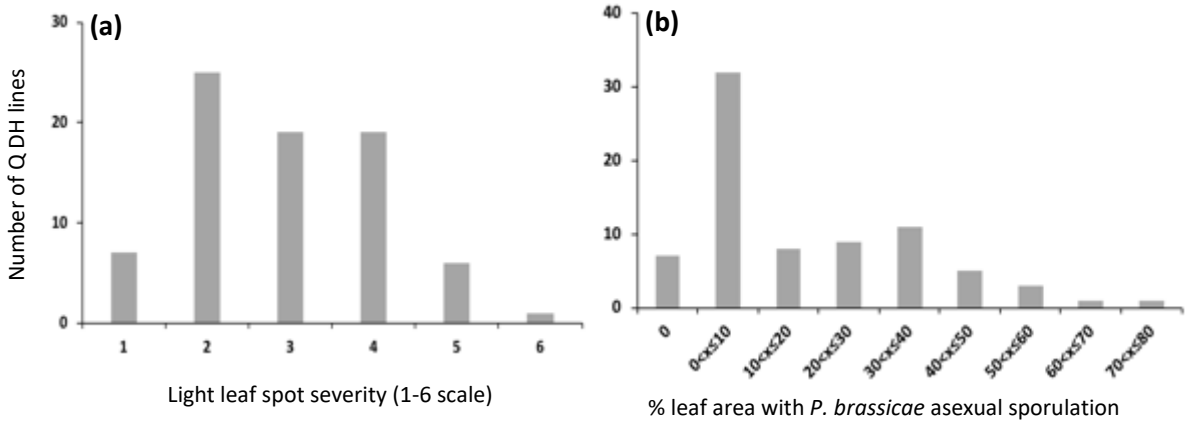

Figure 9: Frequency of Q doubled haploid lines with different light leaf spot severity scores and with different amounts of *P. brassicae* sporulation in a glasshouse experiment

DH lines (84 lines in total) from the Q DH population were phenotyped in a glasshouse experiment for the segregation of resistance against *P. brassicae* (77 Q DH lines were included in the final analysis after excluding seven lines with less than three replicates). (a) The number of Q DH lines in each category on the 1-6 severity scale (where 1 is most resistant) (section 2.5.1). Of these lines, 32 lines were scored as having no disease or traces of disease (light leaf spot scores 1 or 2, respectively). (b) The number of DH lines categorised into different groups based on the % leaf area covered with *P. brassicae* asexual sporulation. A total of 39 lines were recorded to have 10% or less leaf area covered with *P. brassicae* asexual sporulation.

field experiment (phenotypic variation explained ranging from 8.8 to 45.7%). Considering the overall distribution of QTL across different genomic regions and linkage groups, QTL that have been co-localised for the two traits and from different experiments were considered to be more reliable. Based on this, four QTL hotspots were identified in linkage groups C1, C3, C6 and C9. Relatively more QTL were detected in the CC genome than in the AA genome.

4.3. Fine mapping of a major gene locus for resistance against *P. brassicae* and identification of candidate resistance genes

4.3.1. Physical localisation of the closest flanking marker (Na14F11) of the resistance locus on the bottom of chromosome A1 (chrA1)

A DNA fragment of *c.* 250-300 bp corresponding to the Na14F11 marker locus was identified from parental lines of the N26 DH population, which fits with records from previous studies (Sing *et al*. 2011; Boys 2009). Multiple sequence alignment of cloned PCR products showed the polymorphism in repeat motifs, $(GT)_7$. Cloning sequences obtained from cv. Imola indicated heterozygosity in this marker locus. BLAT (BLAST-like alignment tool) search of SSR marker sequence homology in the *B. napus* genome has identified an unlocalised DNA fragment that has not been assembled into a corresponding reference chromosome. However, BLAST search against the *B. rapa* genome identified several sequence matches to the Na14F11 marker sequence and the genome coordinates at the bottom of *B. rapa* chrA01 were identified as the physical location of this marker. In *B. rapa*, the genomic region between the marker locus and the telomere spans *c.* 0.48 Mbp. Comparison of the gene content of *B. rapa* in this genomic region with published data on homologous gene sets among *B. oleracea*, A and C sub-genomes of *B. napus* and their putative orthologs in *A. thaliana* enabled the identification of gene synteny of the corresponding chromosomal regions between *B. rapa* and *B. napus* genomes. Comparison of *B. rapa* and *B. napus* chrA1 sequences also indicated a good collinearity between the two genomes in the chromosomal region containing the SSR marker, Na14F11. A block of genes that included the gene overlapping Na14F11 marker sequence in *B. rapa*, where their homologous genes in *B. napus* have been assigned to chromosomal fragments, was identified. Therefore, the first gene locus upstream to this gene block where the *B. napus* gene homolog has been assigned to chrA1 was considered as the closest proximity to the Na14F11 marker locus on *B. napus* chrA1 (Figure 10). The chromosomal region between this locus and the telomere spans *c.* 1.08 Mbp.

4.3.2. Fine mapping of the major gene locus for resistance against *P. brassicae*

Based on the *B. napus* SNP information published by Schmutzer *et al*. (2015), > 3000 SNP loci have been identified in the chromosomal region between the upstream flanking region of the resistance locus and the telomere of chrA1. There were 38 highly unique SNP loci, to which single blast hits against the *B. napus* chrA01 was found. However, there were some SNP loci where more than one blast hit against the *B. napus* genome occurred, yet the genomic region flanking the SNP loci was unique on chrA1. SNPs associated with highly homologous matches were filtered and the rest were selected as putative SNPs to be used in this study. This consisted of < 7% of the SNPs initially identified in this chromosomal region and appeared in small clusters distributed across the bottom of the chrA1 (Figure 11a). Considering the SNP distribution, a sub-set of 56 SNP were selected

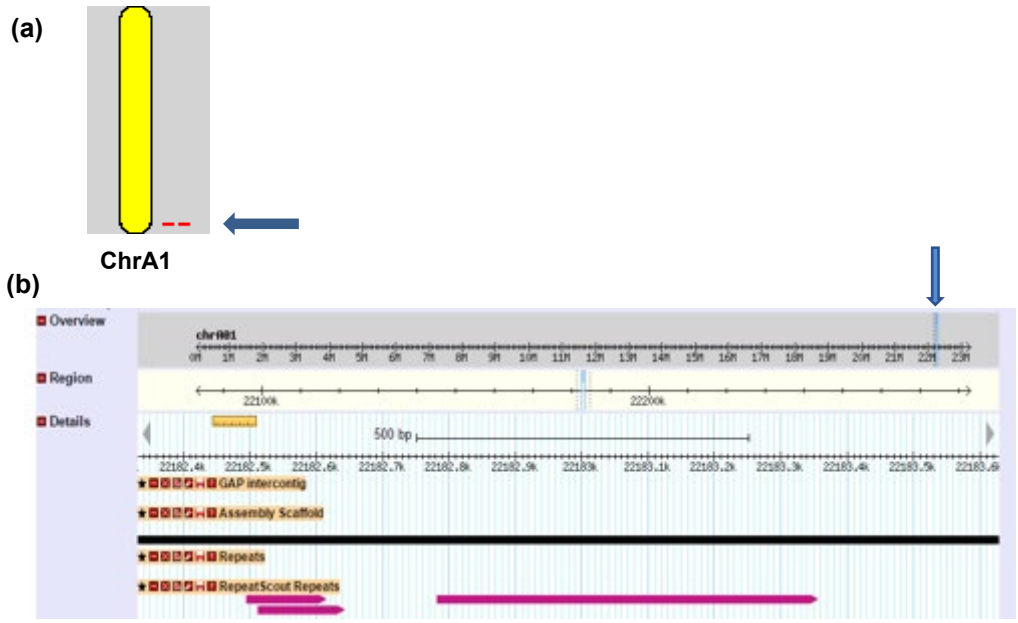

Figure 10: Physical location of the SSR marker Na14F11 on *B. napus* chrA1 and detailed view of the chromosomal region

(a) The *B. napus* gene, which has been recognised upstream to the Na14F11 locus, was used to identify the approximate physical location of Na14F11 on *B. napus* chrA1. (b) The chromosomal region between this flanking locus and the telomere spans *c.* 1.08 Mbp.

for the KASP marker development, providing maximum marker coverage on the corresponding chromosomal region (Figure 11b).

Of the 56 KASP markers developed, 46 were used for further genotyping analysis, which were tested in two batches of 14 and 32 markers, respectively, and the genotyping data were analysed together. Various genotyping calls resulted from the genotyping of the segregating N26 DH population (267 DH lines and the two parental lines). Of the 46 KASP markers used, 12 markers showed polymorphisms, which belonged to one of the three categories; both the parental lines containing opposite alleles, one parent with a heterozygous allele (other parent with a homozygous allele) and one parent with 'no call' genotype (other parent with either homozygous or heterozygous allele). Thirty-two markers were monomorphic between the parental lines and two markers failed in the KASP genotyping assay. Monomorphic markers included two categories in which both the parental lines were homozygous or heterozygous.

Monomorphic markers (homozygous) were subjected to bi-filtering analysis by including genotype data for these markers from all the DH lines in a two-dimensional matrix according to the method described by Cai *et al.* (2015). Percentage of non-parental genotypes (PNPG) quantified in the vertical direction ($PNPG_{DH}$) suggested the presence of a few unauthentic DH lines. Calculation of the percentage of non-parental genotypes in the horizontal direction ($PNPG_{SNP}$) showed differences between different SNP loci. PNPG for monomorphic SNP loci ranged from 0 to 0.15, with an average of 0.02. These values suggested that the SNP detection system (KASP marker analysis)

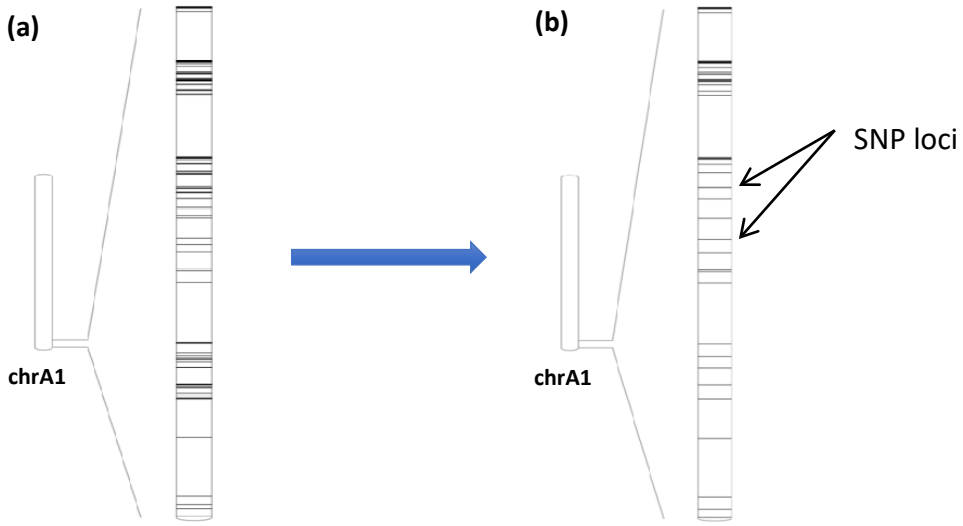

Figure 11: Single nucleotide polymorphisms (SNPs) specific to the *B. napus* chromosomal region flanking the locus for resistance against *P. brassicae* and the distribution of KASP markers developed

(a) Single nucleotide polymorphism (SNP) data for *B. napus* chrA1 were obtained from the e!DAL-electronic data archive library (https://doi.ipk-gatersleben.de/DOI/61c4eb77-1d00-48ff-8f1c-37cf9ddcc58a/b46e79d7-80f0-460a-b3b8-2e0429f25a18/2) (Schmutzer *et al*. 2015). SNPs that lack highly homologous flanking loci on the corresponding chromosomal region were identified using a custom developed bioinformatics workflow in Galaxy (https://usegalaxy.org/). (b) Distribution of a subset of SNPs selected for the development of KASP™ (Kompetitive allele specific PCR) markers in the corresponding chromosomal region. (c) Distribution of KASP™ markers used for the genotyping of the N26 DH population that segregate for a major gene locus for resistance against *P. brassicae*. Diagrams were produced in PhenoGram visualisation software (Wolfe *et al.* 2013) (http://visualization.ritchielab.org/phenograms/plot).

remained stable and was reliable. Of the 46 KASP markers used for the genotyping of the N26 DH population, 12 markers appeared to be polymorphic. Marker genotypes were scored as ‘a’ for maternal (resistant parent - Imola) allele, ‘b’ for paternal (susceptible parent - line 218-11) and non-parental genotypes were treated as missing data and labelled as ‘u’. Due to the large amount of missing data (ambiguous or non-parental genotypes), two of the markers were excluded from linkage mapping. For six markers, the segregation ratio of the two parental alleles (a:b) was significantly different from the expected 1 : 1 ratio (Table 6). Genotyping data included in the linkage mapping consisted of marker genotyping information retrieved from Boys (2009) (simple sequence repeat (SSR) markers and black flecking phenotype scored and mapped as qualitative phenotype controlled by single genomic locus) and 10 KASP markers developed in this study.

Linkage mapping analysis was done using MSTmap online software, with map distances estimated with Kosambi mapping function and with no mapping distance threshold of 15 cM. All the KASP markers developed in this study were observed to be in one cluster and the mapping order of those markers was mostly in agreement with the order expected from their positions on the physical map. Genotyping results for BnaA01_4X84, BnaA01_7X91, and BnaA01_4X44 markers were identical for all individuals analysed, so they were located at the same position in the genetic map. There seemed to be a change of the order of the two closest SSR markers, sN0810 and Na14F11, to the north of the resistance locus. Fine mapping has located the resistance locus, *PBR2*, between the markers

sN0810 and BnaA01_3X30. Compared to the genetic map constructed by Boys *et al.* (2012), total map length (104.8 cM vs 249.0 cM) was significantly improved with the genetic linkage map constructed in the present study. Sequence of the marker sN0810 (provided by Dr. Rachel Wells) did not yield sequence homology in *B. napus* chrA01. Therefore, the corresponding genomic region on the physical map was checked using the sequence of the next closest marker to the north, Na14F11, and the KASP marker BnaA01_3X30, which corresponded to c. 42 Kbp region based on the published *B. napus* Darmor-*bzh* genome sequence.

Table 6: Segregation ratios of polymorphic KASP markers in the N26 DH *B. napus* population

Marker ID	Parental genotype ratio (a:b)*	X^2 ±	*P* value
BnaA01snp_2X47	2.0	19.45	< 0.001
BnaA01snp_3X95	1.0	0.03	0.855
BnaA01snp_1X95	0.8	2.89	0.089
BnaA01snp_3X30	1.2	1.35	0.245
BnaA01snp_4X84	1.8	15.85	< 0.001
BnaA01snp_7X91	1.8	15.85	< 0.001
BnaA01snp_8X39	1.0	0.02	0.897
BnaA01snp_8X51	1.8	14.70	< 0.001
BnaA01snp_2X19	2.0	19.02	< 0.001
BnaA01snp_4X44	1.8	16.35	< 0.001

* Genotypes of DH lines were scored as ‘a’ for the allele from the resistant parent (cv. Imola) and ‘b’ for the allele from the susceptible parent (line 218-11).

± The allele ratio for each marker was tested against the 1:1 ratio with a chi-squared test and the *P* value is given.

4.3.3. Identification of candidate resistance genes

There were 10 genes predicted in the *B. napus* Darmor-*bzh* genome sequence (http://www.genoscope.cns.fr/brassicanapus/cgi-bin/gbrowse/colza/?name=chrA01) within the genomic region determined by fine mapping. However, according to the physical localisation of the resistance locus, there were telomeric sections of chrA1 that appeared to be present in unannotated random DNA/chromosomal fragments in the published genome. For instance, the gene overlapping to the Na14F11 marker locus was identified in a random chromosome fragment. Therefore, in addition to the genes predicted in the *B. napus* Darmor-*bzh* genome sequence, *Brassica* A and C pan-transcriptome data (He *et al.* 2015) were used to guide the identification of candidate resistance genes. Collinearity of the *B. napus* ZS11 genome sequence with *Brassica* A genome coordinates indicated the presence of 32 gene predictions within the genomic region determined by fine mapping. Considering the functional annotations of *Arabidopsis thaliana* orthologues of these genes, there may be six genes related to resistance against pathogens. These include genes harbouring both

leucine-rich repeat (LRR) and nucleotide binding site (NBS) domains. Four of the genes code for protein kinases.

5. Discussion

5.1. Specific host-pathogen interactions in the *Brassica napus-Pyrenopeziza brassicae* pathosystem

According to the observations made in glasshouse and controlled environment experiments, there were two main phenotypes of resistance, formation of black necrotic flecking and limitation of *P. brassicae* asexual sporulation (acervuli), which often appeared to be related. This observation supports the previous findings that proposed a resistance response against *P. brassicae* that was associated with black necrotic flecking in oilseed rape and limited growth of the pathogen (during the endophytic growth phase) and no asexual sporulation (Boys *et al.* 2012) or black flecking associated with limited asexual sporulation of the pathogen (Bradburne *et al.* 1999). There were differences between lines/cvs both in terms of *P. brassicae* asexual sporulation and in other symptoms associated with light leaf spot disease, such as leaf curling and leaf distortion (collectively called leaf deformations). Normally, the light leaf spot disease is measured as the percentage leaf area covered with *P. brassicae* asexual sporulation, mostly in controlled environment experiments and also in field experiments such as the Recommended List (RL) trials. *P. brassicae* asexual sporulation is an important measure of the disease severity as this contributes to the inoculum for polycyclic disease spread in winter oilseed rape crops. However, other symptoms related to plant growth are also important for the yield loss and, hence, they also need to be considered. There was a poor correlation between light leaf spot severity (measured as *P. brassicae* asexual sporulation or light leaf spot score) and the amounts of leaf deformation.

In the present study, analysis of the individual leaves and shoot tips indicated that the pathogen may be carried upwards by stem extension. However, there were differences between different cultivars/lines in the amounts of *P. brassicae* DNA detected in shoot tips, indicating that resistant hosts may be able to recover from the pathogen infection better than susceptible hosts. Moreover, single spore inoculum was able to differentiate between oilseed rape lines/cvs with different levels of resistance. This study included a limited number of lines/cvs, but with a range of resistance/susceptibility to *P. brassicae*. Interestingly, cv. Imola and the DH lines from the Q population (Q83 and Q69) showed better resistance against *P. brassicae* than any of the commercial cultivars tested. Large scale assessment of different oilseed rape lines/cvs would provide more evidence of differential interactions and would help to identify potential new sources of resistance.

5.2. Segregation for resistance against *Pyrenopeziza brassicae* in the Q doubled haploid (DH) population

Analysis of the Q mapping population DH lines using controlled environment, glasshouse and winter oilseed rape field experiments confirmed the segregation of resistance against *P. brassicae* in this population. There appeared to be differences between different lines in the amounts of *P. brassicae* asexual sporulation, which may or may not be associated with the presence of black necrotic flecking. Therefore, it can be assumed that there are two main phenotypes of resistance; black necrotic flecking and reduced *P. brassicae* asexual sporulation. The average sporulation observed in DH lines with the black flecking phenotype was significantly less than that of DH lines without black flecking. Molecular diagnostic methods such as quantitative PCR (qPCR) can be utilised for pathogen detection during the *P. brassicae* asymptomatic growth phase (Boys *et al.* 2007, 2012). Quantification of pathogen biomass over time can provide insights into potential mechanisms of resistance. For example, positive correlation between a small *P. brassicae* biomass and a small resistance rating in lines could indicate host resistance responses preventing pathogen colonisation. However, this was not the case for the disease severity (% leaf area with *P. brassicae* sporulation) in all the DH lines tested, suggesting that resistance lines may not prevent the pathogen colonisation completely and resistance might operate at the time of asexual sporulation. Similar observations have been reported in resistance against *R. commune* in barley (Thirugnanasambandam *et al.* 2011).

Composite interval mapping analysis identified 17 QTL across 10 chromosomes using data for the % leaf area covered with *P. brassicae* sporulation and five QTL across five chromosomes were identified with the *P. brassicae* DNA data. All the QTL identified appeared to have a moderate effect. Resistance QTL identified using the phenotype data from three different experiments suggested that some of the resistance QTL may be environmentally sensitive. However, there were some QTL hotspots where QTL from different experiments and/or traits were co-located. These include loci on chromosomes C1, C3, C6 and C9. Q DH lines with a good resistance against *P. brassicae* can be incorporated into oilseed rape breeding programmes to develop new cultivars.

5.3. Fine mapping of a major resistance locus against *P. brassicae* and identification of candidate resistance genes

The physical location of the flanking marker (Na14F11) to the north of the resistance locus (*PBR2*) was identified at the bottom of *B. napus* chrA1, which agrees with the description of the marker overlapping region by Boys (2009). Fine mapping of this resistance locus with KASP markers led to the identification of markers to the south of the resistance locus and narrowed down the corresponding chromosomal region from >1.2 Mbp to *c.* 42 Kbp in the published Darmor-*bzh* genome sequence. According to the experimental evidence provided, cv. Imola remained the most resistant

host when inoculated with single spore isolates or with populations of *P. brassicae*. Therefore, the development of diagnostic molecular markers for *PBR2* is very useful for oilseed rape breeders to use in breeding programmes. Nevertheless, the identification and cloning of *PBR2* is of great scientific interest. There appeared to be six candidate resistance genes in the corresponding chromosomal region determined by previous and newly added markers flanking the resistance locus. The list of candidate genes consisted of two nucleotide-binding site leucine rich repeat genes (NLRs) and four receptor-like kinase genes (RLKs). Since *P. brassicae* is an apoplastic (extracellular) pathogen, it can be assumed that the pathogen recognition occurs outside host cells and is mediated by membrane-located receptors (Stotz *et al.* 2014).

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7. Appendices

Appendix 1: Light leaf spot severity assessment scale

A 1-6 point disease severity scale (1 is most resistant) was used for the assessment of light leaf spot. This assessment scale was derived from the AHDB Cereals and Oilseeds recommended list trial light leaf spot assessment key (http://cereals.ahdb.org.uk/varieties/running-the-recommended-lists.aspx) and a 9-point assessment scale for *in situ* light leaf spot assessment described by Boys 2009.

Score	Disease severity
1	No disease
2	Traces of disease
3	Plants with few scattered lesions and/or less than 20% of leaf area affected
4	Plants with several scattered lesions and/or 20-40% of the leaf area affected
5	Plants with many small lesions/scattered large lesions and/or 40-60% of the leaf area affected
6	Severely diseased plants, more than 60% of the leaf area affected