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Evolution of virulence in fungal plant pathogens: exploiting fungal genomics to control plant disease

Barbara J. Howlett[1]
School of Botany, the University of Melbourne, VIC 3010, Australia

Rohan G.T. Lowe[2]
School of Botany, University of Melbourne, VIC 3010, Australia

Stephen J. Marcroft
Marcroft Grains Pathology, Grains Innovation Park, Horsham, VIC 3400, Australia

Angela P. van de Wouw
School of Botany, the University of Melbourne, VIC 3010, Australia

Abstract: The propensity of a fungal pathogen to evolve virulence depends on features of its biology (e.g. mode of reproduction) and of its genome (e.g. amount of repetitive DNA). Populations of *Leptosphaeria maculans*, a pathogen of *Brassica napus* (canola), can evolve and overcome disease resistance bred into canola within three years of commercial release of a cultivar. Avirulence effector genes are key fungal genes that are complementary to resistance genes. In *L. maculans* these genes are embedded within inactivated transposable elements in genomic regions where they are readily mutated or deleted. The risk of resistance breakdown in the field can be minimised by monitoring disease severity of canola cultivars and virulence of fungal populations using high throughput molecular assays and by sowing canola cultivars with different resistance genes in subsequent years. This strategy has been exploited to avert yield losses due to blackleg disease in Australia.

Key words: avirulence, *Brassica napus*, effector, *Leptosphaeria maculans*

Introduction

Plant pathogenic fungi evolve in concert with their hosts to invade and overcome innate and induced defense responses. These disease processes are complex and vary among different plant-fungal interactions, but generally molecules termed effectors are involved. Effectors include small secreted proteins, which facilitate infection and/or induce defense responses (Hogenhout et al. 2009). One function of effectors is as avirulence proteins, which in gene for gene interactions render the pathogen unable to attack plant genotypes with the corresponding resistance proteins. Other effectors such as proteinaceous toxins confer susceptibility on plant cultivars with the corresponding susceptibility gene in an inverse gene for gene interaction (Oliver and Solomon 2010).

Improved technologies for functional analyses of genes, as well as a plethora of sequenced fungal genomes, have greatly aided studies aimed at identifying disease-related genes, including effectors. Such research can give clues about the evolution of virulence in fungal populations. It also can lead to practical outcomes for disease control. In this paper, which is based on the 2013 John Karling lecture delivered by Barbara Howlett, we describe how application of knowledge about fungal genome structure, avirulence genes and biology of blackleg disease has enabled a multidisciplinary team to minimize yield losses in the oilseed crop, canola, in Australia.

Blackleg disease of canola

The dothideomycete, *Leptosphaeria maculans*, is a member of a complex of closely related species (*L. maculans*/*L. biglobosa* species complex) and causes blackleg, the major constraint to production of canola (*Brassica napus)* worldwide (Howlett 2004). The primary inoculum is from ascospores released from pseudothecia on infested canola stubble (residue or trash) (Fig. 1). In Australia this generally occurs during rainfall that coincides with the sowing period in late autumn. Ascospores land on cotyledons and leaves, germinate and invade the plant via wounds or via the stomatal aperture, where gaseous exchange occurs. The fungus then colonizes intercellular spaces and lesions are visible within about 10 d. After growth down the petiole and stem in a visually symptomless manner, the fungus finally invades and kills cells of the cortex, resulting in a blackened canker that may girdle the base of the stem; hence the name ''blackleg''. Often plants then fall over and seeds cannot be harvested. Sexual crossing occurs on stubble over summer and the resultant windborne spores infect next season's crop (Fig. 1).

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[1] Corresponding author. E-mail: bhowlett@unimelb.edu.au
[2] Current address: Department of Biochemistry, La Trobe University, Melbourne VIC 3086, Australia

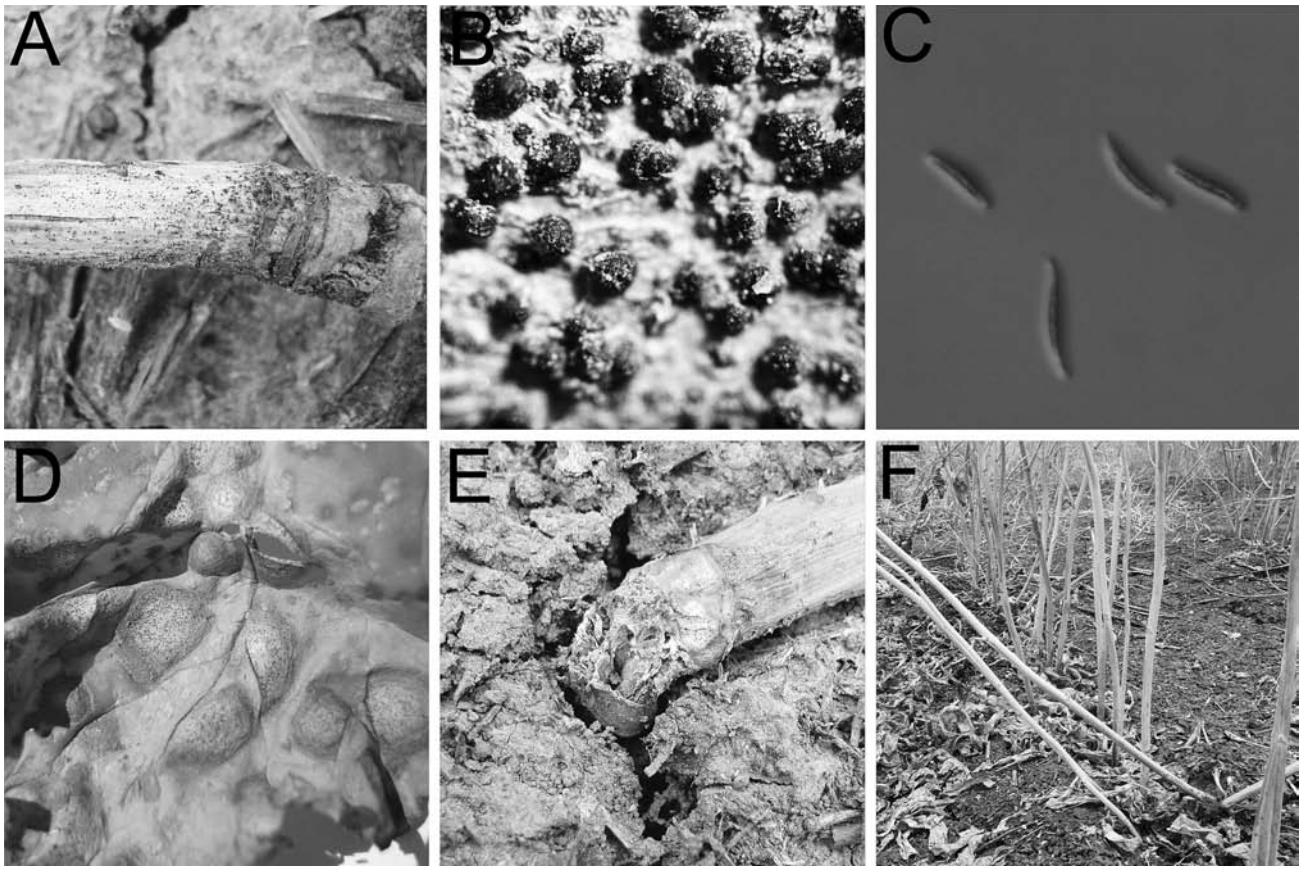

FIG. 1. Life cycle of *Leptosphaeria maculans* on canola (*Brassica napus*) in Australia. Pseudothecia on infected canola stubble (A, B) release windborne ascospores (C) that infect cotyledons and leaves where pycnidia are produced (D). The fungus grows down the petiole and stem. Cankers often girdle the stem, and plants fall over (E, F).

Blackleg disease is controlled by minimizing exposure of new crops to stubble from previously sown crops. This is done by crop rotation (i.e. growing canola, then cereals or pulses in subsequent years within an individual field). In Australia it is recommended that new canola crops be sown at least 500 m from stubble of a canola crop from the previous year (Marcroft et al. 2003). Also seed treatment with fungicide and foliar spraying can control disease. However, the major approach to disease control is breeding and sowing disease-resistant cultivars, and this strategy is the focus of this paper. For many plant pathogens there are two types of resistance: seedling (major or single gene) and adult plant (minor or quantitative). For seedling resistance *L. maculans* has a gene for gene interaction with *B. napus*. Several resistance genes have been mapped, but only one, *LepR3*, has been cloned (Delourme et al. 2006, van de Wouw et al. 2009, Long et al. 2011, Larkan et al. 2013). Five avirulence genes have been cloned in *L. maculans* (*AvrLm1, AvrLm4-7, AvrLm6, AvrLm11, AvrLmJ1*) (Gout et al. 2006, Fudal et al. 2007, Parlange et al. 2009, Balesdent et al. 2013, van de Wouw et al. 2014a). The molecular basis of the interaction between these key proteins in the fungus and the plant is as yet unknown.

BREAKDOWN OF BLACKLEG DISEASE RESISTANCE

The long-term effectiveness of resistance genes in crop cultivars is influenced by biology of the pathogen and its propensity to undergo recombination and mutation, particularly of avirulence genes. Pathogens that pose a high risk for overcoming resistance are those with sexual and asexual reproduction, effective spore dispersal and large populations (McDonald and Linde 2002). *Leptosphaeria maculans* is in this high risk category, in that it outcrosses prolifically in the field, windborne ascospores are dispersed up to 8–10 km and large numbers of lesions with pycnidia are present on leaves and cotyledons (Howlett et al. 2001). Extensive sowing of canola cultivars with major gene resistance results in strong selection pressure upon fungal populations.

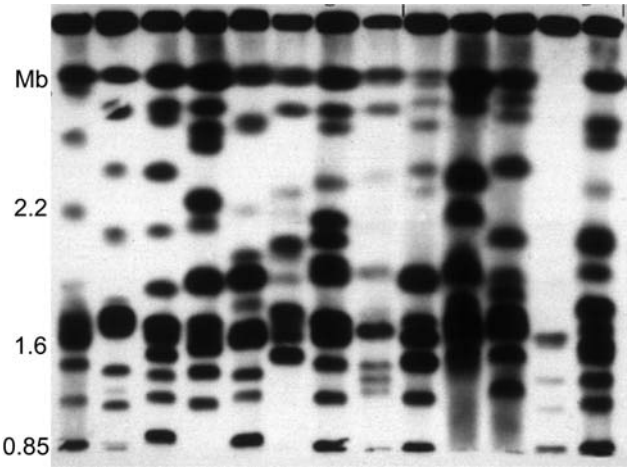

FIG. 2. Chromosomal length polymorphisms in *Leptosphaeria maculans*. Chromosomal DNA was prepared from a range of individual *L. maculans* isolates and subjected to pulsed field gel electrophoresis in a contoured clamped homogeneous electric field (CHEF). Conditions: 0.9% agarose gel, 0.5× Tris Borate EDTA buffer; switching interval 500 s for 72 h at 80 V followed by 420 s for 48 h at 100 V. The gel was blotted and probed with a 32^P labeled fragment of repetitive DNA, which binds to all *L. maculans* chromosomes. Sizes (Mb) of chromosomal DNAs are marked.

The frequency of virulent isolates increases and resistance can be overcome or breakdown within a few years of release of a cultivar. Two such breakdowns have been reported in commercial crops. In France 1996–1999, resistance conferred by resistance gene *Rlm1* was overcome within 5 y of release (Rouxel et al. 2003).

A more dramatic breakdown of resistance occurred in Australia in 2003. Three years after release, cultivars harboring ''sylvestris'' resistance conferred by two major genes (*Rlm1*, *LepR3*) sustained devastating yield losses on the Eyre Peninsula, a high rainfall region where these cultivars had been grown extensively (Sprague et al. 2006a, b). This led to losses of AUD $5 million–$10 million in just a single season and withdrawal of these cultivars from sale across Australia. However, trial sites were maintained on the Eyre Peninsula and by 2006 the frequency of *L. maculans* isolates that were virulent on these cultivars had declined appreciably, as reflected by reuction of disease (Marcroft et al. 2012b). This situation is a classic boom and bust plant pathogen cycle. In such a case cultivars with a newly introduced resistance gene that perform well and do not succumb to disease are sown extensively in subsequent years—the boom phase of the cycle. The pathogen population then changes under this selection pressure and the frequency of isolates with virulent alleles increase and high levels of disease occur. The bust then comes when farmers stop planting the cultivar, and over time in the fungal population the frequency of isolates with virulent alleles decrease. Examples of boom and bust cycles have been documented for diseases of major agricultural crops, including rusts and powdery mildew of cereals (McDonald and Linde 2002).

In view of the rapid evolution of blackleg fungal populations, we attempted to manipulate the frequency of virulence in local populations. Canola cultivars with different complements of major resistance genes, as shown by their reactions to a differential set of isolates with known avirulence genotypes, were sown in subsequent years in pots and small-scale field plots (0.5 ha) (Marcroft et al. 2012b). These plants were exposed to blackleg-infested stubble derived from cultivars with different complements of major resistance genes. Mortality, as well as disease severity (percentage of cross section blackening at the crown), was significantly reduced when plants were exposed to stubble from a cultivar with a different complement of resistance genes, compared to exposure to stubble of a cultivar with the same resistance genes. This is the first report that sowing of canola cultivars with different complements of resistance genes in subsequent years (i.e. rotation of resistance genes) can influence the frequency of virulence in fungal populations and can minimize disease. The properties of the fungal genome responsible for this rapid adaptation to selection pressure are discussed in the next section.

MOLECULAR GENETIC ANALYSIS OF *LEPTOSPHAERIA MACULANS*

Over the years tools have been developed and applied to study *L. maculans* in the laboratory (reviewed by Howlett et al. 2001 and Rouxel and Balesdent 2005). This fungus grows on defined media, and sexual crossing can be carried out in vitro; thus classical genetics can be pursued. Agrobacterium-mediated transformation is efficient, but gene knockout via homologous recombination is difficult to achieve (Gardiner and Howlett 2004). Gene silencing is often pursued to functionally analyze genes (Elliott et al. 2011).

Application of pulsed field gel electrophoresis (electrophoretic karyotyping) revealed results that were surprising at the time (the early 1990s). *Leptosphaeria maculans* isolates have different-sized chromosomes, and progeny of a cross can have different-sized chromosomes to those of the parents (FIG. 2) (Plummer and Howlett 1993, 1995). We postulated that these chromosomal length polymorphisms were due to different amounts of repetitive DNA in

homologs and that slippage (unequal pairing) during meiosis led to generation of length polymorphisms (Plummer and Howlett 1995). Since that time chromosomal length polymorphisms have been reported in a number of other filamentous fungi (Stukenbrock and Kroll 2014).

Another interesting finding from analyzing progeny of crosses was that repeat induced point (RIP) mutation is active (Idnurm and Howlett 2003). This mutation process is a pre-meiotic repeat-inactivation mechanism that occurs in several ascomycetes (Selker 1990) and has been reported in basidiomycetes (Horns et al. 2012). RIP is thought to be a genome defense strategy that hypermutates repetitive DNA, such as multicopy transposable elements and which then limits their accumulation and movement throughout the fungal genome. Transitions from C:G to T:A base pairs occur, often introducing stop codons within genes. A RIP signature as a previous historical event can be deduced by bioinformatics analysis of genome sequences (Hane and Oliver 2008). *Leptosphaeria maculans* is one of the few fungi where RIP mutation has been observed during sexual crossing to inactivate genes, rather than having been predicted as a historical event (Idnurm and Howlett 2003).

These observations of chromosomal length polymorphisms and RIP mutations were confirmed when the *L. maculans* genome was sequenced and annotated (Rouxel et al. 2011). Unlike most other dothideomycetes whose genomes have been sequenced, *L. maculans* has a high amount (33%) of repetitive DNA and a correspondingly large genome (45 Mb). The genome is organized in an unusual patchwork fashion with GC-rich blocks that are gene rich, and AT-rich blocks that are gene poor, with relatively sharp demarcation between blocks. Such an arrangement is known as an isochore structure (Rouxel et al. 2011). The AT-rich, gene-poor regions house mainly class I long terminal repeat (LTR) retrotransposons that have been degenerated by RIP mutation. Such regions contain 3.5% of the total genes in the genome, but 20% of these genes encode small secreted proteins, including effectors such as avirulence genes. The genomic location of effector genes embedded within and between degenerated transposable elements enables them to be easily lost or inactivated by RIP mutation during sexual reproduction, which occurs prolifically on canola stubble. This genomic plasticity appears to be the basis of the rapid evolution of virulence in populations of this fungus and the consequent propensity of the fungus to cause resistance breakdown.

In view of the unusual isochore structure of the genome of *L. maculans*, we were curious to know whether the genomes of four other members of the *Leptosphaeria maculans/L.biglobosa* species complex had a similar structure. This species complex comprises lineages that infect cruciferous plants. At least two lineages also infect *B. napus*, for example *L. biglobosa* ''brassicae'' and *L. biglobosa* ''Canadensis'', but the symptoms are much less severe than that those caused by *L. maculans* ''brassicae'' (*L. maculans* ''brassicae'' is termed *L. maculans* for simplicity in the preceding and following sections), and stem cankering is seldom reported (Mendes-Pereira et al. 2003, Voigt et al. 2005).

In collaboration with colleagues in France, USA and Canada, genomes of *L. biglobosa* ''brassicae'', *L. biglobosa* ''Canadensis'', *L. biglobosa* ''thlaspi'', *L. maculans* ''lepidii'' and another isolate of *L. maculans* ''brassicae'' were sequenced (Grandaubert et al. 2014). The genomes all encode similar numbers of small secreted proteins (ca. 650–750), which include putative effectors. However, *L. maculans* ''brassicae'' is the only member with high amounts of repetitive DNA and an isochore structure. The genomes of the four other members are compact (30–32 Mb), with transposable elements (< 4% of the genome) mainly restricted to chromosome ends. The transposons identified across the Leptosphaeriacae contain both class I (LTR retrotransposons) and class II (DNA transposons) types, with a similar number of families in each class (Grandaubert et al. 2014). Analysis of a range of ascomycete genomes showed that these transposable element sequences are restricted to the dothideomycete order Pleosporales, with the exception of two families. Alignment of 19 protein sequences from 55 fungal taxa revealed that the *Leptosphaeria* species diverged from sampled members of Pleosporales about 73 000 000 y ago (MYA). The time required for acquisition of transposable elements during species divergence was determined (FIG. 3). Phylogenetic analyses of the transposable element families revealed a relatively constant rate of introduction of families during species divergence over the past 100 000 000 y. At some point after the divergence of *L. maculans* ''brassicae'' from *L. maculans* ''lepidii'' (5.1 MYA) four transposon families proliferated within the *L. maculans* ''brassicae'' genome (FIG. 3). If the repeat sequences are ignored, these two fungi have nearly perfect chromosomal synteny. It is unclear what triggered the rapid proliferation of transposons in *L. maculans* ''brassicae''; three of these four families are found across the Pleosporales but in relatively low numbers (Grandaubert et al. 2014). The differences in genome structure, particularly in terms of transposable element content and distribution, correlates with the ability of *L. maculans* ''brassicae'' to evolve, cause

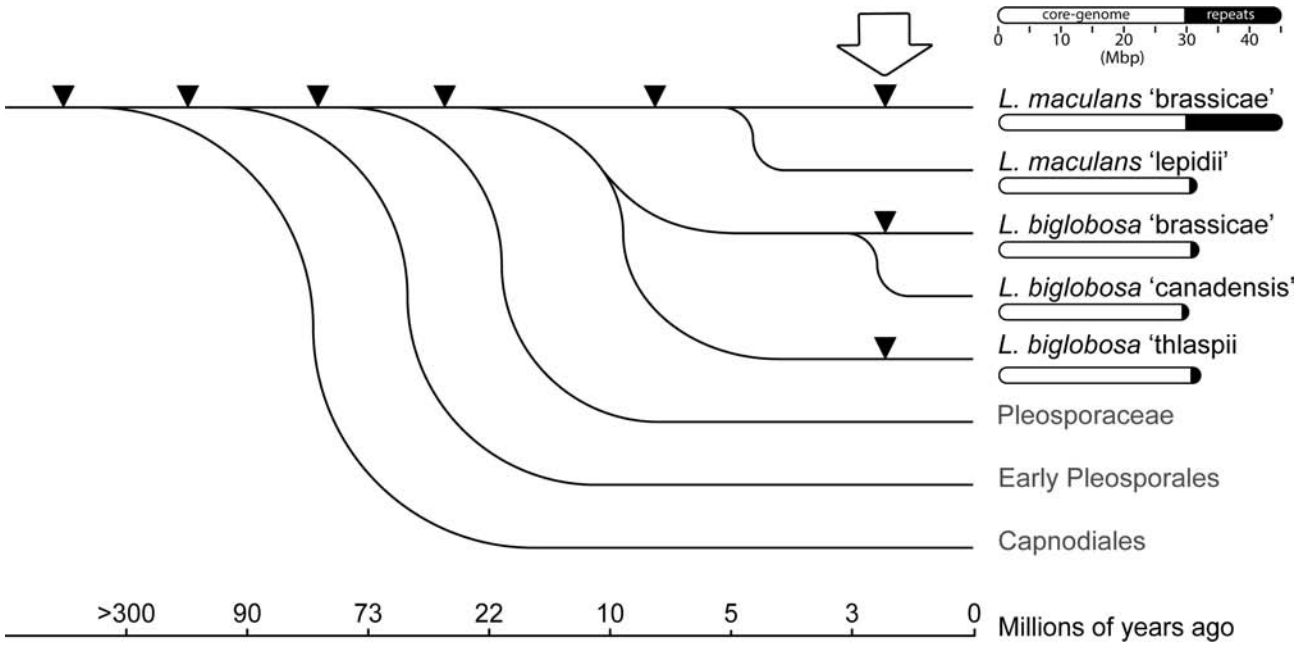

FIG. 3. Timing of divergence of species and invasion of transposons in five members of the *L. maculans/L.biglobosa* species complex. Each introduction of one or more transposon families is marked with a single triangle on the phylogeny diagram. A major invasion/proliferation (indicated by arrow) of four families of transposons into *L. maculans* ''brassicae'' occurred after divergence of this species from *L. maculans* ''lepidii''. The amount of repeat sequence in each genome is drawn below each species name; white shading indicates the non-repetitive core regions of the genome and black shading represents repetitive sequences comprising mainly transposable elements. Approximate timing of divergence events is marked on a nonlinear scale bar. All data are from Grandaubert et al. (2014).

disease and adapt better to its hosts than other members of this species complex.

MOLECULAR BASIS OF BREAKDOWN OF RESISTANCE IN CANOLA CULTIVARS SOWN ON THE EYRE PENINSULA, AUSTRALIA

With the knowledge gained about genome structure of *L. maculans,* we were curious to determine what molecular mechanisms were responsible for the breakdown of resistance in ''sylvestris'' canola cultivars that were sown on the Eyre Peninsula in 2003. The avirulence gene (*AvrLm1*) corresponding to one of the resistance genes (*Rlm1*) in these cultivars was cloned in France (Gout et al. 2006). This avirulence gene is located in a recombination-deficient, transposon-rich region of the genome (Gout et al. 2007) and is linked to another avirulence gene, *AvrLm6,* as well as to five other genes. Analysis of 295 *L. maculans* isolates collected from canola stubble on Eyre Peninsula before and after the resistance breakdown showed that polymorphisms in these genes arose through deletions, amino acid substitutions and Repeat-Induced Point mutations (van de Wouw et al. 2010a). As well as targeting repetitive DNA, RIP mutations also were present in single-copy noncoding and coding regions. The location and degree (intensity) of RIP mutations within these sequences was determined in the 295 isolates with RIPCAL software (Hane and Oliver 2008). The *AvrLm6* gene, which was adjacent to a single-copy non-coding sequence at the 3′ end, had six different RIP alleles conferring virulence. The degree (intensity) of RIP mutation in these single-copy sequences was proportional to the proximity of flanking repetitive DNA that had undergone RIP mutation (FIG. 4). The potential leakage of RIP mutations into closely linked genes highlights the power of RIP to lead to major evolutionary changes to genes such as effectors that play an important role in fungal living strategies.

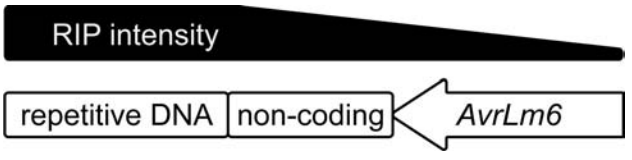

FIG. 4. Repeat induced point (RIP) mutation in *Leptosphaeria maculans* leaking from repetitive DNA into adjacent single copy sequences. A gradient of RIP intensity extends into an adjacent single copy noncoding region and beyond into the single copy *AvrLm6* gene Based on van de Wouw et al. (2010a).

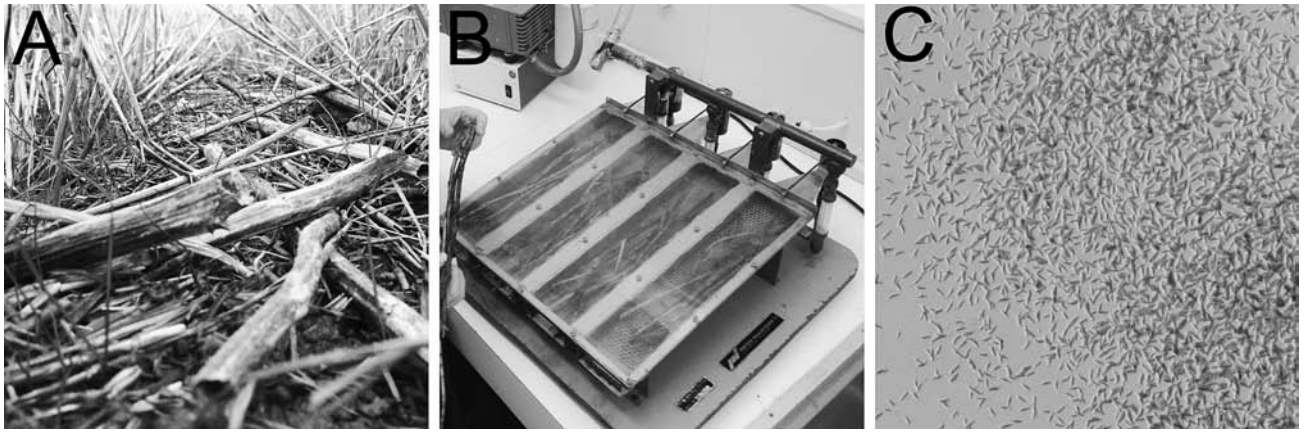

FIG. 5. Equipment used for high throughput assays that determine frequencies of alleles of *Leptosphaeria maculans* avirulence genes. Infected stubble (A) collected from field sites around Australia, thus representing fungal populations from that region, is wetted and placed in a wind tunnel with a clear perspex lid (B). Air passed over the stubble released ascospores (C) onto tape from which fungal DNA is extracted and subjected to molecular assays such as quantitative PCR or pyrosequencing.

The breakdown of ''sylvestris'' resistance was associated with an eightfold increase in frequencies of isolates lacking *AvrLm1*. Although this gene was embedded in repetitive DNA, no RIP alleles were identified. To our surprize, the frequencies of virulence alleles (both deletion and RIP mutation) of *AvrLm6* increased sixfold, even though cultivars with the complementary resistance gene, *Rlm6,* had not been sown on Eyre Peninsula (van de Wouw et al. 2010a). The close linkage and genomic location of *AvrLm1* and *AvrLm6* might have led to a selective sweep, whereby selection at *AvrLm1* affected the frequency of alleles of *AvrLm6* through hitchhiking (Barton et al. 2013). Thus strong selection imposed by widespread deployment of one resistance gene also may lead to breakdown of resistance conferred by another gene, if the two complementary avirulence genes are closely linked. Clearly the genomic environment, as well as extent of exposure to resistance genes in *B. napus* cultivars, affects evolution of avirulence effectors in this fungal pathogen.

MONITORING CHANGES IN VIRULENCE FREQUENCIES IN AUSTRALIAN POPULATIONS OF THE BLACKLEG FUNGUS

Armed with knowledge about the field biology and genomic analysis of *L. maculans*, we set out to monitor changes in frequency of virulence of fungal populations across Australia with the aim of preventing another breakdown of disease resistance. Since 2009 cultivars with different complements of resistance genes sown in 32 trial sites covering canola-growing regions of Australia have been assessed for disease severity. Resistance genes in canola cultivars are identified by the use of 12 differential *L. maculans* isolates (with different sets of avirulence genes) in glasshouse infection trials. On this basis of responses (susceptible or resistant) to these isolates, seven resistance groups (A–G) that include a range of resistance genes have been defined (Marcroft et al. 2012a). Stubble from each site represents local fungal populations, which can be analyzed in high throughput laboratory assays to quantify regional frequencies of alleles of avirulence genes. In these assays blackleg-infested stubble is wetted and placed in a wind tunnel (FIG. 5). This triggers release of ascospores, which are deposited onto tape from which ascospore DNA is extracted and analyzed. quantitative polymerase chain reaction (qPCR) detects the frequency of alleles of particular avirulence genes (*AvrLm1*, *AvrLm6*), where virulence is due to deletion, while PCR and pyrosequencing detects single nucleotide base-pair changes where virulence is due to a particular point mutation (*AvrLm4*) (van de Wouw and Howlett 2012, van de Wouw et al. 2010b). The total number of ascospores is estimated by qPCR of the internal transcribed spacer region of the ribosomal DNA, a genomic region present in all isolates. This is the first example of a high throughput molecular assay that can distinguish genotypes of airborne spores. This type of assay could be applied to other diseases that involve airborne inocula and where the genetic basis of virulence in the pathogen has been identified.

Analysis of data on changes over time in disease severity of cultivars, and in frequencies of avirulence/virulence alleles in populations released from canola stubble, allows predictions of risk of disease outbreaks in different geographic regions. If an epidemic is

predicted, farmers are advised to plant a different canola cultivar with a different complement of resistance genes. A successful application of this approach is discussed in the next section.

AVERTING CANOLA YIELD LOSSES DUE TO BLACKLEG ON THE EYRE PENINSULA

Monitoring disease in canola cultivars and virulence of fungal populations at the field sites across Australia let us identify geographic regions where dramatic changes in disease severity had occurred over time. In Oct 2011 we noticed that cultivar Hyola50 sown at a field on the Eyre Peninsula had high disease severity, which had increased each year from 2009. Ascospores from cv. Hyola50 stubble caused large stem lesions on this same cultivar but not on cultivars with a different complement of resistance genes. This suggested that if cultivars with Hyola50 resistance were sown in 2012 on Eyre Peninsula, large yield losses would be incurred. Based on our findings, in Feb 2012 the Grains Research and Development Corp., a farmer-financed body that invests in grains research, warned farmers to avoid growing cultivars with the resistance gene complement of cv. Hyola50. Cultivars with a different complement of resistance genes were recommended for sowing. This advice was widely accepted, as reflected by seed sales on the Eyre Peninsula (van de Wouw et al. 2014b). Although farmers did not sow Hyola50 cultivars, these cultivars were sown in field trials in this region. When (Oct 2012) these trials were assessed, Hyola50 cultivars had a mean disease severity score of $> 85\%$ while all other cultivars had disease severity scores $< 35\%$, thus validating our prediction. At seven other trial sites across canola-growing regions in Australia where Hyola50' cultivars had not been grown extensively in previous years, disease severity of these cultivars was low ($< 10\%$).

Inheritance of avirulence and resistance in fungal and canola crosses, respectively, suggest that at least two major resistance genes are present in cv. Hyola50. *Rlm1* appears to be one of these genes because transformation of *AvrLm1* into a Hyola50-attacking isolate resulted in isolates with an avirulent phenotype on Hyola50 (van de Wouw et al. 2014b). Also the finding that frequency of the virulent allele of this gene (*AvrLm1*) increased markedly in populations from the Eyre Peninsula 2009–2011 supported this hypothesis—that *Rlm1* was present. Of note, both resistance genes in cv. Hyola50 became ineffective within three years of commercial production, as was the case with the ''sylvestris'' breakdown (Sprague et al. 2006a). This indicates that even when several resistance genes are bred into a cultivar (i.e. stacked or pyramided), resistance can become ineffective in a relatively short time with this plant-fungal interaction, again reflecting the high evolutionary potential of *L. maculans*.

This avoidance of resistance breakdown saved canola growers on Eyre Peninsula about $13 million in a single year. This value is based on conservative estimates of area that would have been sown to cultivars at risk of resistance breakdown (24 000 ha), predicted yield loss (60% of 2 ton/ha) and current canola prices ($600/ha). By sowing alternative canola cultivars, not only did farmers avoid devastating yield losses, but plant breeding companies were able to sell these locally banned cultivars in other canola-growing regions, where resistance breakdown was not predicted. This was a win-win situation for farmers and seed companies.

EVOLUTION OF VIRULENCE IN FUNGAL PLANT PATHOGENS

The findings described above strongly support the hypothesis that fungal virulence can evolve quickly in agricultural systems (McDonald 2014). Major gene resistance coupled with genetic uniformity of crop plants provides strong selection pressure for pathogen populations to overcome disease resistance, as described above for blackleg resistance of canola. Furthermore, the high density sowing of crops lets canopies overlap and enable ready transmission of inocula between plants, thus increasing disease pressure. In contrast, plants in natural environments generally have high genetic diversity and often are sparsely distributed without overlap of canopies.

The accumulation of genomic sequence data from many fungi has revealed relationships between particular genome features and parasitic life strategy (for reviews see Raffaele and Kamoun 2012, Stukenbrock and Kroll 2014). For instance, several plant pathogenic fungi have significantly larger genomes than related saprophytes. This increased genome size generally is due to the presence of repetitive DNA that comprises inactivated or truncated transposable elements, which often house effectors, as is the case for *L. maculans*. Other plant pathogens with large numbers of repetitive DNA include *Mycosphaerella fijiensis, Cladosporium fulvum, Blumeria graminis* and the oomycetes, *Phytophthora infestans* and *Hyaloperonospora arabidopsidis* (Raffaele and Kamoun 2012).

Evolution of virulence in fungal pathogens of crops can take place over different timeframes. The increase in frequency of virulence in populations of *L. maculans* on the Eyre Peninsula in the two resistance breakdowns described above took 3 y; i.e. over three annual sexual cycles of the fungus. Virulence can evolve in a single event when pathogens acquire

virulence by horizontal gene transfer. One well described example of this is the acquisition of a gene encoding a peptide toxin effector, ToxA, a major determinant of virulence, by *Pyrenophora tritici-repentis*, which causes tan (yellow) spot of wheat. This disease became economically important worldwide in the 1940s (Friesen et al. 2006). So far the only other dothideomycete reported to have a homolog of *ToxA* is *Parastagonospora nodorum*, which causes necrotic leaf, stem and glume blotches of wheat.

The *ToxA* gene is in a 11 kb region with a high degree of sequence similarity in *Py. tritici-repentis* and *Pa. nodorum* supporting the hypothesis of horizontal gene transfer (Friesen et al. 2006). Because *ToxA* in *Pa. nodorum* is highly polymorphic between isolates and monomorphic in *Py. tritici-repentis*, the direction of transfer is assumed to be from *Pa. nodorum* to *Py. tritici-repentis,* perhaps on wheat stubble co-infected with the two fungi in the early 1940s, resulting in the selection of an aggressive strain of *Py. tritici-repentis* (Stukenbrock and McDonald 2007). The region flanking *ToxA* includes 7 kb of noncoding DNA and a putative transposase, which probably could facilitate gene transfer.

Knowledge of the *ToxA* sequence has led to development of high throughput screening assays for wheat lines resistant to these diseases (Oliver et al. 2012). This assay takes advantage of the inverse gene for gene relationship between the toxin gene and a corresponding susceptibility gene in wheat. Wheat breeders apply fungal culture filtrates to wheat lines and the presence of necrosis indicates that the line has the corresponding susceptibility gene and should be excluded from further selection. This is one of several approaches where fungal genomics and effectors are now being used as tools in breeding for disease resistance in crops (for review see Vleeshouwers and Oliver 2014).

Recycling resistance genes and pathogen fitness

When disease resistance breaks down in a crop plant, the resistance gene responsible is not usually reused/recycled in new germplasm selected by plant breeders. An exception to this is that the *Rlm1* gene was present in both sylvestrisa and Hyola50 canola cultivars. This probably was because breeders who developed cv. Hyola50 were unaware of its presence, in that until recently the identity of resistance genes in most canola cultivars were unknown. Resistance genes potentially could be recycled if mutation to virulence in the corresponding avirulence gene in the pathogen imposes a fitness cost on the pathogen. Successful recycling would be achieved by ensuring that cultivars with that resistance gene are not sown extensively in a particular region for several seasons. Being able to reuse resistance genes is of great economic benefit to breeding companies and farmers in that each resistance gene costs a significant amount to develop and release in a cultivar.

Zhan and McDonald (2013) observed that fitness cost of mutation of avirulence genes of fungi can affect the effectiveness of recycling the corresponding resistance gene in crops. Fitness costs are associated with mutation of *AvrLm1* and *AvrLm4* in *L. maculans* (Huang et al. 2010). This is consistent with our observations of much higher frequencies of *AvrLm1* in fungal populations in regions in Australia where cultivars with *Rlm1* resistance have not been sown, compared to areas where these cultivars have been extensively sown. A similar situation is seen for *AvrLm4* (Marcroft et al. 2012b). We are continuing to monitor disease severity of cv. Hyola50 and changes in frequency of *AvrLm1* alleles in isolates cultured from cv. Hyola50 stubble in trial sites on the Eyre Peninsula to see whether resistance becomes effective again in the absence of selection pressure from wide spread acreage of crops of cv. Hyola50.

Molecular data support the roles of *AvrLm1* and *AvrLm4* in virulence of *L. maculans*, particularly at early stages of infection where disease symptoms are not visually apparent. Global transcriptome RNA-seq data revealed that three avirulence genes, including *AvrLm1* and *AvrLm4*, are among the 20 most highly upregulated genes 7 d after inoculation of canola cotyledons with a virulent isolate of *L. maculans* (Lowe et al. 2014). Transcript levels of these genes were much lower at 14 d post inoculation, when extensive necrosis was established. The role of avirulence genes as virulence factors in several other fungal-plant systems including *Cladosporium fulvum* and tomato has been reported (e.g. Mesarich et al. 2014).

In conclusion

Genome structure of fungal pathogens can affect evolution of virulence as effectors including avirulence genes are often housed in dynamic genomic regions such as inactivated transposable elements where they can be readily mutated or deleted. The approach of monitoring virulence in fungal populations and then withdrawing particular canola cultivars from sale on a regional level has increased the commercial life of cultivars in Australia, despite blackleg resistance being overcome in one geographic region. This approach of monitoring disease and selectively withdrawing cultivars that harbor genes at risk of being defeated should be applicable to manage diseases of other crops, particularly those where the

pathogen, like *L. maculans*, has high evolutionary potential and where resistance genes have been identified in the crop plant.

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Author/s:
Howlett, BJ; Lowe, RGT; Marcroft, SJ; van de Wouw, AP

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