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Effects of *R* gene-mediated resistance in *Brassica napus* (oilseed rape) on asexual and sexual sporulation of *Pyrenopeziza brassicae* (light leaf spot)

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The phenotype of the *R* gene-mediated resistance derived from oilseed rape (*Brassica napus*) cv. Imola against the light leaf spot plant pathogen, *Pyrenopeziza brassicae*, was characterized. Using a doubled haploid *B. napus* mapping population that segregated for resistance against *P. brassicae*, development of visual symptoms was characterized and symptomless growth was followed using quantitative PCR and scanning electron microscopy on leaves of resistant/susceptible lines inoculated with suspensions of *P. brassicae* conidia. Initially, in controlled-environment experiments, growth of *P. brassicae* was unaffected; then from 8 days post-inoculation (dpi) some epidermal cells collapsed ('black flecking') in green living tissue of cv. Imola and from 13 to 36 dpi there was no increase in the amount of *P. brassicae* DNA and no asexual sporulation (acervuli/pustules). By contrast, during this period there was a 300-fold increase in *P. brassicae* DNA and extensive asexual sporulation in leaves of the susceptible cv. Apex. However, when leaf tissue senesced, the amount of *P. brassicae* DNA increased rapidly in the resistant but not in the susceptible cultivar and sexual sporulation (apothecia) was abundant on senescent tissues of both. These results were consistent with observations from both controlled condition and field experiments with lines from the mapping population that segregated for this resistance. Analysis of results of both controlled-environment and field experiments suggested that the resistance was mediated by a single *R* gene located on chromosome A1.

Keywords: *Brassica napus* (oilseed rape), doubled haploid mapping population, durable resistance, hemibiotrophic crop pathogens, *Pyrenopeziza brassicae* (light leaf spot), quantitative PCR

Introduction

Plant resistance against pathogens of arable crops makes an important contribution to global food security (Beddington, 2010; Brun *et al.*, 2010), especially in areas of the world where subsistence farmers in marginal environments are threatened by devastating epidemics and do not have the option to use fungicides (Flood, 2010; Fitt *et al.*, 2011). It is estimated that crop resistance makes a substantial contribution to disease control that currently benefits food security by 15 kg per person per cropping season (Fitt *et al.*, 2011). To exploit such crop resistance effectively, it is important to understand its phenotype in relation to the life cycle of the pathogen. The phenotype of plant *R* gene-mediated resistance to biotrophic pathogens, such as the polycyclic *Blumeria graminis* (cereal powdery mildews) or *Puccinia striiformis* (wheat yellow rust), often involves a hypersensitive response (rapid localized cell death, <1 day post-inoculation (dpi)) occurring immediately after invasion of host tissue (Table 1; Jorgensen, 1994; Bozkurt *et al.*, 2010). In the case of obligate biotrophs, the pathogen is usually killed as a result of host cell death; it cannot grow or reproduce and epidemic development is stopped. However, operation of this resistance causes rapid selection for pathogen races that can render the resistance ineffective, leading to 'boom and bust' cycles (Stukenbrock & McDonald, 2008).

With hemibiotrophic pathogens, which have both biotrophic and necrotrophic phases in their life cycles (Oliver & Ipcho, 2004; Walters *et al.*, 2008), the phenotype of plant *R* gene-mediated resistance is less clear. Hypersensitive responses do occur with pathogens such as *Rhynchosporium secalis* (barley leaf blotch, 3–4 dpi) and *Leptosphaeria maculans* (oilseed rape phoma stem

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Table 1 Evidence concerning components of the phenotype of *R* gene-mediated resistance operating in leaves of arable crops against biotrophic (b) or hemibiotrophic (h) plant pathogens

Pathogen (biotrophic/hemibiotrophic)[a]	Niche[b]	Host[c]	*R* gene[d]	Phenotype				
				Hypersensitive response[e] (dpi)	Kills pathogen	Limits pathogen biomass[f]	Limits asexual sporulation[g]	Limits sexual sporulation[h]
Blumeria graminis (b)[i]	Intracellular (mesophyll)	*Hordeum vulgare*	*Mla* genes	Yes (<1)	Yes	n/a[j]	n/a	n/a
Puccinia striiformis (b)[k]	Intracellular (mesophyll)	*Triticum aestivum*	*Yr1*	Yes (<1)	Yes	n/a	n/a	n/a
Pyrenopeziza brassicae (h)[l]	Subcuticular	*Brassica napus*	*PBR1/PBR2*?	Yes? (<14)	?	Yes	?	?
Rhynchosporium secalis (h)[m]	Subcuticular	*H. vulgare*	*Rrs1*	Yes (3–4)	No	Yes	Yes	n/a
Leptosphaeria maculans (h)[n]	Intercellular (mesophyll)	*B. napus*	*Rlm6*	Yes (14)	No	Yes	Yes	Yes
Mycosphaerella graminicola (h)[o]	Intercellular (mesophyll)	*T. aestivum*	*STB* genes	No	No	Yes	Yes	?

[a]Hemibiotrophic pathogens are arranged in order with the most 'biotropic' first and the most 'necrotropic' last.
[b]The niche occupied by the biotrophic or hemibiotrophic pathogens after initial infection of leaf tissues at the time when the *R* gene is operating; frequently hemibiotrophic pathogens subsequently switch from a biotrophic to a necrotrophic mode and may occupy niches in other plant tissues.
[c]Host for which the phenotype of the *R* gene was studied; often the pathogen also attacks other related hosts.
[d]Specific *R* gene(s) that have been studied; others may operate in the same way.
[e]Collapse and death of host cells in the plants with the *R* gene (incompatible phenotype) that occurs before any collapse of cells in plants without the *R* gene (compatible phenotype).
[f]Evidence obtained by microscopy or quantitative PCR that the pathogen grows less extensively in host plants with the *R* gene than those without it.
[g]Evidence of no/limited asexual sporulation associated with colonization by the pathogen.
[h]No evidence of sexual sporulation associated with subsequent colonization of senescent leaf tissue by the pathogen.
[i]Colonization of mesophyll cells by means of haustoria. *Mla* genes associated with rapid hypersensitive response (Jorgensen, 1994).
[j]For biotrophic pathogens, because the hypersensitive response generally kills the pathogen, it is not possible to assess these aspects of the resistance phenotype.
[k]Colonization of mesophyll cells by means of haustoria. *Yr1* associated with rapid hypersensitive response (Bozkurt *et al.*, 2010).
[l]Occupies a subcuticular niche (Boys *et al.*, 2007); Bradburne *et al.* (1999) suggest that there are two loci involved in resistance, *PBR1* (on A1) associated with absence of asexual sporulation and *PBR2* (on C6) associated with a necrotic flecking. Other aspects of the phenotype were not described.
[m]Collapse of single/a few epidermal cells occurs in both resistant and susceptible phenotypes but then continues in susceptible and not resistant phenotypes. Asexual conidia are produced on resistant phenotypes, though less extensively than on susceptible phenotypes (Lehnackers & Knogge, 1990; Steiner-Lange *et al.*, 2003; Thirugnanasambandam *et al.*, 2011). Since the teleomorph has not yet been found, it is not possible to assess an effect on sexual sporulation.
[n]Pathogen penetrates through stomata and then grows in intercellular spaces. A hypersensitive response in the resistant phenotype is associated with death of cells around the site of penetration and containment of the pathogen. In the susceptible phenotype, there is extensive cell death, lesion formation and production of asexual spores in pycnidia, followed by spread of the pathogen down the leaf petiole to the stem where sexual sporulation occurs (Huang *et al.*, 2006).
[o]*STB* genes are not associated with a hypersensitive response but do limit pathogen biomass and asexual sporulation (G. Kema Plant Research International, Wageningen, The Netherlands, personal communication).

canker, leaf spot phase, 14 dpi) but much less rapidly than with biotrophic pathogens (Table 1; Lehnackers & Knogge, 1990; Steiner-Lange *et al.*, 2003; Yu *et al.*, 2005; Huang *et al.*, 2006; Thirugnanasambandam *et al.*, 2011). The pathogen is not usually killed, but resistance stops or slows its growth, even if there is no hypersensitive response, as with wheat resistance against *Mycosphaerella graminicola* (G. Kema, Plant Research International, Wageningen, The Netherlands, personal communication). Furthermore, it is not clear how operation of such resistance against hemibiotrophic pathogens interacts with their life cycles. These life cycles often involve initiation of epidemics by sexual or asexual spores produced on crop debris (in autumn for European autumn-sown crops). This is followed by subsequent disease spread by asexual splash-dispersed spores for the polycyclic *R. secalis* and *M. graminicola*, but not the monocyclic *L. maculans* (Fitt *et al.*, 2006; Stukenbrock & McDonald, 2008; Zhan *et al.*, 2008).

This paper characterizes the phenotype of a particular form of resistance against the hemibiotrophic pathogen *Pyrenopeziza brassicae* (Ashby, 1997) that has been introgressed into oilseed rape (*Brassica napus*). *Pyrenopeziza brassicae* causes light leaf spot, a polycyclic disease

initiated in the autumn by airborne ascospores produced following sexual reproduction of the pathogen on senescent plant debris (Gilles *et al.*, 2000a, 2001a). After infection of susceptible winter oilseed rape plants by *P. brassicae* ascospores, there is a long symptomless phase (3–4 months) while the fungus grows biotrophically between the cuticle and the epidermal cells of the leaf. This phase is ended by the onset of asexual sporulation, when the first symptoms are observed (white *P. brassicae* pustules (acervuli) breaking through the leaf surface) (Gilles *et al.*, 2000b). The conidia that they contain are splash-dispersed to cause secondary infections of leaves, stems, meristems and other tissue (Gilles *et al.*, 2001b).

Little is known about the operation of *B. napus* resistance against *P. brassicae* and no clear hypersensitive response to infection has been reported (Boys *et al.*, 2007). Four quantitative trait loci (QTL) involved in polygenic (field) resistance against *P. brassicae* in *B. napus* were reported by Pilet *et al.* (1998), based on assessments of light leaf spot on leaves and stems of the Darmor-Yudal mapping population in field experiments in the 1994–1995 and 1995–1996 cropping seasons in France. In a further report, Bradburne *et al.* (1999) described *R* gene-mediated resistance against *P. brassicae* in *B. napus* and suggested that there were two resistance genes segregating in two mapping populations of doubled haploid (DH) lines produced following introgression of genetic material from *Brassica rapa* (A genome) and *Brassica oleracea* var. *atlantica* (C genome) into *B. napus* (amphidiploid AC genome) via synthetic lines. They suggested that the gene *PBR1* was localized to linkage group N1 (A1) and associated with a phenotype of 'no sporulation' (asexual); the other gene, *PBR2*, was localized to linkage group N16 (C6) and associated with a phenotype of 'necrotic flecking' (Bradburne *et al.*, 1999). Neither of them were co-located with the QTL identified by Pilet *et al.* (1998). These resistance genes were not finely mapped and the resistant phenotypes were not investigated further.

This paper reports work with a resistant winter oilseed rape cultivar derived from the material studied by Bradburne *et al.* (1999), together with a DH mapping population of *B. napus* derived from a cross with this cultivar that segregates for resistance to *P. brassicae*, to characterize the resistance phenotype observed in *B. napus* against *P. brassicae*, as an example for investigating the operation of plant major gene-mediated resistance to hemibiotrophic pathogens.

Materials and methods

Brassica napus material

The resistant oilseed rape cv. Imola was produced from the material studied by Bradburne *et al.* (1999). One of the lines from the AGF32-37 DH population (KWS-UK, Thriplow) was crossed with *B. napus* cv. Navajo. The F_2 and subsequent generations were selected for resistance against *P. brassicae* using pedigree selection to produce cv. Imola. A DH population (N26) was derived from an F_1 cross between cv. Imola and the breeding line 218-11 (a DH breeding line derived from two backcrosses to the susceptible cv. Apex (2001 HGCA Recommended List resistance rating 5 on a 1–9 scale where 1 is susceptible and 9 resistant, http://www.hgca.com/varieties)). Lines derived from this source of resistance have been grown in UK breeding trials each year since 1997. Seeds of cv. Imola, line 218-11 and the N26 DH population were provided by KWS UK Ltd and seeds of cv. Apex were acquired from commercial sources. All seed lots were grown in observation plots at KWS UK Ltd during the 2006–2007 cropping season and found to be pure and conforming to type.

Preparation of *P. brassicae* conidial inoculum

Pyrenopeziza brassicae conidial inoculum was obtained from a selection of winter oilseed rape cultivars grown in two field experiments at Rothamsted (Harpenden, UK). Inoculum from field material was used in preference to an *in vitro* source of inoculum because *P. brassicae* isolates rapidly lose their pathogenicity if they are subcultured *in vitro*. The inoculum used in the first experiment was from the 2005–2006 cropping season; the inoculum used in the second and third experiments was from the 2006–2007 cropping season. Leaves showing *P. brassicae* asexual sporulation were sampled from the experiments and incubated in polyethylene bags for 5 days at 4°C to encourage further sporulation. Leaves showing symptoms of other diseases were discarded and *P. brassicae* conidia were washed from the remaining leaves with sterile distilled water. Conidial suspensions were stored at −20°C until required. The concentration of the inoculum was measured using a light microscope and haemocytometer slide (Weber Scientific International Ltd) and then suspensions were diluted to the required concentration with sterile distilled water. Viability of inoculum was confirmed by assessing percentage germination of spores. Inoculum from the 2006–2007 cropping season was further characterized by RAPD-PCR fingerprinting and mating-type PCR to assess the genetic diversity present. Serial dilutions in sterile water were made of the field-derived *P. brassicae* conidial suspension and were then plated onto antibiotic-amended (streptomycin and kanamycin) 1% malt extract plates; emerging single colonies were excised after incubation at 15°C. DNA was extracted from arising fungal mycelium, using a DNeasy Plant Kit (QIAGEN), and then RAPD-PCR was performed according to Murtagh *et al.* (1999) using primers OPA-05, OPA-09, OPA-20, OPW-05, OPW-06, OPW-09, OPW-10, OPAJ-01 and OPAJ-03 (MWG-Operon). The mating type of each isolate was also determined using the PCR diagnostic developed by Foster *et al.* (2002).

Controlled-environment experiments to investigate the phenotype of the resistance

Three controlled-environment experiments were carried out to characterize the phenotype of resistance against

P. brassicae in *B. napus*. In the first experiment, eight DH lines from the N26 population, the two parents of the DH population (resistant cv. Imola and susceptible line 218-11) and commercial cultivars Elan (2007 HGCA Recommended List resistance rating 8) and Hearty (2004 HGCA Recommended List resistance rating 3) were grown in controlled-environment conditions (14/16°C, 12-h photoperiod, 80–100% humidity) in a randomized complete block design with five replicate blocks. Plants were spray-inoculated at growth stage (GS) 1,04–1,05 (Sylvester-Bradley *et al.*, 1984) with a suspension of *P. brassicae* conidia (10^5 conidia mL^{-1} + 0·005% Tween 80) using a handheld sprayer (Wilkinsons). Plants were sprayed until the leaves were evenly covered with fine droplets and were then kept at 100% relative humidity for 48 h by covering each tray (22 × 35 cm) of six plants with a propagator lid. Light leaf spot symptoms (white *P. brassicae* pustules (acervuli containing asexual spores) erupting through the leaf surface in approximately concentric circles) were visually assessed 16 and 23 dpi by estimating the percentage leaf area covered with the acervuli. Any other symptoms were also recorded.

In a second experiment, the two parents (Imola and line 218-11) of the DH population were grown in the same conditions in a randomized complete block design with six replicate blocks. Plants were point-inoculated at GS 1,04–1,05. Filter paper (Whatman No. 1) was cut into 1-cm^2 pieces, which were soaked for *c.* 15 s in a suspension of conidia (5×10^4 conidia mL^{-1}) and then placed on the leaf surface. Inoculations were made with two pieces of filter paper in one of three positions; along the midrib (vein), *c.* 1·5 cm from the midrib or *c.* 3 cm from the midrib (leaf lamina). Plants were kept at 100% relative humidity for 48 h by covering each tray of four plants with a propagator lid. Plants were assessed 28 dpi for visual symptoms and 1·8-cm-diameter leaf disc samples were cut from leaves at each inoculation site (two discs per site) and frozen as a pair at −20°C to be analysed using quantitative PCR. When the point of inoculation had been approximately 3 cm from the midrib, discs were cut from the point of inoculation, from approximately 1·5 cm from the midrib and from the midrib itself. When the point of inoculation had been approximately 1·5 cm from the midrib, discs were cut from the point of inoculation and from the midrib. When the point of inoculation had been on the midrib, leaf discs were cut from the same position.

In a third experiment, the resistant parent (Imola) and the susceptible cv. Apex (2001 HGCA Recommended List resistance rating 5) were grown in the same conditions in a split-plot design with four replicate blocks. The treatment applied to the whole plots was the time at which the plant was destructively sampled and the treatment applied to the subplots was the cultivar. Plants were spray-inoculated at GS 1,03–1,04. The first true leaf was sampled from each designated plant at alternating 4- and 5-day intervals after inoculation and these samples were frozen at −20°C. The development of *P. brassicae* in each treatment was assessed using quantitative PCR. At 9, 13 and 20 dpi, the second true leaf was also sampled from a number of plants and examined using scanning electron microscopy.

Quantitative PCR

DNA was extracted from 20-mg ground, freeze-dried samples of plant tissue using the DNAMITE Plant Kit (Microzone Ltd). The manufacturer's protocol was used with the following modifications: samples were mixed with solution LA for 30 s in a FastPrep FP120 machine (Qbiogene Inc.) with three ball-bearings (4-mm diameter) in each tube; DNA was resuspended in 100 μL sterile, nuclease-free water. Concentrations of DNA were measured using a NanoDrop-1000 spectrophotometer (NanoDrop) and samples were diluted to 10 ng μL^{-1} with sterile, nuclease-free water.

The amount of *P. brassicae* DNA in a sample with 50 ng total DNA was quantified by quantitative PCR (qPCR) with the *P. brassicae*-specific primers PbITSF and PbITSR (Karolewski *et al.*, 2006). A 20-μL reaction volume was used, consisting of 10 μL SYBR® Green Jumpstart™ Taq Readymix™ (Sigma-Aldrich Company Ltd), 0·1 μL Rox, 0·06 μL 100 μM PbITSF, 0·06 μL 100 μM PbITSR, 4·8 μL sterile nuclease-free water and 5 μL 100 ng μL^{-1} template DNA. Each DNA sample was tested in duplicate in an Mx3000P qPCR System (Stratagene) for 2 min at 95°C followed by 50 cycles of 15 s at 95°C, 45 s at 58°C, 45 s at 72°C, and a final step of 15 s at 84°C during which fluorescence was measured. To check the specificity of the primers, a dissociation curve was included after the final amplification cycle by heating to 95°C for 1 min, cooling to 58°C for 30 s and then heating to 95°C for 30 s, measuring fluorescence at each increase of 1°C.

Six standards, each containing a known amount of *P. brassicae* DNA (a 10-fold dilution series from 10 ng to 0·1 pg) and *B. napus* DNA to give a total volume of 50 ng DNA, were included in duplicate on each 96-well plate. The cycle number at which fluorescence exceeded the threshold (Ct) was calculated for all samples. The Ct of each standard was used to plot a standard curve, from which the amount of *P. brassicae* DNA in the unknown samples could be calculated. Two no-template controls (one with 50 ng *B. napus* DNA and no *P. brassicae* DNA and the other with just nuclease-free water) were also included in duplicate on each plate. The samples, controls and standards were arranged in a randomized design on each 96-well plate, with the duplicates in adjacent wells (i.e. randomization was done over 48 pairs of wells, each pair of adjacent columns was identical).

Scanning electron microscopy

Sections of leaf tissue (*c.* 5 mm × 5 mm) were excised from spray-inoculated leaves (third experiment) using a sterile razor blade. These were quickly mounted onto an aluminium cryo-stub (slotted stubs were used for freeze-fracturing) using Tissue-Tek O.C.T. compound (Sakura

Finetek) and plunged into pre-frozen liquid nitrogen. Samples were transferred under vacuum to the Alto 2100 cryochamber (Gatan UK) with the stage temperature −180°C. Fracturing (where applicable), subliming and gold-coating were done on this stage. The samples were then transferred to the JSM LV 6360 scanning electron microscope (Jeol UK), with the stage temperature maintained at −150°C for examination and imaging.

Pyrenopeziza brassicae sexual sporulation

When the infected leaves from the first controlled-environment experiment had senesced (30–40 dpi), they were removed from the plants and dried for 48 h between sheets of absorbent paper at 20°C. They were then placed in plastic boxes on three layers of filter paper (Whatman No. 1) wetted with rainwater (Gilles *et al.*, 2001a). The boxes were incubated in the dark at 15°C for 14–16 days before being examined using a stereomicroscope.

Controlled-environment and field experiments to investigate the segregation of resistance to *P. brassicae* in the N26 DH population

Two types of experiment were carried out to investigate the genetic basis of the resistance phenotype. Firstly, in a series of 10 controlled-environment experiments (14/16°C, 12-h photoperiod, 80–100% humidity), a subset of 125 lines from the N26 DH population were screened for resistance to *P. brassicae* over a period of 12 months. Sixteen lines/cultivars were tested in each experiment in a balanced incomplete block design with five replicates. At least three lines/cultivars in each experiment were repeated from a previous experiment to allow comparisons to be made across experiments. Plants were spray-inoculated at GS 1,03–1,04. Light leaf spot symptoms were visually assessed 25 dpi by estimating the percentage leaf area covered with *P. brassicae* asexual sporulation. Any other symptoms were recorded. Leaves were then incubated in polyethylene bags for 10 days at 4°C to encourage asexual sporulation before being re-assessed (Fitt *et al.*, 1998).

Secondly, a field experiment was carried out in the 2006–2007 winter oilseed rape cropping season. On 3 October 2006, 272 lines from the N26 DH population (including all the 125 lines used in the CE experiments) were sown in an inoculated field site ('disease nursery') at Barley (near Royston, Hertfordshire, UK: OS Ref TL 402 404) in unreplicated rows (40-cm spacing) with 7-cm seed spacing using a Hege 95B precision drill. The plots were inoculated by spreading diseased oilseed rape stubble from the previous season between the plots on 3 October 2006 and by spraying on 17 October 2006, 17 November 2006 and 1 February 2007 with suspensions of *P. brassicae* conidia obtained from leaves collected from the disease nursery during the previous season and frozen at −20°C (8×10^4–$1{\cdot}6 \times 10^5$ conidia mL^{-1}). The field received no fungicide treatments. *In situ* plot assessments of light leaf spot symptoms were made on 20 April 2007 using a 9-point scale where nine was 'no observable symptoms or sporulation' and one to eight were decreasingly severe visual symptoms (necrosis to limited sporulation) (Table 2).

Statistical analysis of controlled-environment phenotype experiments

Because the three controlled-environment experiments examining the phenotype of this resistance against *P. brassicae* produced data of different types, these data were analysed in different ways. The light leaf spot symptom data (percentage leaf area with *P. brassicae* asexual sporulation) from the first experiment using *P. brassicae* conidial inoculum in controlled-environment conditions were transformed using a logit function $z = \log_e \frac{\text{data}+2}{102-data}$ before analysis of variance (ANOVA). Data from cv. Imola and three of the DH lines (none of which showed any light leaf spot symptoms) were excluded from the analysis. Data from the qPCR analysis of samples from the second controlled-environment experiment were $\log_{10}$-transformed and analysed using a REML (restricted maximum likelihood) analysis, with the inoculation and sampling points treated as a single factor with six levels. This factor and the cultivar factor were included in the fixed model and the blocking structure was included in the random model. Data from the qPCR analysis of samples from the third controlled-environment experiment were $\log_{10}$-transformed and analysed using ANOVA.

Statistical analysis of controlled-environment and field experiments investigating segregation of resistance and mapping position of the resistance locus

Before analysis of data from controlled-environment and field experiments to investigate the segregation of resistance to *P. brassicae* in the N26 DH population, DH lines that showed *P. brassicae* asexual sporulation (scores of

Table 2 Scale (1–9) for the visual assessment of severity of light leaf spot (*Pyrenopeziza brassicae*) symptoms on winter oilseed rape (*Brassica napus*) plots

Score	Description
1	All plants stunted or dead
2	>50% plants stunted or deformed
3	<50% plants stunted or deformed, and >50% plants with *P. brassicae* sporulation
4	35–50% plants with *P. brassicae* sporulation
5	20–35% plants with *P. brassicae* sporulation
6	5–20% plants with *P. brassicae* sporulation
7	1–5% plants with *P. brassicae* sporulation
8	Traces of *P. brassicae* sporulation visible on close inspection
9	No disease observable

This scale is used by KWS UK Ltd for visually assessing plots of winter oilseed rape in spring when symptoms are visible; it is based on a scale developed for visual assessment of light leaf spot in HGCA Recommended List trials (http://www.hgca.com/varieties).

1–8 on the 9-point scale) were classed as susceptible. Plants that never showed any *P. brassicae* asexual sporulation (score of 9 on the 9-point scale) in the field and controlled-environment experiments were classed as resistant. The differences between a 1:1 ratio and the ratios of resistant:susceptible DH lines obtained in the controlled-environment and field experiments were tested using chi-squared tests. A contingency chi-squared test was used to test the association between the dark flecking phenotype observed and the presence or absence of asexual sporulation in the series of controlled-environment experiments. All analysis was done using GenStat® 11th edition (Payne *et al.*, 2008). Graphs were produced using SigmaPlot for Windows Version 10·0 (Systat Software, Inc.).

A set of 92 polymorphic microsatellite SSR (simple sequence repeat) markers with known map positions on *B. napus* chromosomes A1, A7, C1 or C6 were scored using DNA from 267 N26 DH lines. Polymorphic SSR markers were identified by screening a total of 346 SSR markers from the AAFC (Saskatoon Research Centre), Celera AgGen, BBSRC microsatellite programme and IAPB (University of Gottingen) private and public collections. All SSR assays were run at KWS UK Ltd or KWS SAAT. Stepwise mapping efforts identified linkage group A1 as the location of the resistance locus, enabling a final total of 36 markers to be mapped on this key linkage group. A complete marker map was not developed. The qualitative score for the *P. brassicae* resistance was treated as a single locus and mapped by linkage analysis using JoinMap 4 (Van Ooijen, 2006).

Results

Genetic composition of *P. brassicae* inoculum used in controlled-environment experiments

A total of 25 single-spore *P. brassicae* isolates were obtained from the conidial suspension derived from field material from the 2006–2007 cropping season. Analysis by RAPD-PCR identified at least eight unique genotypes amongst the 25 isolates, of which five were mating type *MAT1-1* and three were *MAT1-2* (Boys, 2009). This demonstrated that the inoculum used for controlled-environment experiments represented a range of pathogen genotypes such as might occur in natural conditions.

Visual symptoms in controlled-environment experiments

The main symptoms of light leaf spot in controlled-environment conditions were white *P. brassicae* acervuli (small fruiting bodies containing asexual spores) that erupted through the leaf surface (Fig. 1a). In the first

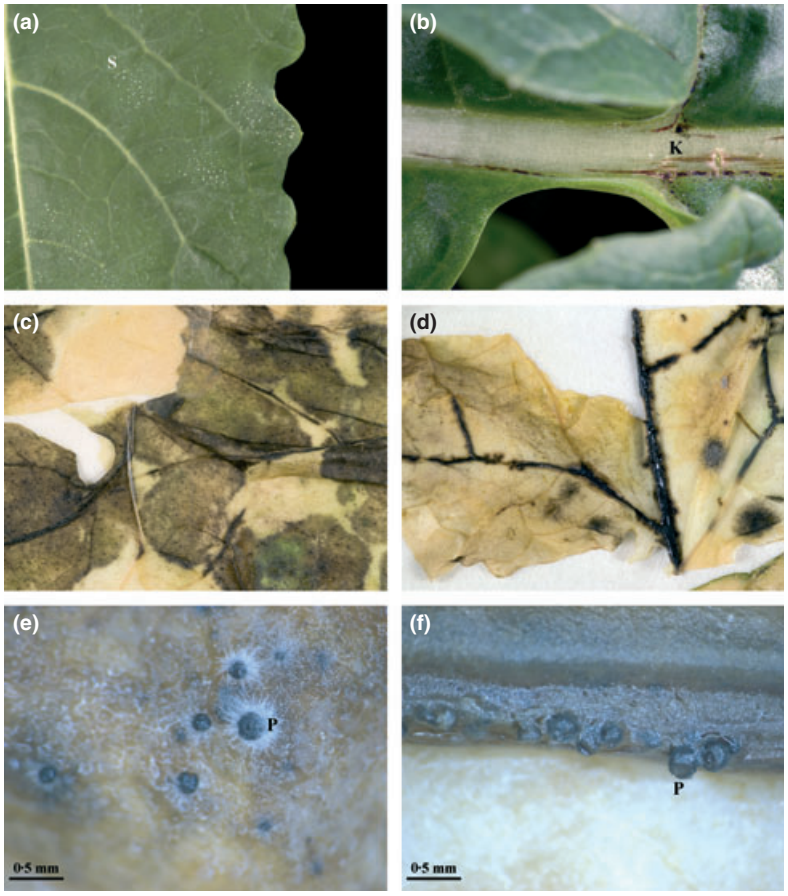

Figure 1 (a) Light leaf spot symptoms (white *Pyrenopeziza brassicae* acervuli; S) on a leaf of the susceptible parent of the N26 doubled haploid population (*Brassica napus* line 218-11) 23 days after spray-inoculation (dpi) with a suspension of conidia from a mixed population of *P. brassicae*; (b) a leaf of the resistant parent of the N26 doubled haploid population (*B. napus* cv. Imola) 23 dpi, showing dark flecking (K) along the midrib (first phenotype controlled-environment experiment); senescent leaves of (c) line 218-11 and (d) cv. Imola, after being spray-inoculated with *P. brassicae* conidia and incubated to encourage the production of apothecia, which have developed in association with the dark staining; *P. brassicae* apothecia (P) on senescent leaves of (e) susceptible line 218-11 and (f) resistant cv. Imola.

experiment, this asexual sporulation was visible from 16 days post-inoculation (dpi) on susceptible plants (five of the eight DH lines, the susceptible parent, line 218-11 and all of the commercial cultivars tested) and increased significantly with time ($F_{1,60}$ = 18·0, P < 0·001). Three of the eight DH lines and the resistant parent (cv. Imola) showed no *P. brassicae* asexual sporulation at any time. A dark flecking phenotype (Fig. 1b) was observed on the resistant plants from *c.* 10 dpi. This was most obvious on leaf vein tissue, but was also present elsewhere on the leaf lamina. Point-inoculation of the two parental lines in the second experiment demonstrated that both the resistant (dark flecking) and susceptible (asexual sporulation) phenotypes were confined to the area of inoculation and did not induce a visible response elsewhere on the leaf.

Symptomless growth in controlled-environment experiments

In the third experiment in controlled conditions, the amount of *P. brassicae* DNA increased significantly with time over the first 13 dpi ($F_{10,30}$ = 5·52, P < 0·001, Fig. 2) and at the same rate in leaves of both cvs Apex and Imola. Then, from 13 to 36 dpi, the amount of *P. brassicae* DNA increased more than 300-fold in leaves of cv. Apex but remained unchanged in those of cv. Imola, resulting in a statistically significant difference between the two cultivars 36 dpi ($F_{1,25}$ = 9·67, P = 0·005, Fig. 2). Finally, between 36 and 45 dpi, when leaves started to senesce, the amount of *P. brassicae* DNA rapidly increased in leaves of the resistant cv. Imola but not in those of the susceptible cv. Apex, such that it reached equal values in both cultivars by 45 dpi.

Analysis of samples from the point-inoculated (second) experiment using quantitative PCR showed a significant interaction between cultivar and the point of inoculation/sampling ($F_{6,42}$ = 3·18, P = 0·012). The amount of *P. brassicae* DNA detected 1·5 or 3 cm away from the point of inoculation was very small in every case (Fig. 3). For susceptible line 218-11, there was no significant difference in the amount of *P. brassicae* DNA detected at the point of inoculation 28 dpi between different points of inoculation (midrib (vein), 1·5 or 3 cm away from midrib (leaf lamina)). For resistant cv. Imola, there was a very small amount of *P. brassicae* DNA detected at the point of inoculation 28 dpi when the point of inoculation was on the lamina not the midrib, whereas there was a signifi-

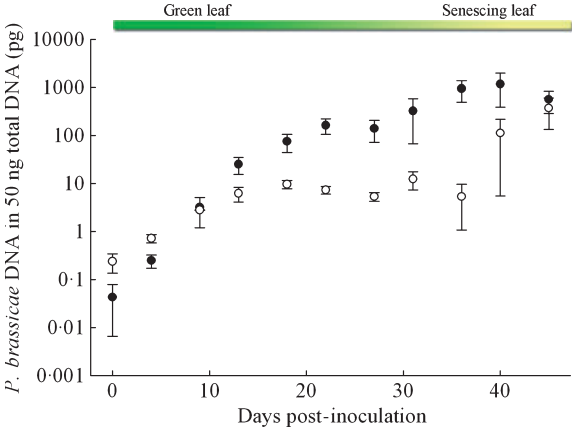

Figure 2 Change in amount of *Pyrenopeziza brassicae* DNA with time in *Brassica napus* leaf samples of cv. Imola (resistant, ○) and cv. Apex (susceptible, ●) after spray-inoculation with a suspension of *P. brassicae* conidia (third phenotype controlled-environment experiment). Error bars are ± one standard error of the mean.

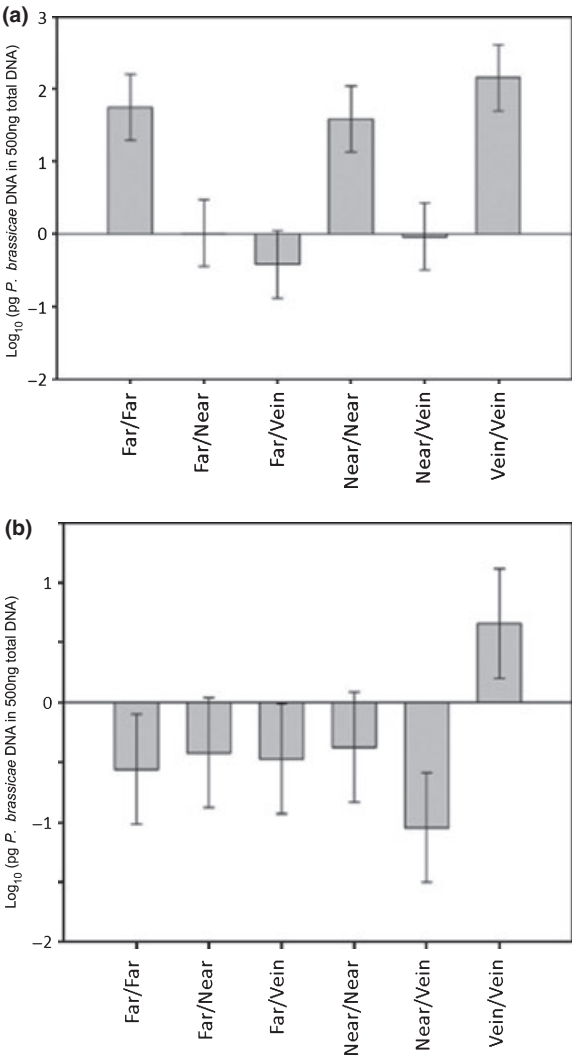

Figure 3 Amount of *Pyrenopeziza brassicae* DNA in 1·8-cm-diameter *Brassica napus* leaf discs after inoculation at different points of the leaf for (a) susceptible (line 218-11) or (b) resistant (cv. Imola) genotypes in a controlled-environment experiment. For statistical analysis, inoculation point and sampling point were treated as a single factor with six levels. This is shown as Inoculation point/Sampling point, where Far was ~3 cm away from the midrib, Near was ~1·5 cm away from the midrib and Vein was on the midrib. Error bars illustrate the standard error of the difference for comparing within levels of the cultivar factor (0·46).

cantly larger amount detected when the point of inoculation was on the midrib (though significantly less than the equivalent amount on the midrib of line 218-11).

Examination of sections of spray-inoculated leaves of cv. Apex (third experiment) under a scanning electron microscope showed how the *P. brassicae* hyphae grew within susceptible leaf tissue (Fig. 4a,c). The hyphae grew between the cuticle and the upper epidermis (Fig. 4a,c), forcing the cuticle upwards slightly so that patterns of hyphal growth could be seen clearly from above (Fig. 4a). Hyphae were observed mostly on the leaf veins and growing outwards from the leaf veins to the surrounding tissue. There was no evidence that *P. brassicae* hyphae penetrated host cells. At 13 dpi, asexual sporulation was not yet visible under a stereomicroscope, but was observed on leaves of cv. Apex under the scanning electron microscope (Fig. 4e). By 20 dpi asexual sporulation was visible on leaves of cv. Apex both unaided and under the scanning electron microscope (Fig. 4g).

Subcuticular hyphal growth occurred similarly in leaves of cv. Imola (Fig. 4b,d). Hyphae were again observed mostly on the leaf veins and there was no observation of cell penetration. The amount of *P. brassicae* hyphal growth was less than that on leaves of cv. Apex, however, and there was less growth out from the leaf veins to the surrounding tissue (Fig. 4b). No asexual sporulation was observed on sections of leaves from cv. Imola, in agreement with visual observations. The dark flecking observed on inoculated leaves of the resistant plants was shown to be caused by the collapse of epidermal cells (Fig. 4d,f,h). This cell collapse was always associated

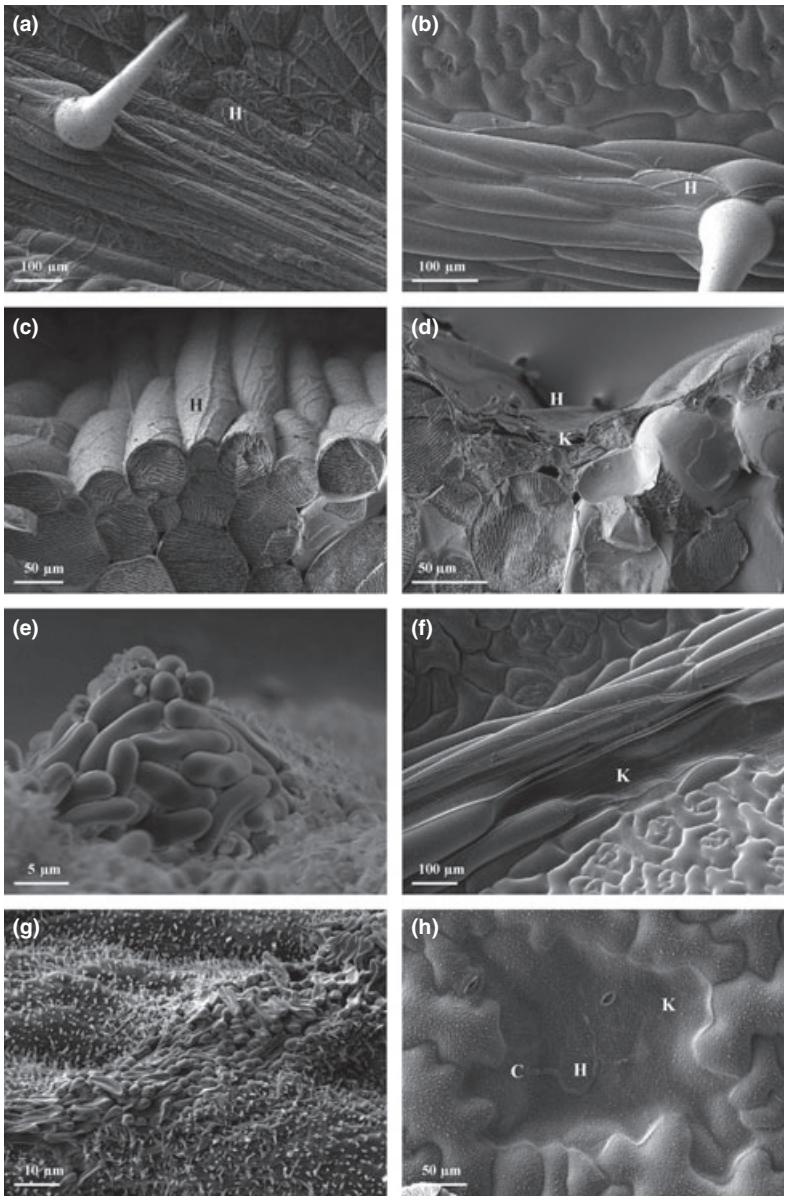

Figure 4 Scanning electron micrographs of leaf sections from (a) *Brassica napus* cv. Apex (susceptible) 13 days post-inoculation (dpi, third phenotype controlled-environment experiment) showing abundant *Pyrenopeziza brassicae* subcuticular hyphal growth (H) on a leaf vein and surrounding tissue; (b) *B. napus* cv. Imola (resistant) 13 dpi showing subcuticular hyphal growth (H) on a leaf vein but not surrounding tissue; (c) cv. Apex 9 dpi showing subcuticular hyphal growth (H) on a leaf vein; (d and f) cv. Imola 9 dpi showing subcuticular hyphal growth (H) on a leaf vein with collapsed longitudinal epidermal cells (K); (e) cv. Apex 13 dpi showing early asexual sporulation; (g) cv. Apex 20 dpi showing later asexual sporulation erupting through the leaf cuticle; and (h) cv. Imola 20 dpi showing a region of collapsed epidermal cells (K) associated with a germinated *P. brassicae* conidium (C) and a small amount of hyphal growth (H).

with the presence of *P. brassicae* hyphae, but the presence of *P. brassicae* hyphae did not always result in cell collapse (Fig. 4b).

Sexual sporulation of *P. brassicae* in controlled-environment experiments

After incubating senescent, infected leaves from the first experiment for 14–16 days, *P. brassicae* apothecia were observed on leaves from both susceptible and resistant plants (Fig. 1e,f). The apothecia were produced in association with a dark discoloration, which was spread over the leaf lamina on the susceptible lines/cultivars (Fig. 1c) but confined to the area on and around the leaf veins on the resistant parent/lines (Fig. 1d).

Segregation of resistance and gene mapping

Analysis of resistance or sensitivity in the N26 DH population showed that the population was segregating for resistance to *P. brassicae* in a ratio of *c.* 1:1. Of the 125 lines tested in the series of experiments in controlled-environment conditions, 66 showed a susceptible phenotype with asexual sporulation and no dark flecking; of the remaining 59 lines, 57 showed the resistant dark flecking phenotype (Fig. 5a). There was no significant difference (5% level) between the ratio of resistant (no asexual sporulation) to susceptible (asexual sporulation) lines and a 1:1 ratio. These results are supported by observations in the field experiment with an extended subset of the N26 population, where 151 out of 272 lines showed no asexual sporulation (Fig. 5b). Those lines that showed a susceptible phenotype in controlled-environment experiments also showed a susceptible phenotype in field experiments. There was a significant association between the no asexual sporulation phenotype and the dark flecking phenotype in controlled-environment conditions (χ^2 = 117, d.f. = 1, $P < 0{\cdot}001$).

The 103-cM linkage marker map was constructed for chromosome A1. When it was compared to the A1 linkage group from the integrated *B. napus* map BnaWAIT (Wang *et al.*, 2011), there was considerable similarity in the orders of the marker loci between the two linkage groups (Fig. 6). Of the 16 marker loci common to both the N26 linkage group and the consensus linkage group, only two were in different positions with regards to marker order (CB10158 and sN1838). Mapping of both the controlled-environment and the field data positioned the resistance locus close to the telomere (end) on the long arm of chromosome A1 (Fig. 6).

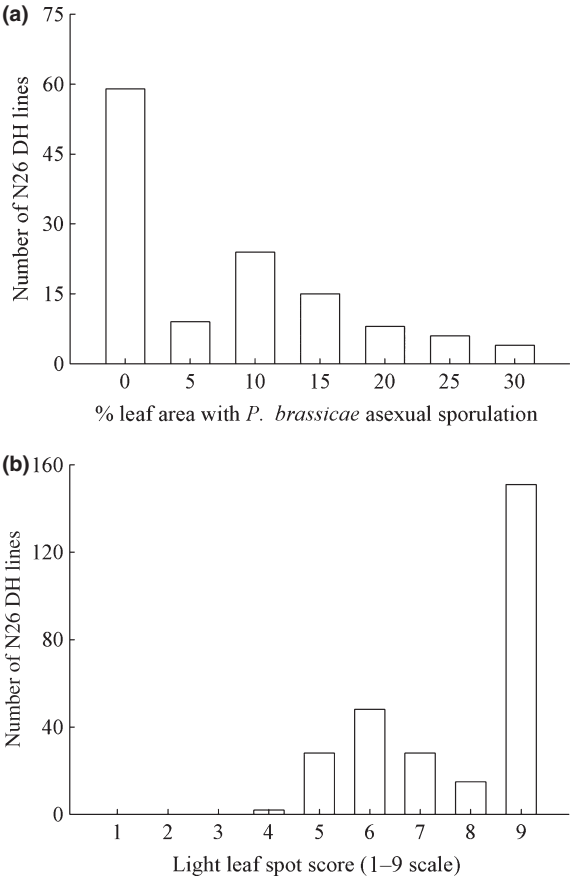

Figure 5 Assessment of light leaf spot symptoms (a) in a series of experiments in controlled-environment conditions with a subset (125 lines) from the N26 doubled haploid mapping population and (b) on 20 April 2007 in field rows of winter oilseed rape (*Brassica napus*) with 272 lines from the population showing that the N26 population is segregating for resistance to *Pyrenopeziza brassicae* in an approximately 1:1 ratio of resistant lines to susceptible lines. In controlled-environment conditions (a), the percentage leaf area covered by *P. brassicae* asexual sporulation was estimated. A 9-point assessment scale was used for field assessments (b), where 9 was 'no observable symptoms or sporulation' and 8 to 1 were increasingly severe visual symptoms (limited sporulation to necrosis) (Table 2).

Discussion

Results from these field and controlled-environment experiments suggest that a novel form of resistance in *B. napus*, mediated by a single *R* gene, can limit asexual sporulation but allow sexual sporulation of the hemibiotrophic pathogen *P. brassicae*. This resistance, introgressed into *B. napus* cv. Imola, differs from *R* gene-mediated resistance operating against biotrophic crop pathogens such as *B. graminis* or *P. striiformis* (Table 1; Jorgensen, 1994; Bozkurt *et al.*, 2010) but shows some similarities to *R* gene-mediated resistance operating against hemibiotrophic crop pathogens such as *R. secalis*, *L. maculans* or *M. graminicola* (Table 1; Lehnackers & Knogge, 1990; Steiner-Lange *et al.*, 2003; Yu *et al.*, 2005; Huang *et al.*, 2006; Thirugnanasambandam *et al.*, 2011; G. Kema, personal communication). For example, operation of the resistance against *P. brassicae* does not kill the pathogen, unlike operation of *R* gene-mediated resistance against biotrophic pathogens. Furthermore, the operation of the resistance in *B. napus* involves a substantial decrease in, but not prevention of, pathogen hyphal growth during a period 13–36 days after inoculation, whilst the biomass

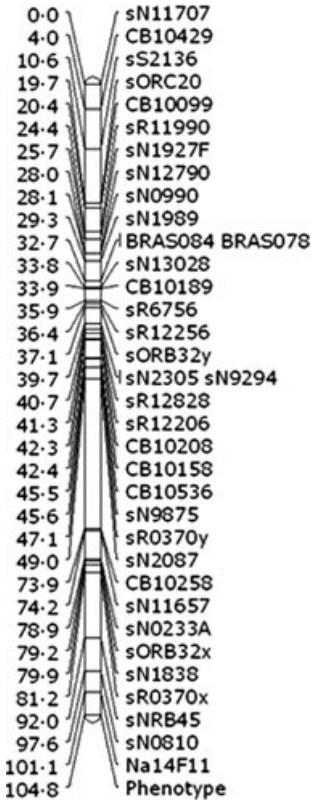

Figure 6 Genetic map for linkage group A1 of the N26 doubled haploid *Brassica napus* population produced using microsatellite SSR markers with the resistance phenotype included as a qualitative trait. Linkage analysis was performed using the JoinMap 4 software (Van Ooijen, 2006) and the Kosambi mapping function was used to estimate map distances (centimorgans). Markers are linked with a LOD (log_{10} of odds) value of 7; other linkage groups are shown in Boys (2009). The eight markers at the bottom of group A1 and the resistance phenotype scored as a qualitative trait remain linked until LOD = 15.

of *P. brassicae* was increasing 300-fold in leaves of the susceptible cultivar (Fig. 2). Both scanning electron microscopy (Fig. 4) and quantitative PCR (Fig. 2) confirmed that, although the pathogen was able to grow within the leaves of resistant plants, this was to a much lesser extent than within the leaves of susceptible plants. Furthermore, during this period substantial asexual sporulation occurred on leaves of susceptible plants but not on those of resistant plants. Operation of *R* gene-mediated resistance against other hemibiotrophic pathogens is also fungistatic rather than fungitoxic. For example, the *LepR2* gene for resistance against *L. maculans* in *B. napus* also reduces but does not prevent hyphal growth, by a mechanism involving callose deposition (Yu *et al.*, 2005). Work with *GFP*-labelled *R. secalis* and *Rrs1* in barley also shows that the operation of the gene is fungistatic (Thirugnanasambandam *et al.*, 2011).

The dark flecking phenotype observed on resistant plants from *c.* 8 days post-inoculation (Fig. 1b) and by Bradburne *et al.* (1999) was shown to be associated with the collapse of epidermal cells in association with *P. brassicae* hyphae (Fig. 4). This cell collapse is typical of a late (3–14 dpi) hypersensitive response observed with operation of *R* gene-mediated resistance against other hemibiotrophic pathogens such as *R. secalis* or *L. maculans* (Lehnackers & Knogge, 1990; Steiner-Lange *et al.*, 2003; Huang *et al.*, 2006; Thirugnanasambandam *et al.*, 2011). However, its function is different from that of early (<1 dpi) hypersensitive responses against biotrophs that kill the pathogen (Jorgensen, 1994; Kombrink & Schmelzer, 2001; Bozkurt *et al.*, 2010). It may be stimulated by changes in the pathogen shortly before the onset of asexual sporulation in order to limit that sporulation. This function of the hypersensitive response may be generic to *R* gene-mediated resistance against other hemibiotrophic pathogens. The *B. napus LepR2* gene for resistance against *L. maculans,* the barley *Rrs1* gene against *R. secalis* and the wheat *STB* genes against *M. graminicola* also inhibit asexual sporulation (Yu *et al.*, 2005; Thirugnanasambandam *et al.*, 2011; G. Kema, personal communication).

However, this is the first evidence that operation of such an *R* gene against a hemibiotrophic pathogen does not prevent sexual sporulation of the pathogen, as observed by the formation of apothecia by *P. brassicae* on senescent debris from resistant plants (Fig. 1d). The results provide evidence that the operation of the *R* gene ends when leaves start to senesce; during this period the biomass of *P. brassicae* increased 300-fold in leaves of the resistant cultivar but did not increase in leaves of the susceptible cultivar, so that there was finally the same biomass available for sexual sporulation in both. Production of apothecia, and associated sexual sporulation (Gilles *et al.*, 2001c), was most abundant on the petiole and leaf midrib tissues where apothecia commonly occur in winter oilseed rape crops (McCartney & Lacey, 1990).

Results from experiments in field and controlled-environment conditions suggest that resistance to *P. brassicae* in the winter oilseed rape cultivar Imola was the result of the action of a single *R* gene. The ratio of resistant to susceptible lines in the N26 DH population derived from crossing cv. Imola and a susceptible breeding line was not significantly different from a 1:1 ratio (Fig. 5). The cultivar Imola was generated from a pedigree that involved interspecific hybridization, with resistant material originally derived from the diploid parents *B. rapa* and *B. oleracea* studied by Bradburne *et al.* (1999). At that time the material was found to segregate for two major genes for resistance against *P. brassicae*. However, only one *R* gene was found in the present study, suggesting that one *R* gene may have been lost during the breeding of cv. Imola. Transmission of the resistance was through a conventional pedigree selection process with phenotypic selection for *P. brassicae* resistance at each generation; loss of one of two major genes can be expected in this situation. The re-mapping during the course of this work did not confirm the location of the QTL identified by Pilet *et al.* (1998) or either of the two resistance loci found by Bradburne *et al.* (1999) in material from the same source but

suggests that the resistance locus responsible for the black flecking (*PBR2*), previously mapped to C6 (N16), in this population is located near the telomere on the long arm of A1. Whilst unexpected, such a rearrangement, including translocation of small chromosome segments, is often observed in material where alien introgression or interspecific hybridization has occurred (Gaeta & Pires, 2010; Szadkowski *et al.*, 2010). It is also conceivable that the use of different sources of *P. brassicae* inoculum might have contributed to these genetic differences. Bradburne *et al.* (1999) used two defined isolates of *P. brassicae* in their pathogenicity work, whereas populations of natural field isolates of *P. brassicae* were used in the present study. Therefore, differences in the genetic background of the pathogen inoculum might have selected a different resistance locus. However, given the ancestry of cv. Imola, the phenotypic similarity in resistance and the fact that the inoculum used in the present study exhibited evident genetic diversity, this alternative explanation seems unlikely. Plants containing this *B. napus R* gene operating against *P. brassicae* have been grown in small-scale breeding trials since 1997 and there has been little evidence of widespread change in pathogen populations to render the resistance ineffective. This can be compared to the *Leptosphaeria maculans–Brassica napus* pathosystem, where resistance that prevents sexual sporulation because it stops the pathogen reaching stem tissues on which sexual sporulation occurs has been rendered ineffective within 4 years (Brun *et al.*, 2000). Thus, the *R* gene operating against polycyclic *P. brassicae* may be more durable than the *R* gene operating against monocyclic *L. maculans*. It is likely that the numbers of ascospores produced as a result of sexual sporulation of *P. brassicae* on this resistant cultivar in a natural epidemic would be considerably smaller than the numbers produced on a susceptible cultivar. This is because the resistance prevents the successive cycles of asexual sporulation that spread the pathogen through the crop (Evans *et al.*, 2003) and thus it would be much less likely that isolates of compatible mating types would meet to allow sexual reproduction.

It is important to consider the best strategy with which to deploy this *R* gene-mediated resistance in commercial cultivars to maximize its durability (Pink, 2002); for example it has been shown that the durability of an *R* gene may be increased when it is introgressed into a resistant or partially resistant genetic background (Palloix *et al.*, 2009; Brun *et al.*, 2010). If this phenotype of *R* gene-mediated resistance is shown to be of generic relevance to other crop–pathogen systems, and it can be deployed effectively, it can make an important contribution to global food security (Brun *et al.*, 2010).

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