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REVIEW

Resistance to infection by stealth: *Brassica napus* (winter oilseed rape) and *Pyrenopeziza brassicae* (light leaf spot)

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Abstract Light leaf spot (*Pyrenopeziza brassicae*) is an important disease on winter oilseed rape crops (*Brassica napus*) in northern Europe. In regions where economically damaging epidemics occur, resistance to *P. brassicae* in commercial cultivars is generally insufficient to control the disease without the use of fungicides. Two major genes for resistance have been identified in seedling experiments, which may operate by decreasing colonisation of *B. napus* leaf tissues and *P. brassicae* sporulation. Much of the resistance present in current commercial cultivars is thought to be minor gene-mediated and, in crops, disease escape and tolerance also operate. The subtle strategy of the pathogen means that early colonisation of host tissues is asymptomatic, so a range of techniques and molecular tools is required to investigate mechanisms of resistance. Whilst resistance of new cultivars needs to be assessed in field experiments where they are exposed to populations of *P. brassicae* under natural conditions, such experiments provide little insight into components of resistance. Genetic components are best assessed in controlled environment experiments with single spore (genetically fixed) *P. brassicae* isolates. Data for cultivars used in the UK Recommended List trials over several seasons demonstrate how the efficacy of cultivar resistance can be reduced when they are deployed on a widespread scale. There is a need to improve understanding of the components of resistance to *P. brassicae* to guide the development of breeding and deployment strategies for sustainable management of resistance to *P. brassicae* in Europe.

Keywords *Cylindrosporium concentricum* · Epidemiology · Host-pathogen genetics · Major gene-mediated resistance · Polygenic resistance · Disease assessment

Abbreviations

ELISA	Enzyme-linked immunosorbent assay
HR	Hypersensitive response
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait loci
R gene	Major resistance gene
SEM	Scanning electron micrograph

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Introduction

This review focuses on resistance to *Pyrenopeziza brassicae* (anamorph *Cylindrosporium concentricum*) in relation to the epidemiology of light leaf spot on winter oilseed rape (*Brassica napus*; canola; rapeseed) in Europe. Light leaf spot is one of the most important diseases of oilseed rape in the UK and northern parts of continental Europe. Seasonal yield losses caused by light leaf spot have been estimated to range from around €9–€32 million in the UK between 2000 and 2005 (www.cropmonitor.co.uk; Sharon Elcock, Central Science Laboratory, York, pers. comm.). Its main effect on yield is through decreasing leaf photosynthetic area and plant vigour in winter, and increasing susceptibility to frost damage (Baierl et al. 2002). Further yield loss results from pod disease, which causes premature ripening and seed shedding from the brittle pods. There have been many light leaf spot epidemics in the UK since 1974 (Majer et al. 1998), particularly in Scotland and the north of England, where the disease is favoured by high rainfall (Figueroa et al. 1995; Karolewski et al. 2002). Since the 1990s, light leaf spot has become increasingly damaging in Poland, where it is a problem after mild winters (Karolewski 1999). France has experienced regular epidemics with serious yield losses during the 1980s and 2000s (Karolewski et al. 2006) and the disease also occurs in Germany. Effective control measures against light leaf spot are needed throughout northern Europe to sustain oilseed rape production, which is predicted to increase as markets for biofuel production expand.

The use of cultivars with resistance to *P. brassicae* is an important method for controlling light leaf spot, being more environmentally acceptable and less expensive than reliance on fungicides (Pilet et al. 1998). Resistance to *P. brassicae* in commercial cultivars varies greatly, and there is a negative correlation between resistance rating and yield response to fungicides (Walker et al. 1995). In regions with weather favourable to the disease, even the most resistant commercial cultivars can still develop light leaf spot if fungicides are not applied (Latunde-Dada et al. 2007). Two major genes for resistance (*R* genes) to *P. brassicae* have been identified in *B. napus* (Bradburne et al. 1999) but have not yet been cloned and fully characterised. Some quantitative trait loci (QTL) involved in resistance have also been identified by assessing light leaf spot severity on leaves and stems (Pilet et al. 1998) but their functions are unknown. The breeding of new resistant cultivars is hindered by both the narrow genetic base of *B. napus* (Chen and Heneen 1989) and the poor understanding of the *P. brassicae—B. napus* interaction in relation to the epidemiology of the disease.

Systemic fungicides are used widely in the control of light leaf spot in the UK, but due to its long symptomless phase (Rawlinson et al. 1978), the first, most important application must often be made before symptom appearance. This has led to concerns that many fungicide applications are unnecessary and that extensive use may result in selection for fungicide-resistant strains in the *P. brassicae* population (Pilet et al. 1998).

To improve understanding of this pathosystem and facilitate the breeding of resistant cultivars, more accurate, reliable methods for the assessment of resistance are needed. Currently, resistance to *P. brassicae* in *B. napus* is assessed by many different techniques, ranging from the use of seedlings in controlled environment experiments to full-scale field experiments over a growing season. It is important to appreciate the different components of resistance and to use appropriate assessment methods to investigate them. The aims of this review are (1) to assess the current knowledge of the components of resistance to *P. brassicae* in *B. napus*; (2) to examine their relative importance in relation to the epidemiology of the disease; and (3) to evaluate the different techniques used to study the resistance of *B. napus* to *P. brassicae*.

Categories of resistance

The phrase ‘‘host resistance to a plant pathogen’’ is used to describe a number of different concepts, ranging from a specific genetic interaction between elicitors and receptors to an overall disease phenotype in crops. Genetic resistance can be categorised as major gene-mediated resistance (also vertical, monogenic or qualitative resistance), which involves one or a few genes, or minor gene-mediated resistance (also horizontal, polygenic, quantitative resistance or basal defence), which is dependent on the additive actions of several genes, each of which

may have little effect alone (Agrios 2005; McDonald and Linde 2002). *R* genes often function by enabling host recognition of a pathogen-produced elicitor, and stimulating a resistance response. In the case of major gene-mediated resistance to many biotrophs, and some hemibiotrophs, this involves a hypersensitive response (HR) shortly after penetration, and failure of the pathogen to colonise or multiply in the host (Nimchuk et al. 2003). Minor gene-mediated resistance does not generally prevent infection, but slows the colonisation of host tissue and thereby the spread of disease and development of epidemics (Agrios 2005), with the genes, for example, encoding hydrolytic enzymes or components of chemical or physical barriers (McDonald and Linde 2002). Major gene-mediated resistance is usually race-specific, whereas minor gene-mediated resistance tends to be race non-specific, i.e. acting against all isolates of a pathogen species (Hammond-Kosack and Parker 2003).

Apparent resistance describes the situation in which susceptible plants remain free from damaging disease, either through tolerance or disease escape (Agrios 2005). Plants that are able to produce a good yield even when infected may be described as tolerant; they are susceptible, but incur very little damage (Clarke 1986). This may be of limited use in crops because, although yield loss is reduced, the pathogen can continue to reproduce (and potentially mutate to produce a more aggressive strain) and produce inoculum for the next season. Disease escape occurs when factors such as canopy structure or spacing of plants mean that a susceptible host, virulent pathogen and favourable environment do not coincide to produce disease.

Potential resistance mechanisms: insights from light leaf spot epidemiology

Adhesion and germination of ascospores on the leaf cuticle (Fig. 1a) is the first stage of the *P. brassicae*—*B. napus* interaction at which minor gene-mediated resistance could act (Fig. 2a). This process occurs in the autumn, when light leaf spot epidemics are initiated by relatively small numbers of air-borne *P. brassicae* ascospores released from infected stem and pod debris from previous crops (Gilles et al. 2001b; Evans et al. 2003). Germination and leaf penetration by *P. brassicae* may be affected by the thickness and topology of cuticular waxes, which form a thick, structured layer on *B. napus* leaves (Fig. 3a), although the thickness, distribution and configuration of these waxes depends greatly on environmental conditions (Koch et al. 2005; Baker 1974). It has been observed that herbicides such as Dalapon (2,2-dichloropropionic acid) that alter the wax structure of the plant favour this pathogen (Rawlinson et al. 1978; Davies 1997). Davies (1997) observed that leaf cuticle topology differed markedly between cultivars, and that cultivars with higher Home-Grown Cereals Authority (HGCA) UK resistance ratings (www.hgca.com) tended to have greater wax coverage than more susceptible cultivars. The relative importance of cultivar and environment in determining wax thickness and topology have yet to be determined, since Davies (1997) worked with seedlings grown in controlled environments. These observations are consistent with our understanding of the *P. brassicae* infection process, since the entry of *P. brassicae* through the leaf surface of *B. napus* is by direct penetration of the cuticle (Li et al. 2003) (Fig. 1a); germ tubes may swell slightly at their apex, but appressoria are not formed (Rawlinson et al. 1978; Maddock 1979; Davies et al. 2000). No entry through stomata has been observed (Rawlinson et al. 1978; Maddock 1979) and scanning electron microscopy has shown infection hyphae, even those adjacent to stomata, apparently avoiding them (Davies et al. 2000) (Fig. 3b).

Evidence indicates that cuticular penetration by *P. brassicae* involves enzymatic degradation (Rawlinson et al. 1978; Maddock 1979; Davies 1997) by an extracellular cutinase, Pbc1 (Davies et al. 2000; Li et al. 2003). Inhibition of cutinolytic activity has been shown to decrease the efficiency with which *P. brassicae* infects *B. napus* (Davies et al. 2000) and disruption of the *Pbc1* gene results in mutants that are unable to penetrate the cuticle or produce disease symptoms (Li et al. 2003). There is potential, therefore, for *B. napus* resistance to *P. brassicae* to function through inhibition of fungal cutinases (Fig. 2a), although no such resistance has yet been reported in commercial cultivars.

A potential recognition point for the operation of major gene-mediated resistance to *P. brassicae* in *B. napus* is immediately after the penetration of the leaf surface (Fig. 2a). Although major gene-mediated resistance acting at the point of penetration is a

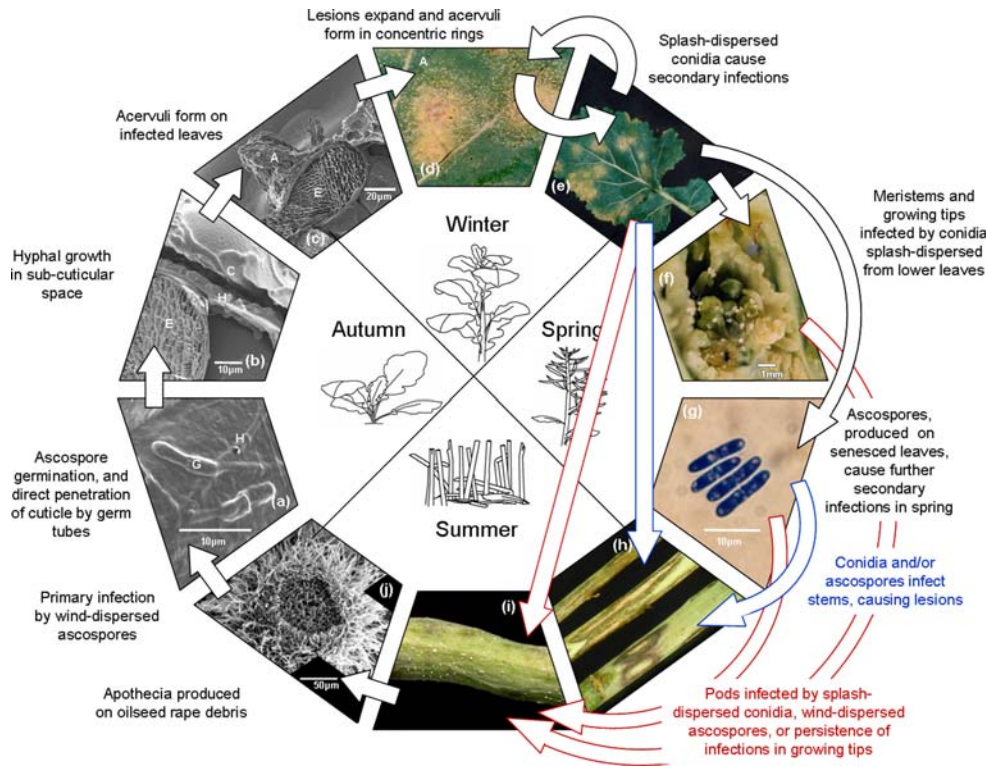

Fig. 1 Life cycle of *Pyrenopeziza brassicae* (light leaf spot) on winter oilseed rape (*Brassica napus*) in Europe. Photographs show: scanning electron micrograph (SEM) of germinating spore on *B. napus* (cv. Bristol) leaf, with germ tube (G) penetrating cuticle (Li et al. 2003) (**a**); SEM of a freeze fractured *B. napus* (cv. Hearty) leaf, with *P. brassicae* hyphae (H) growing in the space between the cuticle (C) and epidermal cells (E) (**b**); SEM of a freeze fractured *B. napus* (cv. Hearty) leaf, with conidiomata rupturing cuticle to form acervuli (A) (**c**); necrotic light leaf spot lesions with many *P. brassicae* acervuli, and dryness and cracking in the centre of the lesion (cv. Eurol) (**d**); *B. napus* (cv. Eurol) leaf with numerous *P. brassicae* lesions (**e**); *B. napus* meristem (cv. Cobra) with *P. brassicae* acervuli (**f**); light micrograph of *P. brassicae* ascospores, stained with cotton blue (**g**); *B. napus* (cv. Jet Neuf) stems with light leaf spot lesions (**h**); *B. napus* (unknown cultivar, June 2005) pod with *P. brassicae* acervuli (**i**); SEM of a *P. brassicae* apothecium on *B. napus* (cv. Bristol) debris, after 14 days of incubation (Gilles et al. 2001a) (**j**)

common resistance mechanism in other pathosystems (Kombrink and Schmelzer 2001), there have been no reports of a HR in *B. napus* immediately after infection by *P. brassicae*. During pathogenesis, *P. brassicae* displays a mode of nutrition typical of the hemibiotrophic plant pathogens within the Ascomycotina (Ashby 1997) and it is possible that through the subtlety of its sub-cuticular growth, *P. brassicae* evades major gene-mediated resistance operating through the immediate recognition of a pathogen elicitor by a host *R* gene.

After penetration, the fungus forms a hypomycelium and hyphae grow slowly through the sub-cuticular space between the cuticle and walls of the epidermal cells (Rawlinson et al. 1978) (Fig. 1b). This early phase of biotrophic growth is very subtle, with no cell penetration or systemic spread and consequently very few signs of infection (Ashby 1997). The inhibition of sub-cuticular growth is a potential mechanism for the operation of both major and minor gene-mediated *B. napus* resistance to *P. brassicae* (Fig. 2a). Quantitative PCR (qPCR) has shown differences in the accumulation of *P. brassicae* biomass between different *B. napus* cultivars (Measures 2006), although these results could be accounted for by differences in the frequency of successful infections.

An understanding of the pathogenicity determinants in *P. brassicae* can suggest targets for genetic resistance breeding (Ashby 1997; De Lorenzo and Ferrari 2002). For sub-cuticular growth, *P. brassicae* requires both space and nutrients. The observation of

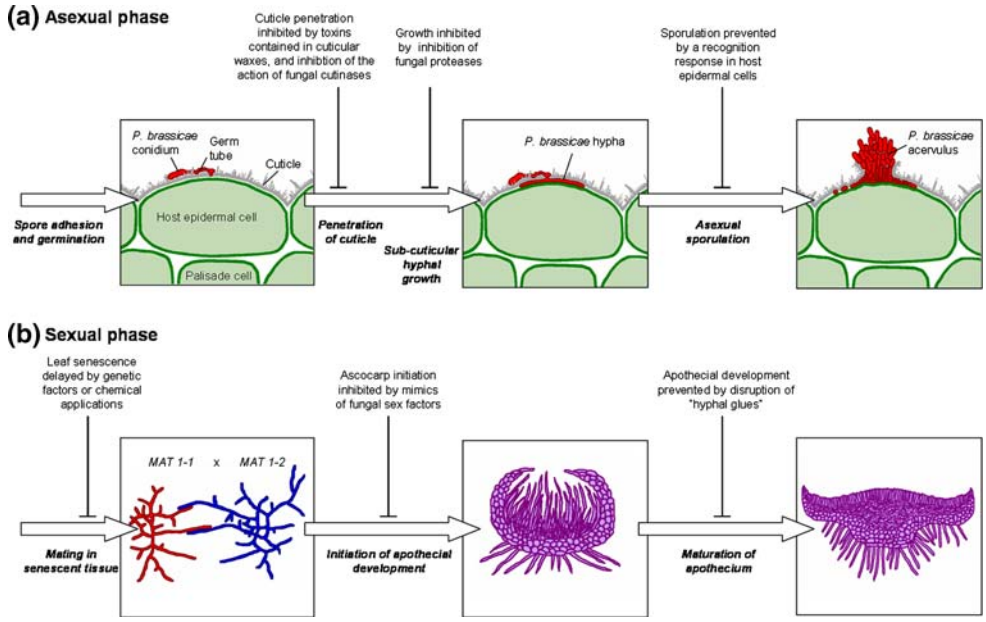

Fig. 2 Potential mechanisms by which *Brassica napus* resistance to *Pyrenopeziza brassicae* could operate, during the asexual phase (**a**) and the sexual phase (**b**) of the *P. brassicae* life cycle

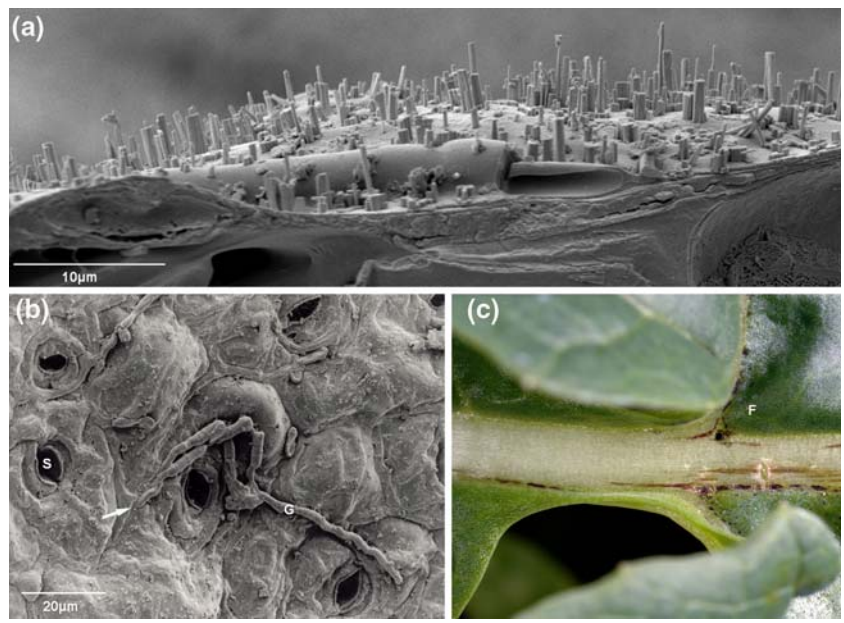

Fig. 3 Scanning electron micrograph (SEM) of the upper surface of a freeze-fractured *Brassica napus* (line from Peter Werner, CPB Twyford) leaf, showing the structure of cuticular waxes (**a**); SEM of *B. napus* (cv. Shogun) leaf, 7 days after inoculation with *Pyrenopeziza brassicae*, showing avoidance of stomata (S) by germ tubes (G) and direct penetration of the cuticle (arrow) (Davies 1997) (**b**); leaf of a *B. napus* line (from Peter Werner, CPB Twyford) with major gene resistance to *P. brassicae*, 23 days after inoculation with *P. brassicae*, showing black necrotic flecking (F) (**c**).

the *Pbc1* transcript during *in planta* growth suggests an additional role for the extracellular cutinase at this stage (Li et al. 2003). An extracellular protease, Psp1, with a role in pathogenicity has also been identified (Ball et al. 1991; Batish et al. 2003) and shown to be present during the growth of *P. brassicae in planta* (Batish et al. 2003). A likely role for Psp1 in pathogenesis is the breakdown of host intercellular matrices, providing space for sub-cuticular hyphal growth. Other possible functions could include the degradation of host signal proteins involved in the HR or the release of nutrients from the apoplast through the degradation of host cell wall or membrane proteins (Ball et al. 1991; Ashby 1997).

Cytokinins have been implicated in the pathogenicity of *P. brassicae* to *B. napus*, with a possible role in mediating the provision of nutrients to the fungus through the alteration of host metabolism and diversion of nutrients to the site of infection, although it is unclear whether *P. brassicae* utilises plant-derived cytokinins or synthesises them de novo (Ashby 1997, 2000; Murphy et al. 1997). An involvement of cytokinins in defence against free radicals, therefore limiting processes that contribute to the HR, has also been proposed (Beckman and Ingram 1994; Ashby 2000). Further evidence for the role of cytokinins in this interaction is provided by the constitutive expression of the *ipt* (isopentenyltransferase: an enzyme catalysing a key step in de novo cytokinin biosynthesis) gene from *Agrobacterium tumefaciens* in *P. brassicae*, which leads to rapid growth of the pathogen and premature rupturing of the *B. napus* cuticle to accommodate the pathogen mycelium (Ashby 2000). The identification of the extracellular protease and cytokinins as key pathogenicity components (Ball et al. 1991; Ashby 1997) suggests possibilities for disease control, for example, through the development of transgenic lines constitutively expressing cutinase or protease inhibitors, or through the modification of the protease substrate to prevent cleavage (Ashby 1997).

Asexual sporulation (Fig. 1c–d) marks the end of the subtle, symptomless phase of *P. brassicae* growth. Induction of sporulation may be biomass- and/or space-dependent, with limitation leading to a stress response in the pathogen and the onset of asexual sporulation (Ashby 1997). Some downward growth of hyphae between cells of the upper mesophyll has been observed beneath acervuli (Maddock 1979) and it is possible that receptors within the mesophyll could recognise *P. brassicae* hyphal components: a potential point for major gene-mediated resistance to act (Fig. 2a). Resistance preventing asexual sporulation in commercial cultivars would be likely to have a considerable effect on the development of light leaf spot epidemics in winter oilseed rape crops, as the disease is polycyclic, spreading throughout the winter and spring by splash dispersal of conidia (Gilles et al. 2001b) (Fig. 1d–i). Conidial infection would be subject to the same potential resistance mechanisms at the points of adhesion, germination and penetration as those for ascospore-initiated infection described above.

Further potential targets for resistance to *P. brassicae* occur in the sexual phase of the pathogen life cycle (Fig. 2b). Production of ascospores on senescent infected leaves in the spring may contribute to late-season leaf, stem and pod infections (Fig. 1g), while ascospores released in the autumn from oilseed rape stem and pod debris provide the main source of inoculum to initiate epidemics in the new crops (Gilles et al. 2001b) (Fig. 1j). The release of ascospores in spring could be decreased by delaying leaf senescence, through genetic or physiological factors in the crop (Chandlee 2001) or by the application of chemicals with a 'greening' effect, such as strobilurin fungicides. Once leaf senescence has occurred, it is possible that factors could be generated in the host to inhibit apothecial development by mimicking fungal sex factors (Ashby 1997) or disrupting the pathogen 'glues' required for hyphal aggregation (Ashby 1998).

QTL involved in minor gene-mediated resistance to *P. brassicae* have been mapped by Pilet et al. (1998). Their experiments on a *B. napus* doubled haploid mapping population involved visual assessment of light leaf spot severity on leaves at the stem extension stage and then on stems when the pods were formed (Pilet et al. 1998). Genomic regions contributing to resistance to *P. brassicae* were identified at each assessment point, but results were inconsistent between seasons (Pilet et al. 1998). Disease escape and tolerance may also play a role in limiting the effect of *P. brassicae* on *B. napus*, but little work has been done to assess their importance relative to genetic resistance. Tolerance could occur in cultivars that are particularly frost-resistant, which would incur less yield loss due to infection by *P. brassicae* in the winter months. Disease escape could be related to characteristics such as canopy structure, plant height, speed of plant growth and timing of key developmental stages. If the early infection of the meristem is an important route for pod infection, then cultivars with leaves that initially grow very close to the meristem could be protected from this initial meristem infection and thus avoid pod damage later in the season. Also, tall cultivars could potentially develop less light leaf spot than shorter cultivars through the avoidance of the splash-dispersal of conidia from the lower leaves to upper leaves, flowers and pods (Pielaat et al. 2002).

The relative importance of different components of resistance depends on the epidemiology of light leaf spot on winter oilseed rape, and particularly which stages of the *P. brassicae* life cycle contribute most to yield loss. There has been little research into the main causes of yield loss (Gilles et al. 2000). It is generally assumed that most yield loss arises through a decrease in leaf photosynthetic area and plant vigour, but pod disease (Fig. 1i), which can result in seed-loss from pods before or during harvest and a reduction in pod canopy size, may also be important (Fitt et al. 2003). The main route for infection of flowers and pods is unclear (Gilles et al. 2000) (Fig. 1). They could become infected by splash-dispersed conidia from lower leaves or other infected plants, or through air-borne ascospores released from leaf debris in April and May (McCartney and Lacey 1990), or by persistence of earlier infections of the meristem, which are then carried upwards by stem extension into the developing pods. qPCR data have shown that there is a measurable concentration of *P. brassicae* DNA present in the oilseed rape meristems in the winter, which relates to levels of light leaf spot disease observed at a field-site later in the spring (Latunde-Dada et al. 2007). If the main route by which pods are infected is through ascospores or conidia, then resistance operating to prevent initial penetration or asexual sporulation would be most effective, whereas if colonisation of the meristems provides the main route for infection of pods, the inhibition of sub-cuticular growth is likely to be more important.

Major gene-mediated resistance to *P. brassicae* in *B. napus*

The first report of major gene-mediated resistance to *P. brassicae* in *B. napus* was by Bradburne et al. (1999). They introgressed resistance from wild accessions of *Brassica oleracea* (CC) and *Brassica rapa* (AA) into a standard winter oilseed rape background, producing a set of doubled haploid lines. Two different resistance phenotypes were observed, corresponding to those of the original parents: no visible symptoms/sporulation (*B. rapa* parent) and black necrotic flecking (Fig. 3c) accompanied by limited sporulation (*B. oleracea* parent) (Bradburne et al. 1999). Linkage analysis and the study of inheritance patterns suggested that these two responses were controlled by two unlinked genes (Bradburne et al. 1999). Few markers were used, but the positioning of each gene did correspond to linkage groups from the A and C genomes, as predicted from the parental phenotypes (Bradburne et al. 1999).

PBR1, positioned on linkage group N1, was associated with a symptomless resistance phenotype after infection (Bradburne et al. 1999). The mode of action of this gene is as yet unknown. It could operate by preventing initial infection or sub-cuticular growth, but Bradburne et al. (1999) did not report any associated hypersensitive response or investigate the effect of the gene on pathogen growth in planta.

The second gene (*PBR2*) was positioned on linkage group N16 (Bradburne et al. 1999). This locus was associated with the black necrotic flecking phenotype, due to the necrosis of epidermal cells (Measures 2006), accompanied by little or no sporulation observed on inoculated cotyledons (Bradburne et al. 1999). Recent work on related material, involving scanning electron microscopy, qPCR to measure amounts of pathogen DNA and the re-isolation of viable *P. brassicae*, indicates that this gene does not operate to prevent infection, but does inhibit in planta proliferation and asexual conidiospore production (Measures 2006). The major gene-mediated resistance reported by Bradburne et al. (1999) offers the potential for effective resistance to *P. brassicae* to be incorporated into commercial oilseed rape cultivars. However, as this gene does not prevent infection or kill the pathogen hyphae, sexual reproduction is still possible on senescent debris from 'resistant' plants whose leaf surfaces had been artificially inoculated with conidia (E. Boys, Rothamsted Research, UK, unpubl.). Although this implies that 'resistant' cultivars possessing this gene could still contribute ascospore inoculum to epidemics in the spring, this may be negligible due to the much lower level of natural inoculum and the inability of the fungus to multiply during its asexual phase.

Measurement of *B. napus* resistance to *P. brassicae*

Different techniques are suitable for studying different aspects of resistance to *P. brassicae* in *B. napus* (Table 1). Large scale field experiments are a well-established and valuable tool for assessing overall

resistance under the environmental conditions to which commercial oilseed rape crops are exposed. Such experiments are essential for producing recommended cultivar lists, such as those published by the HGCA in the UK (www.hgca.com/varieties) and GEVES in France (www.geves.fr). Field experiments can be exposed to natural inoculum, especially in areas like Scotland where there are large amounts of natural inoculum each season. One potential practical problem with large, naturally inoculated field experiments is that of sampling. Early in the season, the distribution of infected plants tends to be random because primary infection is by air-borne ascospores. As epidemics progress, however, the short-distance splash-dispersal of conidia results in disease spatial patterns that become more aggregated (Evans et al. 2003). To estimate disease incidence accurately, early in the season, large numbers of samples are then required (Evans et al. 2003). To avoid these problems, field experiments can be inoculated with infected oilseed rape debris from previous seasons scattered within plots to ensure measurable levels of disease and/or by spraying with suspensions of *P. brassicae* conidia, although this is generally only possible in small plot experiments. The main limitation of field experiments is that they do not allow different components of genetic resistance to be easily distinguished, and differentiating genetic resistance from tolerance or disease escape can be impossible.

A practical problem that may affect all field experiments is the accurate assessment of disease: in situ plot disease assessments, commonly used in field experiments, are open to environmental

Table 1 An evaluation of the different types of experiment and methods available for assessing resistance of *Brassica napus* to *Pyrenopeziza brassicae*

Technique	Advantages	Disadvantages
Field experiment[b, f, h, i]	Testing cultivars in the environment they will be grown in commercially	Different components of resistance cannot be separated
	Plants can be grown through to maturity	Large numbers of samples are required to counteract aggregation of disease unless experiment is artificially inoculated
Polyethylene tunnel experiment[a]	Testing plants in near commercial environment	Less control than controlled environment
	More control than in the field	Conditions not reproducible
	Plants can be inoculated Cheaper than controlled environment	
Controlled environment experiment[a, b, f]	No sampling problems	Not representative of commercial growth conditions
	Reproducible conditions	
	Smaller, quicker and easier than field experiments	Potential problems with comparing seedling resisitance to that of adult plants
	Variables can be manipulated to investigate different components of resistance	Cannot investigate tolerance or disease escape
In situ visual assessment of symptoms[a, c, f, h]	Quick and easy	Unreliable, particularly in field experiments, because acervuli may be washed off by rain, or produced in low numbers under low humidity
Sampling, incubation, then visual assessment of symptoms[a, b]	Promotes sporulation which leads to a more reliable assessment of infection than direct in situ assessment	Time-consuming
PCR assessment of *P. brassicae* DNA[d–f, g, i]	Can detect early, symptomless infection and is more sensitive than visual assessment	No information on the effect of the disease on the crop or yield
	Can differentiate between mating types and quantify pathogen DNA	No distinction between living and dead hyphae

[a] Bradburne et al. (1999); [b] Fitt et al. (1998a); [c] Fitt et al. (1998b); [d] Foster et al. (1999); [e] Foster et al. (2002); [f] Karolewski et al. (2006); [g] Latunde-Dada et al. (2007); [h] Pilet et al. (1998); [i] Thomas et al. (2004)

influence, although it may be possible to overcome problems by applying irrigation when conditions do not favour the disease. The main symptoms of light leaf spot are the white *P. brassicae* acervuli produced on leaves, but the spores may be washed off by rain (Fitt et al. 1998a) or produced in low numbers under low humidity. As these symptoms are unreliable in the field, it is often necessary to resort to more subjective measures, such as the assessment of frost-scorch, appearance of wetness films or pale and faint yellow blushing on the leaf, which may be confounded by physiological characteristics. A more reliable alternative to in situ plot assessment is the collection of samples from the field and their assessment after several days of incubation at high humidity to promote sporulation (Fitt et al. 1998a). Karolewski et al. (2006) found that, in field experiments, there was a poor correlation between results of in situ plot assessments and assessments after incubation, except in severe epidemics; particularly in dry periods, in situ plot assessments failed to detect widespread infection. A comparison of assessment methods by Fitt et al. (1998b) suggested that the assessment of percentage plants with light leaf spot was a reliable disease assessment method and that the more time-consuming assessment of percentage leaves affected added little extra information and the difficult, subjective assessment of percentage leaf area affected was inconsistent (Fitt et al. 1998b). For cultivar evaluation work requiring high throughput visual assessment, objective keys have, however, proved effective in generating more consistent data (http://www.defra.gov.uk/planth/pvs.VCU_DUS.htm). Cultivar yield data may also be collected, but the assessment of resistance to *P. brassicae* from these data is difficult because separation of effects on yield of light leaf spot from those of other diseases, unrelated cultivar traits and environmental factors is complex.

Experiments in polyethylene tunnels or glasshouses allow more control than field experiments while still maintaining 'near commercial' conditions. They allow plants to be individually inoculated with a uniform inoculum and enable environmental conditions, such as water status, to be more easily monitored and manipulated. The conditions in these experiments are not reproducible, but they provide a less expensive alternative to controlled environment experiments.

Unlike field experiments, artificially inoculated experiments in controlled environment conditions allow different stages of the pathogen life cycle and different components of resistance to be studied in isolation, through the manipulation of variables such as plant age at inoculation and source of inoculum. They also reduce variation due to environmental factors and eliminate problems of sampling strategy. For studies on gene-for-gene interactions, inoculum from single-spore isolates of *P. brassicae* is necessary and the OREGIN project (www.oregin.info) is producing a collection of single-spore isolates to help facilitate this (Latunde-Dada et al. 2006). This form of inoculum produced in culture may be less infective than that produced on plants, so it may be necessary to regularly passage isolates through plants. To investigate minor gene-mediated resistance in different cultivars, a natural inoculum, typically a conidial suspension derived from field populations of *P. brassicae* sporulating on different cultivars, should be more informative. Controlled environment experiments on young plants (Karolewski et al. 2006) or cotyledons (Bradburne et al. 1999) are generally smaller, quicker and easier than large-scale field trials. They are likely to be more useful in studying the genetic basis of resistance than for assessing the effects of resistance on development of epidemics in crops (Karolewski et al. 2006), although if components of resistance that have a large impact on infection levels in the field, such as the length of the latent period, can be identified in controlled environment experiments, a good correlation with field data could be expected.

Results of controlled environment experiments using seedlings often correlate poorly with those from field experiments (Bradburne et al. 1999; Karolewski et al. 2006). For many pathosystems, such as *Leptosphaeria maculans—B. napus*, different components of resistance are thought to operate in seedlings and in older plants (Ballinger and Salisbury 1996). This may also be true for *P. brassicae—B. napus*, although it is possible that the large effect of environmental factors on the incidence of light leaf spot in crops contributes to the poor correlation. The differences between resistance components operating in seedlings and older plants (Karolewski et al. 2006) have yet to be fully explained. The differences in morphology and gene expression between cotyledons, leaves and pods make it likely

that differences in resistance exist between these organs.

New molecular techniques offer alternatives to the visual assessment of cultivar resistance to *P. brassicae* in both field and controlled environment experiments. Primers have been developed for use in PCR, thus allowing the detection of *P. brassicae* within *B. napus* tissue (Foster et al. 1999, 2002; Latunde-Dada et al. 2007). PCR is more sensitive than visual assessment, enabling the detection of infection in crops up to two months earlier, even when visual assessments are made after incubation to promote sporulation (Foster et al. 2002; Karolewski et al. 2006). The PCR technique can also be extended to provide more information than simply the presence or absence of *P. brassicae* within tissue. A three-primer technique has been developed that allows the determination of mating types present within a sample of *B. napus* tissue (Foster et al. 1999, 2002) and qPCR can be used to assess the amount of *P. brassicae* DNA in a sample (Thomas et al. 2004; Latunde-Dada et al. 2007). qPCR has potential for use in predicting severity of light leaf spot epidemics, if the amount of *P. brassicae* DNA present early in the season correlates with severity of epidemics later (Kenyon et al. 2004; Latunde-Dada et al. 2007).

A disadvantage of using PCR to assess resistance to *P. brassicae* is that it is not possible to determine whether the DNA detected is from living or dead pathogen tissue. It is also possible that the highly sensitive reaction could detect DNA from spores on the surface of the plant in addition to hyphae within the tissue, potentially resulting in the classification of resistant plants as susceptible. However, in controlled environment conditions this could be compensated for by the use of appropriate controls, and it may not be a problem in samples from field experiments because inoculum concentrations are very low (Karolewski et al. 2006). PCR assessment is particularly useful for detecting symptomless infection. When used in conjunction with visual disease assessments, it is a valuable tool for investigating how resistance operates. In isolation, however, it cannot provide all the information needed by breeders, growers and recommended list compilers, such as the effects of the disease on crop development and yield.

Antibody-based techniques such as ELISA (enzyme-linked immunosorbent assays), although often used in plant pathology (e.g. Balesdent et al. 1995), have yet to be successfully applied to the *P. brassicae—B. napus* pathosystem. Attempts have been made to apply ELISA to the diagnosis and quantification of *P. brassicae*, but problems were encountered with identifying novel *P. brassicae* genes and expressing candidate proteins at levels suitable for antibody screening (S. J. Foster, The Sainsbury Laboratory, UK, unpubl.). Although ELISA is a less expensive technique with more potential to be easily used in the field, since it is chiefly useful for quantifying pathogen biomass, the development of reliable qPCR protocols for *P. brassicae* reduces the necessity for ELISA methods to be developed.

Towards sustainable resistance to *P. brassicae* in *B. napus*

Pyrenopeziza brassicae is polycyclic, with both sexual and asexual reproductive systems, a wide host range among the brassicas and a potentially high population size on volunteer *B. napus* plants (Maddock et al. 1981). All these characteristics increase the potential for the pathogen to evolve to render major gene-mediated resistance ineffective or gradually erode minor gene-mediated resistance (McDonald and Linde 2002). Majer et al. (1998) reported considerable genetic diversity among field isolates of *P. brassicae*, implying frequent sexual reproduction, and both mating types have been found in the UK, Ireland, Poland and France (Lacey et al. 1987; Ball et al. 1990; Majer et al. 1998; Karolewski et al. 2004; K. Downes, Rothamsted Research, UK, unpubl.).

Major gene-mediated resistance is usually rendered ineffective in a 'boom-and-bust' manner, as the widespread growth of a cultivar with a single *R* gene imposes strong directional selection on the pathogen population (McDonald and Linde 2002). Examples of this can be found in the *L. maculans—B. napus* pathosystem (Rouxel et al. 2003; Sprague et al. 2006). Minor gene-mediated resistance is predicted to be more durable (Hammond-Kosack and Parker 2003; Agrios 2005) because it is effective against all strains of a pathogen (Lindhout 2002; Parlevliet 2002). However, it is likely that populations of pathogens, such as *P. brassicae*, could also evolve to erode the efficacy of minor gene-mediated resistance (McDonald and Linde 2002).

Variability in cultivar resistance to *P. brassicae* has been observed in the UK national trial system (www.hgca.com), where cultivars are grown in different regions or are included in field experiments inoculated with a natural mixture of isolates. Some cultivars which appeared to have relatively good resistance when initially evaluated showed a decline in resistance over time. Figure 4a, b shows the severity of light leaf spot, measured as percentage leaf area affected, on cvs Apex and Bristol over a period of fifteen seasons. Cultivar Apex dominated the UK winter oilseed rape market during the 1990s (Fig. 4a), while cv. Bristol declined in market share from the early 1990s onwards (Fig. 4b). Both cultivars remained in some trials until 2000. Disease severities were initially similar, before cv. Bristol appeared to become much more susceptible in 1993–1994 (Fig. 4b). This sudden shift in resistance suggests cv. Bristol carried a major resistance gene that was rendered ineffective. Cultivar Apex retained a moderate level of resistance until the late 1990s, when disease severity in national trials increased steadily over several seasons (Fig. 4a). The more gradual shift in resistance observed for cv. Apex suggests that this may be the gradual erosion of resistance mediated by several minor genes. For both cultivars, the increase in susceptibility to *P. brassicae* occurred after the cultivars had been grown very widely across the UK, suggesting that the high selection pressure exerted on *P. brassicae* caused directional changes to render the resistance of the cultivars ineffective. While these changes occurred over a relatively long period, other shifts have occurred more quickly; for example cv. Recital

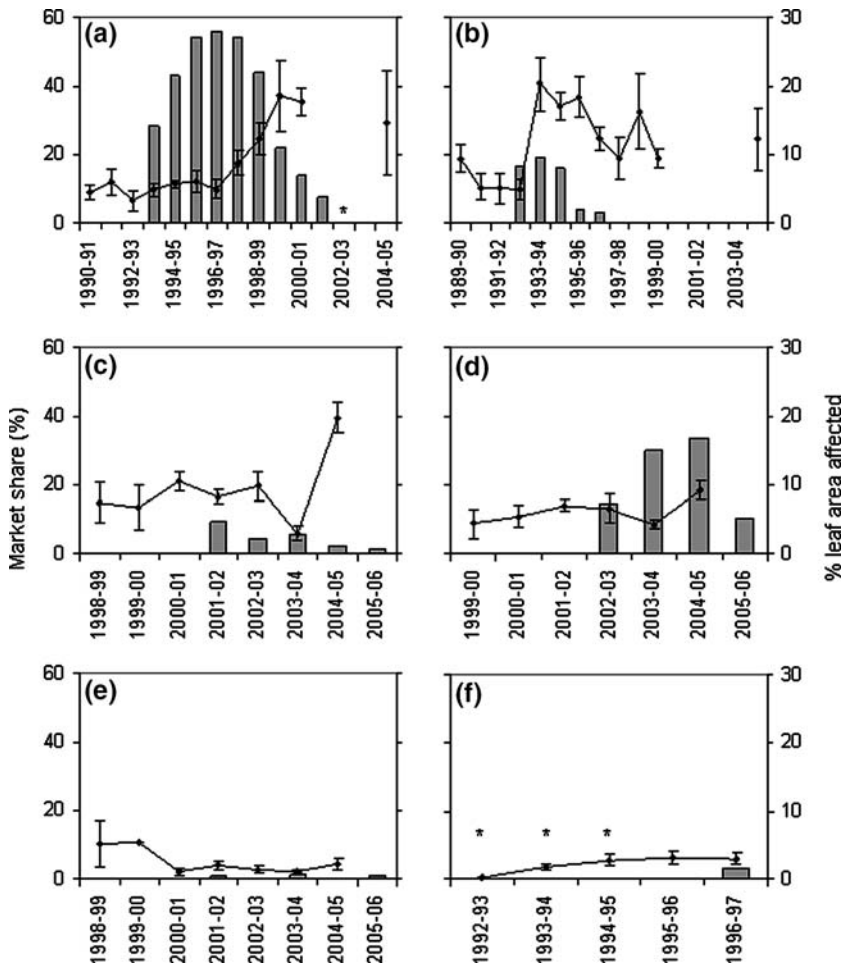

Fig. 4 Changes over time in the susceptibility to *Pyrenopeziza brassicae* (light leaf spot) of oilseed rape cultivars (–♦–)Apex (**a**), Bristol (**b**), Recital (**c**), Winner (**d**), Elan (**e**) and Nickel (**f**), in relation to their UK market share (filled bars). * indicate missing data on market share. Market share data are based on weights of certified seed available for sowing in autumn, and do not include farm-saved, imported or held-over seed. Disease data (% leaf area affected with light leaf spot assessed each season) from HGCA/NIAB Recommended List trials at sites around the UK, with each data point the mean of 1–22 sites. Error bars indicate ± 1 standard error of mean leaf area affected by *P. brassicae*. Current Recommended List data can be found at www.hgca.com/varieties

appeared to become suddenly more susceptible after about six seasons, despite having a relatively small market share (Fig. 4c). One explanation for this could be the extensive growth of related cultivars with the same resistance genes.

In contrast to these decreases in resistance, cv. Winner, with a large market share from 2002 onwards, has not yet appeared to change notably in resistance (Fig. 4d). This could be due to heterogeneity within the cultivar. Cultivars Elan and Nickel have also remained resistant during a relatively long period in trials, though neither of them achieved a large market share (Fig. 4e, f), and hence will not have exerted such a strong selection pressure on the *P. brassicae* population. Although not achieving a large market share in the UK as a whole, cv. Elan does have a significant local market in Scotland and the resistance still appears to be stable, which may be because cv. Elan is a hybrid unlike the other cultivars discussed.

Improving resistance to *P. brassicae* is an important objective for oilseed rape breeders. The introgression of genes from other *Brassica* species using synthetic lines is possible and can provide a useful source of novel resistance, but it can be difficult to recover agronomically acceptable cultivars because undesirable traits are introduced with the target genes (Chen and Heneen 1989; Bradburne et al. 1999). Furthermore, deployment of major gene-mediated resistance derived from wild *Brassica* species may offer only a short-term yield benefit, as virulent pathogen strains are already likely to exist, as seen in the *L. maculans*—*B. napus* pathosystem (Li et al. 2005; Rouxel et al. 2003; Sprague et al. 2006).

Although few *R* genes have been identified for the *P. brassicae*—*B. napus* interaction, QTL involved in minor gene-mediated resistance have been mapped (Pilet et al. 1998). Sequence information from markers flanking these regions could be usefully combined with the improved knowledge of synteny between the *B. napus* and *Arabidopsis thaliana* genomes to identify potential resistance genes and to aid the development of effective markers for gene introgression (Snowdon and Friedt 2004). Genetic markers for resistance genes can be used to assist both screening and the combination of genes to create a more durable resistance (Snowdon and Friedt 2004).

Genetic engineering potentially offers an alternative to traditional breeding methods, enabling the insertion of cassettes of linked resistance genes (McDonald and Linde 2002). The identification of key *P. brassicae* pathogenicity determinants offers clear targets for genetic engineering. However, in addition to a potential cost of genetic resistance to yield in the absence of the pathogen (Brown 2002), genetic engineering of resistance is currently not a viable solution in Europe because of legislation and consumer acceptance issues.

There are various techniques that can be used to limit the likelihood of resistance being rendered ineffective. One possibility is the deliberate selection of *R* genes for which the corresponding virulence mutations impose a large negative fitness cost on the pathogen (McDonald and Linde 2002). It is also possible to 'pyramid' several resistance genes in a single cultivar, meaning that the pathogen would have to undergo several corresponding mutations to acquire virulence (McDonald and Linde 2002). It may also be beneficial to deploy both major and minor gene-mediated resistance in a single cultivar. As proposed for *L. maculans*, these approaches should be combined with good management practices (Li et al. 2005), which aim to limit the selection pressure imposed on the pathogen and to decrease the size of the pathogen population (Aubertot et al. 2006). Such management practices could include the rotation of resistant cultivars with different resistance genes in both time and space to create a disruptive selection pressure (McDonald and Linde 2002).

Tackling infection by stealth: can resistance to *P. brassicae* be sustainable?

A greater understanding of mechanisms of *B. napus* resistance to *P. brassicae* and the deployment of a number of different mechanisms may help to provide sustainable resistance to *P. brassicae* in Europe. Study of the epidemiology of light leaf spot can provide insights into potential resistance mechanisms, which can guide the development of more resistant winter oilseed rape cultivars. Further study of the symptomless stage of the pathogen life cycle would be valuable, to investigate the extent of physical and chemical interactions between the

pathogen hyphae and host tissue. Further investigation of the major gene-mediated resistance reported by Bradburne et al. (1999), to identify the mode of action of each gene is needed to predict the likelihood of this resistance being rendered ineffective if deployed commercially.

A range of techniques and molecular tools are required to investigate both the early symptomless colonisation by *P. brassicae* and the array of resistance mechanisms that together contribute to the phenotypes observed in *B. napus* crops. Seedling experiments in controlled environment conditions should be used to investigate major gene-mediated resistance. Field experiments over a growing season are required to investigate other components of resistance and the overall disease phenotype of cultivars in commercial cropping conditions. It is also important to appreciate the shortcomings of different symptom assessment methods, particularly in situ visual assessments, and to combine them with more sensitive techniques.

The potential for the *P. brassicae* population to evolve rapidly to render major gene-mediated resistance ineffective suggests that minor gene-mediated resistance is likely to play an important role in sustainable resistance. Minor gene-mediated resistance may not be completely durable (Fig. 4b), but it is likely to be more durable than major gene-mediated resistance (Lindhout 2002; Parlevliet 2002) (Fig. 4a). For resistance management strategies (e.g. Aubertot et al. 2006) to be effectively applied to the *P. brassicae—B. napus* pathosystem, further study of the genetic variation in the *P. brassicae* population is needed to determine whether specific pathotypes/races exist, and to assess their potential to render ineffective the components of resistance. Controlled environment experiments with susceptible cultivars could then be used to establish whether there is a fitness cost to virulence. It is thought that loss or mutation of avirulence alleles by pathogens to render resistance ineffective usually carries a fitness cost, and although it has been demonstrated less frequently for fungal pathogens than for bacteria (White et al. 2000), this phenomenon has been demonstrated in the *L. maculans—B. napus* pathosystem (Huang et al. 2006). It is useful to know whether this is the case for a particular resistance gene when deciding on the best resistance management strategies by which to deploy it (Pietravalle et al. 2006) with the aim of improving resistance to *P. brassicae* in commercial oilseed rape cultivars and decreasing fungicide use.

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