Pathogenic and genetic variation in *Leptosphaeria maculans* in Australia.

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Abstract

Blackleg disease of canola (*Brassica napus*) is caused by the ascomycete *Leptosphaeria maculans*. Isolates of the fungus were collected from across Australia between 2001 and 2002 and their virulence, mating type and population genetic structure were assayed. The isolates were hierarchically sampled from pycnidia within lesions on the cotyledons of newly sown canola plants, or pseudothecia in canola stubble left in the field from the previous year’s crop. The virulence of two hundred isolates of *L. maculans* was determined upon cultivars with resistance derived from *Brassica juncea* and *B. rapa* subsp. *sylvestris*. The isolates displayed a range of pathogenicity, with lesion scores between 0 and 9, when tested on the cotyledons of *B. napus* (cultivars Westar, Dunkeld, Surpass 400) and *B. juncea* cultivar Stoke. Most isolates could not attack *B. juncea* cultivar Stoke, resulting in a hypersensitive response or small necrotic lesion (score 3). A similar infection response was observed on *B. napus* cultivar Surpass 400. Highly virulent isolates were defined as those that resulted in mean lesion scores greater than 3 on *B. juncea* cultivars Stoke and Surpass 400. These highly virulent isolates fell into three classes, with some isolates able to attack both cultivars. Stoke and Surpass 400 equally, while others attacked one cultivar but not the other. The mating type (*MAT1–1* or *MAT1–2*) of each isolate was determined by a PCR assay, which revealed an even distribution of the two mating types, regardless of whether the isolates originated from different cotyledon lesions or different stubble stems within a field. Microsatellite markers have been developed to study genetic variation in the Australian *L. maculans* population.

Keywords: *Brassica napus*, canola, mating type, virulence, microsatellites.

Introduction

Blackleg disease epidemics severely affected canola production in the early 1970s in Australia (Howlett *et al.* 1999). *L. maculans* races are being characterised in Europe on the basis of interactions with *B. napus* cultivars (cv.) with defined disease resistance genes (Balesdent *et al.* 2002). In contrast, there is little knowledge about resistance genes in Australian *B. napus* cultivars.

New Australian cultivars of *B. napus* have been bred with resistance to *L. maculans*, derived from related *Brassica* species *B. rapa* subsp. *sylvestris*, and released by Pacific Seeds (Hyola and Surpass cultivars). Lás *et al.* (2003) reported the appearance of stem cankers in a field plot of Surpass 400, and showed that isolates cultured from the cankers caused lesions on Surpass 400 cotyledons in glasshouse experiments. Pacific Seeds also issued a media release in March 2003, detailing the discovery of *L. maculans* infected ‘Blackleg Tested 9.0’ (Hyola and Surpass) cultivars at two locations in Australia. The durability of these new cultivars is now uncertain, particularly given the ability for *L. maculans* to change in response to selection pressure (Brun *et al.* 2000). For the improvement of canola cultivars it is thus essential to understand the virulence of *L. maculans* in Australia upon cultivars derived from the resistant species *Brassica juncea* and *B. rapa* subsp. *sylvestris*.

*L. maculans* is haploid and outcrossing, thus both mating types (*MAT1–1* and *MAT1–2*) must be present for sexual reproduction. Pseudothecia (sexual fruiting bodies) are formed in infected stems of *B. napus* over the summer, releasing ascospores (sexual spores), which are windborne and infect newly sown crops in late autumn. By determining the distribution of the two mating types within populations of the fungus, we can identify if new virulence genes may spread quickly. The occurrence of recombination can also be measured in populations by studying the level the gametic disequilibrium or linkage between genes.

Microsatellites (simple sequence repeats) are molecular markers based upon tandem arrays of short nucleotide motifs (1–6 bp). Variation occurs
when organisms differ in the number of tandem repeats detected by polymerase chain reaction (PCR) at a locus. Microsatellites have been developed for genetic diversity studies of L. maculans to determine the amount and distribution of genetic variation of populations (genetic structure) of L. maculans. They can also be used to study gametic disequilibrium and gene flow between populations. Better disease management strategies can be designed from studying the virulence and population genetics of L. maculans. In this paper we report on virulence, mating type distribution and the development of microsatellite markers for L. maculans.

Methods
Isolate collection

In 2001, one field at Wonwondah, Victoria was intensively sampled for L. maculans. Isolates were hierarchically sampled from both infected canola stubble left in the field from the previous year’s crop, and from different pycnidia within lesions formed on the cotyledons of newly sown canola plants. In 2002, isolate collections were expanded to sample L. maculans from stubble left in canola fields in Thuddungra in New South Wales, Lake Bolac and Wonwondah in Victoria, Mount Barker in Western Australia, and Struan in South Australia.

All isolates were cultured from single spores or hyphal tips to ensure that only one individual had been cultured. Single pycnidia from infected leaves were isolated as described by Barrins et al. (2001) and then hyphal tipped. Single ascospores were isolated from infected stubble as described by Plummer and Howlett (1993).

Virulence tests

The virulence of a subset of isolates (45 from the 2001 collection, and 155 from 2002) was tested on the cotyledons of B. napus (cvs. Westar, Dunkeld, Surpass 400) and B. juncea cv. Stoke. Westar is a susceptible cultivar, Dunkeld is a commercial cultivar, and Surpass 400 has resistance derived from B. rapa ssp. sylvestris. Inoculations were done as described by Purwantara et al. (1998). Responses were scored on a scale of 0 to 9 depending on lesion size and appearance (Koch et al. 1991), 12 days post-inoculation. Significant differences between disease infection scores were tested between isolates on the four different cultivars by analysis of variance (ANOVA).

Mating type assay

Mycelia were harvested following growth in 10% V8 liquid media at 26°C for 10 days. Mycelia were freeze dried, and DNA extracted as described by Sexton and Howlett (2000). The distribution of the two mating types within the 2001 collection (Wonwondah) was determined for 160 isolates by a PCR assay developed by Cozijnsen and Howlett (2003).

Microsatellite marker development

A limited amount of L. maculans genomic sequence was examined for DNA sequences containing microsatellites. The program Tandem Repeat Occurrence Locator (TROLL) identified perfect repeat motifs in the genomic sequence. Sequences containing at least six dinucleotide, four trinucleotide or three pentanucleotide repeats were identified as target microsatellites. Primers were designed between 18 and 22 nucleotides for the microsatellite flanking regions to yield amplification products of 100–500 bp. All primers were tested for amplification, band number and size, and polymorphisms against a test set of 14 L. maculans isolates from Australia and overseas. PCR products were size fractionated in 2% agarose gels with an internal lane size marker.

Results

One hundred and seventy isolates of L. maculans were collected intensively from one canola field at Wonwondah, Victoria in 2001. Two hundred and thirty isolates of L. maculans were collected from many different canola fields around Australia in 2002. Isolates from Wonwondah (2001) have been characterised for their mating type, virulence, and microsatellite analysis is in progress. Isolates from the Australian collection (2002) have been virulence tested and molecular analyses of mating type and population structure are currently underway.

Virulence tests

Two hundred isolates of L. maculans (50 from Wonwondah, 150 from around Australia) were tested to determine their virulence upon cultivars with different levels of resistance. The isolates displayed a range of pathogenicity, with lesion scores between 0 and 9, when tested on the cotyledons of B. napus (cvs. Westar, Dunkeld, Surpass 400) and B. juncea cv. Stoke. Significant differences were observed between isolates in their ability to infect each of the four cultivars (P<0.001, df = 463).

Most isolates could not attack B. juncea cv. Stoke, resulting in a hypersensitive response or small necrotic lesion (score 3). A similar infection response was observed on B. napus cv. Surpass 400. Highly virulent isolates were defined as those that resulted in mean lesion scores greater than 3 on Stoke and Surpass 400. These highly virulent isolates fell into three classes, with some isolates able to attack both cultivars equally, while others were host specific to
either Surpass 400 or Stoke, attacking one resistant cultivar but not the other, as shown in the different quadrants of Figure 31.

Mating type assays

The distribution of the two mating types of \textit{L. maculans} was assessed for 160 isolates from one field at Wonwondah, using a PCR assay. An even ratio of both mating types was detected (57:43%), regardless of whether the isolates originated from different cotyledon lesions or different stubble stems within the paddock. Isolates collected from different fields around Australia in 2002 are currently being tested.

Mating type assays

The distribution of the two mating types of \textit{L. maculans} was assessed for 160 isolates from one field

Microsatellite marker development

Primer pairs were designed for 35 sequences containing microsatellite motifs, and screened across 14 \textit{L. maculans} isolates from Australia and overseas. Thirty-two microsatellite loci were amplified, with five primer sets producing multi-copy banding patterns and 25 primer sets amplifying single copy loci. Six polymorphic single copy microsatellite loci, with two to three alleles, were identified. Sequence data confirmed that allele size polymorphism for the six loci were due to variation in the number of motif repeats. Mendelian segregation of alleles detected by microsatellite primer pairs was demonstrated for locus \textit{SSR8} in F1 progeny of a pair cross of \textit{L. maculans}.

\textit{L. maculans} microsatellite markers are now being used in conjunction with a \textit{L. maculans} minisatellite marker (Attard et al. 2001) to determine the amount and distribution of genetic diversity in 160 \textit{L. maculans} isolates collected from one canola field at Wonwondah in 2001. DNA is currently being extracted from 230 isolates of \textit{L. maculans} collected from around Australia in 2002. The microsatellite markers and minisatellite marker will then be used on these isolates to determine the population genetic structure of \textit{L. maculans} in Australia.

Discussion

This research is aimed at understanding the virulence and genetic diversity within \textit{L. maculans} in Australia, across different hierarchical scale from field to different growing regions in NSW, Victoria, South Australia and Western Australia. Isolates from one field, Wonwondah (2001) displayed a range of virulence, but were mostly low in virulence on cv. Surpass 400 and cv. Stoke. This pattern was also observed for isolates collected from the different states in 2002.

Several isolates observed to be highly virulent in pathogenicity tests, differed in their ability to attack cv. Stoke or Surpass 400, demonstrating host specificity on these cultivars, and possibly differences
in their virulence genes. Several isolates had the ability to form lesions on the cotyledons of Surpass 400, including isolate M1 which was cultured from canola stubble about 15 years ago (Chen et al. 1996). This demonstrates a gene that overcomes cotyledonary resistance has been present in the Australian L. maculans population long before Hyola and Surpass cultivars have been sown commercially. We are currently determining the ability of these isolates to cause stem cankers, the major cause of yield loss, in glasshouse tests.

Isolates were much less virulent on B. juncea than on B. napus cv Surpass 400, giving encouragement to alternative sources of resistance for B. napus breeding, but also demonstrating that greater knowledge of resistance genes and genetic background of Australian canola cultivars is required for disease management strategies to be employed in response to pathogen change.

L. maculans undergoes both asexual and sexual reproduction during infection of canola. Each of the spore types produced has different implications for disease epidemiology, with ascospores having an important role as they mature over summer in pycnidia and infect newly planted crops. The finding of an even ratio of the two mating types in field isolates suggests there is no fitness penalty associated with either mating type.

Microsatellite analysis of population structure will allow us to study isolates derived from ascospores and pycnidiospores within one field (at Wonwondah), giving insight to the epidemiology of the fungus. It will also allow us to determine if L. maculans exists as one population that has been spread across Australia since canola production began in the different regions, or if different populations exist in each area which may then be considered as different management units. Together the knowledge gained from the population studies and the virulence testing can be utilised to develop better disease management strategies that may reduce disease spread of L. maculans.

References


