2017 Daniel McAlpine Memorial Lecture

A ‘genome to paddock’ approach to control plant disease

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Abstract:

Pathogenic fungi evolve in concert with their plant hosts to invade and overcome defence responses. A detailed knowledge of these processes is essential for integrated disease management strategies. Blackleg caused by the fungus, *Leptosphaeria maculans*, is the major disease of canola (*Brassica napus*) worldwide. In this article, I describe the development of a multidisciplinary research program that has been applied to control this disease in Australia. Information about the biology, molecular genetics and genomics of the blackleg fungus coupled with knowledge of disease incidence and severity in the field has been exploited to avert yield losses due to disease.

Key Words:

Leptosphaeria maculans; *Brassica napus*; blackleg

24 **Choosing a research problem**

25 The McAlpine memorial lecture provides an opportunity for the awardee to outline
26 circumstances and opportunities leading up to tackling a research problem. Accordingly, in
27 this article I include some biographical details that shaped decisions I have made during my
28 career. When I completed my undergraduate Honours degree, I was unsure about what I
29 wanted to do next. Coming from a farm, I knew that I wanted do something with agricultural
30 relevance. Early in my career, due to personal circumstances I had several short term
31 contracts working on diverse projects (tapeworms, self incompatibility in plants, bacterial
32 chemotaxis, nitrogen fixation). Rather than being a drawback, I now feel that having
33 experience in a range of research areas can be beneficial for tackling new problems.

34 I first became interested in plant pathology when I was a post doctoral fellow with Adrienne
35 Clarke at the University of Melbourne studying stem rot of cowpea, which is caused by the
36 oomycete, *Phytophthora vignae*. At the time, *Phytophthora* species were intractable to
37 genetic manipulation, and I was making little headway at understanding how this pathogen
38 caused disease. Fortuitously, due to my husband taking a sabbatical leave in San Francisco, I
39 then spent eight months at Stanford University where I acquired skills in fungal genetics. In
40 the laboratory of David Perkins, I studied *Neurospora crassa*, the bread mould, which is a
41 model fungus for classical and molecular genetics. In an adjacent laboratory, a technique
42 was being developed to separate chromosomal DNA of fungi. The technique, pulsed field
43 gel electrophoresis, is now widely used and I was fortunate to be one of the first people to
44 learn it.

45 When I returned to Australia in 1989 I decided to focus on a plant pathogen that was more
46 amenable to genetic manipulation than *Phytophthora*, and one that caused significant crop
47 losses. I reasoned that such a situation would enable me to be competitive for research
48 funding from industry. Upon advice from John Irwin, University of Queensland, with whom

I had collaborated on *P. vignae*, I choose *Leptosphaeria maculans*, which causes blackleg of canola. Canola was first grown in Australia in the late 1960s, but cultivars grown at that time had no resistance to blackleg disease and consequently severe epidemics occurred in the 1970s. During the 1980s extensive breeding programs incorporated resistance into cultivars and by the end of that decade, the acreage sown to canola was beginning to increase steadily. However, it seemed likely that blackleg disease would be a continual threat to the industry (which indeed it became and still is). *Leptosphaeria maculans* is an outcrossing ascomycete and was likely to be amenable to the genetic approaches I learnt from studying *Neurospora* at Stanford. I was fortunate to receive funding to study blackleg. I won an Australian Research Council Fellowship for five years, grants from the Oilseed Research Council and later investment by the Grains Research and Development Corporation (GRDC) to study blackleg. Since then with some excellent students and staff, I have developed molecular approaches and undertaken field studies to analyse the interaction between the blackleg fungus and canola.

Breaking bad: the blackleg fungus can breakdown resistance bred into canola

Blackleg disease is often severe in Australia, as canola is grown during mild winters over a long period, which allows enough time for the fungus to colonise and complete its life cycle in the plant. During summer, the fungus undergoes prolific sexual reproduction on stubble producing large populations of windborne recombinant ascospore inoculum, which is released during rainfall events the following year at seedling emergence. Ascospores infect cotyledons then hyphae grow through xylem to the crown, which leads to lodging and yield loss. The disease is controlled by cultural practices that minimise exposure to *L. maculans* - infested canola stubble from the previous year's crop, fungicide applications and sowing cultivars with disease resistance (Van de Wouw et al. 2016).

In the 1990s, as acreage of canola sown increased and rotations with cereals became tighter (1 in 2 or 3 years, instead of 1 in 4 years), blackleg disease pressure increased. In 2003 a dramatic case of resistance breakdown occurred on the lower Eyre Peninsula, South Australia. Three years previously, ‘sylvestris’ cultivars with a new blackleg resistance gene (*LepR3*) were released and for the first two years few lesions were evident on plants, so the cultivars were widely taken up in this region. However, in the summer of 2003, crops (about 20,000 ha) were decimated by blackleg. Total losses to farmers were about \$30 million and the cultivars were removed from the Australian market. During the next two years, my Ph.D student, Steve Marcroft, observed many cankered plants in field plots in the region, but by 2006, few plants of this cultivar were diseased. In contrast, cultivars with a different source of resistance that were sown extensively in the region after the ‘sylvestris’ breakdown showed significant disease symptoms in the field plots (Sprague et al. 2006). These findings led us to propose that *Leptosphaeria maculans* is a high risk pathogen, in terms of breakdown of resistance. Extensive sowing of cultivars with major gene resistance had resulted in strong selection pressure towards isolates virulent to that resistance gene and consequently the frequency of virulent isolates in fungal populations had increased. We were then interested to determine features of the fungal genome that allow virulence to evolve so rapidly, and in particular, changes in avirulence genes responsible for breakdown of ‘sylvestris’ resistance.

Understanding the ‘enemy’: molecular analysis of the blackleg fungus

Even before the breakdown of ‘sylvestris’ resistance it was apparent that to understand how *L. maculans* caused disease, we needed to be able to manipulate this fungus in the laboratory. *Leptosphaeria maculans* has several features that make it attractive for molecular analysis. It grows on defined media and sexual crossing can be carried out *in vitro*; thus

classical genetics can be pursued. Over the years we have developed several techniques and tools including *Agrobacterium*-mediated transformation. However, functional analysis of genes by gene knockout via homologous recombination is difficult to achieve; the reasons for this are described later.

The first molecular technique we applied to *L. maculans* was pulsed field gel electrophoresis. Kim Plummer, a Ph.D student, made a surprising finding that isolates have different sized chromosomes. Furthermore, she showed that progeny of a cross can have different-sized chromosomes to those of the parents (Plummer and Howlett 1993, 1995). We suggested that these chromosomal length polymorphisms were due to different amounts of repetitive DNA in homologs and that unequal pairing during meiosis led to generation of length polymorphisms.

A few years later, another Ph.D student, Alex Idnurm, while analysing progeny of crosses, showed that Repeat Induced Point (RIP) mutation is active in *L. maculans* (Idnurm and Howlett 2003). This mutation process is thought to be a genome defence strategy that mutates repetitive DNA, such as transposable elements. This then limits their accumulation and movement throughout the fungal genome. A common outcome of RIP mutation is the generation of stop codons within genes, which leads to gene inactivation. *Leptosphaeria maculans* is one of the few fungi where RIP mutation has been actually observed to inactivate genes, rather than the process having been predicted as a historical event by bioinformatic analysis of RIP ‘signatures’ (Hane and Oliver 2008).

By 2010 there was a revolution in fungal genetics. The genomes of several filamentous fungi including some important plant pathogens (for example, *Magnaporthe grisea*, *Stagonospora nodorum*) were now publicly available (Dean et al. 2005; Hane et al. 2007) and many exciting features of genomes that impacted on disease mechanisms were being discovered. Accordingly we were very keen to sequence *L. maculans*. Fortuitously we were

approached by Thierry Rouxel, INRA, France and asked to submit a joint project to Genoscope, the French sequencing agency. This project was successful and the genome was sequenced and annotated by an international team (Rouxel et al. 2011).

Several features of the genome, for instance, the high level (33%) of repetitive DNA, were consistent with our previous findings; eg. chromosomal length polymorphisms, active RIP mutation and low degree of homologous recombination. The genome is organised in an unusual 'patchwork' pattern with GC-rich blocks that are gene-rich, and AT-rich blocks that are gene-poor. The AT-rich regions house mainly transposable elements that have been degenerated by RIP mutation. Only 3.5% of the total genes in the genome are present in these regions, but 20% of these genes encode small secreted proteins, including avirulence proteins. The genomic location of avirulence genes embedded between and within transposable elements enables these genes to be easily lost or inactivated by RIP mutation during sexual reproduction. These features can explain the rapid evolution of virulence in populations of this fungus and the consequent ability to breakdown resistance. Having the genome sequence, we now could attempt to determine changes in the avirulence genes responsible for the breakdown of 'sylvestris' resistance.

To make headway, we needed to consider the genetics of the interaction between *L. maculans* and *B. napus*. Resistance genes expressed by the seedlings are complementary to avirulence genes in the fungus in a 'gene for gene' relationship (Balesdent et al. 2001). Over the years several avirulence genes have been cloned, including *AvrLm1*, which is complementary to resistance gene *Rlm1* (Gout et al. 2006). A post doctoral fellow, Angela Van de Wouw, found that *Rlm1*, as well as *LepR3* is present in 'sylvestris' cultivars (Van de Wouw et al. 2009). She then set about to determine if mutations/deletions in *AvrLm1* were responsible for breakdown of 'sylvestris' resistance. She had hundreds of isolates collected from canola stubble from the Eyre Peninsula before and after the breakdown of resistance

and she sequenced *AvrLm1* and a closely linked gene, *AvrLm6*, as well as regions flanking them in these isolates.

The breakdown of ‘sylvestris’ resistance was associated with an eight-fold increase in frequencies of isolates lacking *AvrLm1*. Virulence at this locus was only conferred by complete deletion of this gene, not by mutation. The frequencies of virulence alleles (both deletion and RIP mutation) of *AvrLm6* increased six-fold, even though cultivars with the complementary resistance gene, *Rlm6*, had not been sown on Eyre Peninsula (Van de Wouw et al. 2010a). We concluded that close linkage of *AvrLm1* and *AvrLm6* and their location in a recombination-deficient region of the genome may have resulted in selection at *AvrLm1* affecting allele frequency of *AvrLm6*. Thus wide-spread deployment of one resistance gene had led to breakdown of resistance conferred by another gene because the two complementary avirulence genes are closely linked.

Keeping them working: maximising effective life of resistance genes in canola

It is important to enhance the durability of resistance genes given that developing and commercialising such genes costs companies several millions of dollars. The finding that resistance of ‘sylvestris’ cultivars in field plots in the Eyre Peninsula became effective several years after the resistance breakdown, led us to predict that rotating cultivars with different resistances, from season to season, would minimise disease and thus enhance the life of these resistance genes. Marcroft – now with a Ph.D and having set up a small company, Marcroft Grains Pathology, carried out glasshouse and field plot experiments, which confirmed this assumption. Cultivars sown into stubble of a cultivar with the same resistance gene developed significantly more disease than when they were sown into stubble of a cultivar with a different resistance gene (Marcroft et al. 2012b). These data suggested

that rotation of resistance genes from season to season can minimize blackleg disease and hence maximize the durability of resistance genes.

To achieve this outcome on a wider scale, we first needed to know the complement of resistance genes in the cultivars sown in Australia. Van de Wouw and Marcroft now classify cultivars prior to commercial release into resistance groups (A-H) based on reactions (resistant or susceptible) in glasshouse infection trials to *L. maculans* isolates with different sets of avirulence genes (Marcroft et al. 2012a). Additionally, cultivars from different resistance groups sown in 32 trial sites covering canola-growing regions of Australia are assessed for disease severity by members of the GRDC-funded National Canola Pathology Program. Stubble from each site can be analysed in high throughput molecular assays to quantify regional frequencies of alleles of avirulence genes. These data can be used to determine the risk of breakdown of resistance of particular cultivars. Van de Wouw has developed assays whereby blackleg-infested stubble is wetted and placed in a wind tunnel. Released ascospores are captured on tape from which DNA is then extracted and analysed. Quantitative Polymerase Chain Reaction detects the frequency of alleles of avirulence genes (*AvrLm1* and *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).

By analysing data on changes over time in disease severity of cultivars, and in frequencies of avirulence/virulence alleles in fungal populations released from canola stubble, we can predict risk of disease outbreaks in different geographic regions. If an epidemic is predicted, then farmers are advised to plant a different canola cultivar with a different complement of resistance genes. In general grain growers are advised to only grow cultivars from a particular resistance group for up to three years and then to switch to a cultivar from a different group (Marcroft et al. 2012a). Information on resistance groups, as well as advice

about other farming practices, is released biannually in the GRDC Blackleg Management Guide (<https://grdc.com.au/resources-and-publications/all-publications/publications/2017/09/blackleg-management-guide>). A successful application of the rotation of resistance genes is discussed in the next section.

Averting another resistance breakdown

On the Eyre Peninsula in October 2011, Marcroft, Van de Wouw and Andrew Ware, a local agronomist/pathologist, noticed that crops and field plots of cultivar Hyola50 had high disease severity scores (defined as percentage of blackening of the cross section at the base of the stem). Furthermore, this score had increased each year from 2009. Additionally, in glasshouse studies, ascospores from cv. Hyola50 stubble caused large stem lesions on this same cultivar, but not on cultivars with a different complement of resistance genes. Marcroft and colleagues predicted significant yield losses would occur on the Eyre Peninsula if cultivars with ‘Hyola50’ resistance were sown in 2012, due to resistance breakdown. In February 2012 GRDC warned farmers not to sow cultivars with the resistance gene complement of cv. Hyola50, but to sow cultivars with a different complement of resistance genes. This advice was widely accepted and Hyola50 cultivars were only sown in small field plots in this region. When these plots were assessed later that year, ‘Hyola50’ cultivars had a mean disease severity score of > 85% whilst 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 < 10% (Van de Wouw et al. 2014).

This avoidance of resistance ‘breakdown’ saved canola growers on Eyre Peninsula about \$13 million. By sowing cultivars with different resistance genes, not only did farmers avoid devastating yield losses, but breeding companies could sell these locally ‘banned’ cultivars

in other canola-growing regions. This was a ‘win-win’ situation for farmers and seed companies.

Staying Ahead of Blackleg

Based on the findings described above, it is obvious that blackleg cannot be eradicated; when resistance genes are deployed, strong selection pressure is exerted on fungal populations leading to evolution of virulence. Hence advice to Australian grain growers is to ‘stay ahead of blackleg’, and minimise rather than eliminate disease.

New challenges in disease control have arisen in the last few years in Australia. Firstly, new disease symptoms are apparent, probably due to the early sowing of cultivars, which is now a relatively common practice. Crops are undergoing stem elongation in June or July, which is earlier than previously, and lesions are developing on the upper parts of the plant including flowers, stems, branches and pods. The effect of these symptoms on yield is currently being determined. Secondly, the practice of incorporating (stacking) of several resistance genes into a cultivar presents challenges for durability of resistance. Although having several resistance genes in a single cultivar may seem attractive, populations of the blackleg fungus can overcome multiple resistance genes simultaneously, as shown by the breakdown of two resistance genes in the ‘sylvestris’ cultivars (Van de Wouw 2010a). Furthermore, this practice reduces the pool of cultivars available for rotation, as farmers cannot sow cultivars that contain any of the two individual resistance genes in future years. Thirdly, the widespread use of fungicides may lead to development of fungicide resistance. Most canola seeds are treated with Jockey® (fluquiconazole) and fertiliser dressing often contains Impact® (flutriafol). Foliar fungicide, Prosaro® (prothioconazole and tebuconazole), is also used widely. These three products are all azoles and so there is concern that fungal populations might develop azole resistance. In 2014 the first case of

fungicide ‘tolerance’ in *L. maculans* in Australia (or elsewhere) was observed. Some isolates derived from canola stubble could infect canola seedlings grown from Jockey-treated seed (Van de Wouw et al. 2017). The extent and nature of this tolerance is not known and a large scale survey of stubble from regions across Australia is being undertaken.

In the laboratory, my successor, Alex Idnurm who is an Australian Research Council Future Fellow, is continuing and indeed has revitalised the molecular aspects of blackleg research. For instance, he has applied a new technology, CRISPR-Cas9, to mutate several key fungal genes and with a team of new students is exploiting this breakthrough to analyse several novel aspects of fungal virulence.

In summary, I feel very privileged to have been part of a team that has taken our findings from the laboratory and applied them to the field to control disease. Being involved in this multidisciplinary research has been an immensely rewarding experience.

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