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Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia

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Abstract

Resistance of *Brassica napus* (oilseed rape, canola) conferred by three different major resistance genes has been overcome by changes in virulence of *Leptosphaeria maculans* populations in France and Australia. In South Australia where *B. napus* cultivars with major gene resistance derived from *Brassica rapa* ssp. *sylvestris* were grown extensively, resistance was rendered ineffective within 3 years of commercial release of the cultivar. Disease severity was higher on cultivars with *sylvestris*-derived resistance than cultivars with polygenic resistance. This Australian situation is compared to that in France, where resistance conferred by the *Rlm1* gene was overcome nation-wide in 5 years under commercial cropping practices, and also where a source of resistance introgressed into *B. napus* from *B. juncea* was rendered inefficient in 3 years in experimental field plots near Rennes.

Introduction

Phoma stem canker (blackleg), caused by the fungus *Leptosphaeria maculans*, is the most important disease of *Brassica napus* worldwide (for reviews see Howlett, 2004; Fitt et al., 2006). As discussed by Delourme et al. (2006) and Aubertot et al. (2006), the main strategies for control of the disease are cultural practices and effective deployment of resistance genes. The durability of particular sources of resistance can vary depending on the biology of the pathogen. Pathogens that pose the greatest risk of overcoming resistance conferred by single or few genes (major gene resistance) are those with a high evolutionary potential; i.e. those that reproduce both sexually and asexually, have effective spore dispersal and large population sizes (McDonald and Linde, 2002). *Leptosphaeria maculans* has a high potential for overcoming major gene resistance since it undergoes an annual cycle of sexual recombination to produce widely dispersed wind-borne ascospores, and it also produces large numbers of conidia spread by rain splash (Fitt et al., 2006).

In accordance with this high evolutionary potential of *L. maculans*, major gene resistance has been overcome in field experiments in France (Brun et al., 2000) and in nation-wide surveys of commercial fields and trial sites (Rouxel et al., 2003). Oilseed rape breeding programs in Australia have focussed on developing cultivars displaying adult plant resistance, which provides protection only against stem canker, whilst leaves develop sporulating lesions. This non-specific

resistance is probably conferred by several genes and is hereafter referred to as polygenic (for review see Delourme et al., 2006). In 2000, cultivars with specific resistance conferred by a single dominant gene were released by Pacific Seeds Pty Ltd (Li and Cowling, 2003). This resistance gene was derived from *Brassica rapa* ssp. *sylvestris* by Crouch et al. (1994) and hereafter resistance conferred by this gene is referred to as *sylvestris*-derived resistance. When these cultivars were first released, they showed few phoma leaf spots or stem canker symptoms (Sosnowski et al., 2004) and were given an Australian Blackleg Rating of 9 on a scale where 1 is highly susceptible and 9 is highly resistant (http://www.canolaaustralia.com). However, in 2002 canker symptoms were identified on such cultivars at two locations remote from each other in South-eastern Australia (Andrew Easton, Pacific Seeds Pty Ltd, Australia, pers. comm.). In addition, isolates in Western Australia capable of attacking these cultivars were identified (Li et al., 2003a; 2005).

In this paper, we report in greater detail the decline in efficiency of resistance in *B. napus* cultivars with *sylvestris*-derived resistance to *L. maculans* in South Australia and compare it to the decline in efficiency of resistance in France.

Materials and methods

Survey of commercial oilseed rape crops and trial sites in South Australia for canker severity

Following the release of cultivars with *sylvestris*-derived resistance in Australia in 2000, field surveys were conducted to detect any changes in host resistance and in *L. maculans* populations in response to the large-scale use of these cultivars. In 2003, the incidence and severity of phoma stem canker were examined in crops at different distances from a trial site on the Lower Eyre Peninsula in South Australia where diseased plants were first observed in 2002. The 2002 site (0.5 ha) had been used for the previous 3 years to assess resistance to *L. maculans* in oilseed rape lines and is in a region that had been cropped intensively with cultivars with *sylvestris*-derived resistance for the previous 3 years, in rotation with cereal or grain legume crops. Thirty-one oilseed rape crops of cultivars with *sylvestris*-derived resistance were examined within a 100 km radius of this site. These cultivars with *sylvestris*-derived resistance included Surpass 400, Surpass 501TT and Hyola 501.

In a separate experiment in 2004, disease severity in cultivars with either polygenic or major gene *sylvestris*-derived resistance was examined at 14 sites in South Australia. At one of these sites (Struan), there had been significant yield loss in cultivars with *sylvestris*-derived resistance in 2003 (Trent Potter, South Australian Research and Development Institute, Australia, pers. comm.). At most sites, the moderately resistant cv. Beacon (with polygenic resistance) and cv. Surpass 501TT were examined. Where these cultivars were not present, cultivars with a similar level of polygenic or *sylvestris*-derived resistance were assessed.

In the two experiments described above, the incidence and severity of phoma stem canker were assessed on 60 mature plants per site just before wind-rowing (swathing). Plants were cut transversely at the crown and then visually assessed for disease severity, which was scored as a percentage (0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100) of the cross section with internal necrosis (Marcroft et al., 2004). The average disease severity and incidence of plants with disease severity ≥5% were then calculated for each field and trial site.

Virulence testing of L. maculans *isolates*

Stubble pieces bearing *L. maculans* pseudothecia from cultivars with polygenic resistance or with *sylvestris*-derived resistance were collected from Lower Eyre Peninsula and Struan in South Australia in 2002 and 2003. Individual ascospores were cultured and isolates derived from these ascospores (35 isolates from 2002 stubble and 49 isolates from 2003 stubble) were tested for virulence on the cotyledons of cv. Surpass 400. Symptoms were scored 12 or 14 days after inoculation on a scale of 0 (no darkening around wounds) to 9 (large grey-green lesions with profuse sporulation) (Koch et al., 1991). Isolates were classified as producing low (score 0 to 2), moderate (2 to 4), high (4 to 6) or very high (6 to 9) lesion scores. The percentage of isolates in each category was determined for both years.

Statistical analyses

Stem canker severity and incidence data collected at different distances from the original site where

disease was observed on the Lower Eyre Peninsula in 2003, were square-root transformed and arcsin-transformed, respectively prior to analysis with REML, the algorithm for unbalanced data sets. A *t*-test was used to compare the severity of disease in cultivars with polygenic resistance to that in cultivars with *sylvestris*-derived resistance at the same site. The severity of cotyledonary lesions caused by isolates collected in 2002 and 2003 was compared using the *t*-test. All analyses were performed using Genstat version 6.1 (Payne et al., 1995).

Results and discussion

The incidence and severity of phoma stem canker in cultivars with *sylvestris*-derived resistance were examined on the Lower Eyre Peninsula in South Australia. At the sites examined there were no significant differences in the incidence and severity of disease, even at distances up to 100 km from the original 0.5 ha site where stem cankers were first observed in 2002 (Figure 1). This finding implies that individual *L. maculans* genotypes capable of overcoming *sylvestris*-derived resistance did not arise at this one location, or alternatively, that the spread of virulent isolates of the fungus was very efficient. However, it is most likely that such isolates were present over the whole area at a low frequency and were selected for by increased sowing of cultivars with *sylvestris*-derived resistance. Indeed, an isolate collected in Millicent, South Australia before cultivars with *sylvestris*-derived resistance were developed (isolate IBCN18 collected in 1986) is able to produce stem cankers on these cultivars (data not shown).

In 2004, severity of phoma stem canker was assessed on 60 mature plants at each of the 14 South Australian sites. The mean disease severity in cultivars with *sylvestris*-derived resistance was 51.7, significantly greater ($P < 0.01$) than the mean of 25.3 recorded on cultivars with polygenic resistance (Figure 2). At six of the sites, canker severity was similar in cultivars with *sylvestris*-derived and polygenic resistance, although disease severity was extremely low at two sites (Figure 2). However, at eight of the sites the pathogen populations showed host specificity whereby canker severity was higher on cultivars with *sylvestris*-derived resistance than on cultivars with polygenic resistance. This finding of populations adapted to cause cankers on cultivars with *sylvestris*-derived resistance is consistent with the extensive use of

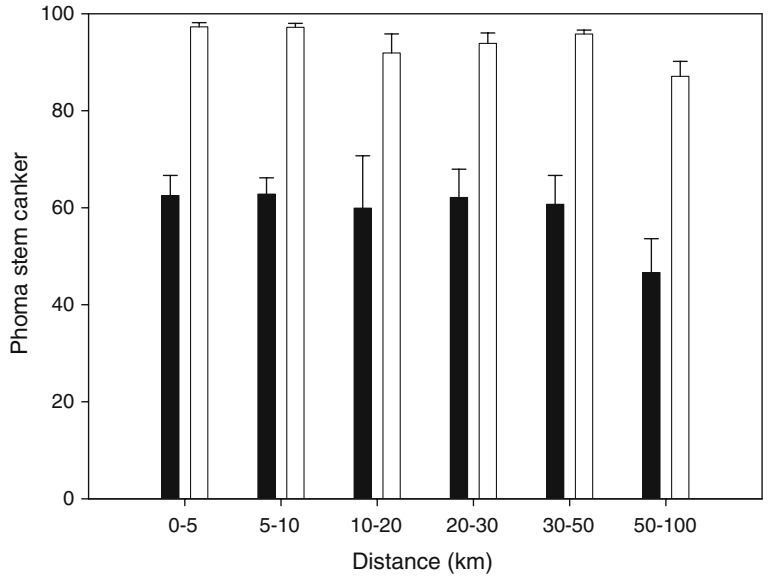

Figure 1. Severity (■) and incidence (□) of phoma stem canker in 31 commercial crops of *Brassica napus* cultivars with resistance derived from *B. rapa* subsp. *sylvestris* in 2003 at several distances from the South Australian site where plants with stem canker were first observed in 2002. Results are presented as mean ± SE.

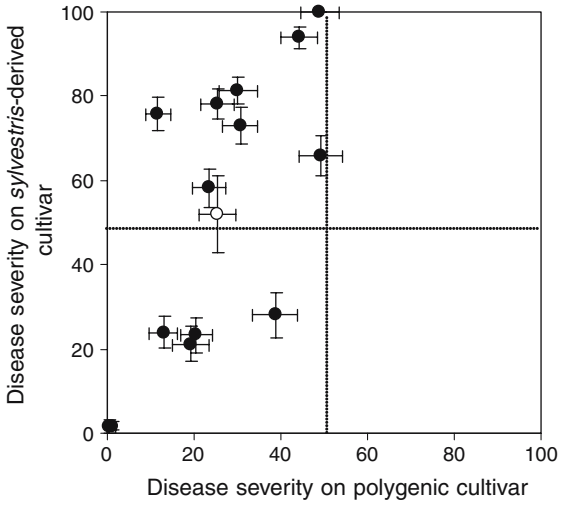

Figure 2. Severity of phoma stem canker in *Brassica napus* cultivars with either polygenic or *sylvestris*-derived resistance at 14 sites in South Australia in 2004. Each closed point (●) represents data (mean ± SE of disease severity) collected from 60 plants at one site. The open point (○) represents the mean (±SE) disease severity across all sites. Disease severity was significantly ($P < 0.01$) higher in cultivars with *sylvestris*-derived resistance than in cultivars with polygenic resistance.

such cultivars in these regions, compared to cultivars with polygenic resistance.

Individual isolates cultured from stubble of cultivars with polygenic resistance or with *sylvestris*-derived resistance were tested for their ability to cause cotyledonary lesions on cv. Surpass 400, which has *sylvestris*-derived resistance. Isolates collected in 2003 produced higher disease scores than isolates collected in 2002 (Figure 3). The mean disease score for isolates inoculated on Surpass 400 in 2003 was 4.3, significantly higher ($P < 0.001$) than the 2002 mean of 2.3 (data not shown). Although the number of isolates tested was small, this finding supports the hypothesis that the virulence of *L. maculans* isolates against *sylvestris*-derived resistance has changed. Surveys in New South Wales and Victoria have identified some fields with high levels of phoma stem canker in cultivars with *sylvestris*-derived resistance (S.J. Marcroft and S.J. Sprague, Australia, unpubl.). Isolates from Western Australia capable of overcoming the *sylvestris*-derived resistance in glasshouse trials have been described (Li et al., 2003a; 2005), but field surveys of disease severity and yield loss in this state have not yet been reported.

The situation in South Australia can be compared to the situation in France, where major gene resistance has been reported to be rendered inefficient. Brun et al. (2000) carried out a field experiment at Le Rheu over a 4-year period (1992–1995), where oilseed rape lines with major gene resistance introgressed from two different Brassica species were sown into *L. maculans*-infested stubble of the respective lines to assess the durability of the resistance sources. After 4 years of recurrent selection, the fungal populations had adapted to overcome resistance in a cultivar-specific manner. Under high inoculum concentration and in the presence of *L. maculans* populations selected recurrently on an oilseed rape line (MX) with the *Rlm6* resistance gene from *B. juncea*, resistance of this line was overcome after three seasons. In contrast, the MX-line was much more resistant to

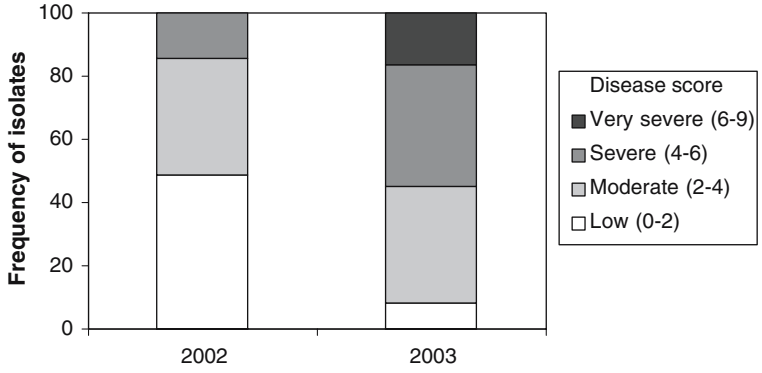

Figure 3. Frequency of *Leptosphaeria maculans* isolates collected from South Australia in 2002 and 2003 causing different levels of disease symptoms (low to very high disease scores) on the cotyledons of *Brassica napus* cv. Surpass 400. The mean disease score for isolates inoculated on Surpass 400 in 2003 was 4.3, significantly higher ($P < 0.001$) than the 2002 mean of 2.3 (data not shown).

populations selected on stubble of *B. napus* and an oilseed rape addition line (LA4+) with chromosome 4 introgressed from *B. nigra*. The resistance of the LA4+ line was maintained in the presence of *L. maculans* populations from stubble of plants with *B. juncea* or *B. nigra* resistance. Both the MX and LA4+ lines exhibited a high level of resistance when exposed to *L. maculans* populations from stubble of *B. napus* cultivars.

These recurrent selection experiments suggest that *L. maculans* populations can rapidly shift under selection pressure exerted by a new resistance gene. The *Rlm6* gene from *B. juncea* seems to have given a selective advantage to virulent isolates that pre-existed within the fungal population, as previously shown by Somda et al. (1999). Consequently, the selection pressure within the field experiment rendered the *Rlm6* resistance source inefficient after only three seasons. Unlike the *Rlm6* gene, which was introduced into a susceptible genetic background, the more stable source of resistance from *B. nigra* was introduced into the LA-line, which has polygenic resistance at the adult plant stage. Some isolates produced leaf spots but there was little canker at the stem base (data not shown). These results are consistent with the hypothesis that the durability of major resistance genes can be increased in the presence of a genetic background with polygenic resistance (Kiyosawa and Shiyomi, 1976). An alternative hypothesis is that isolates virulent on the *B. nigra* recombinant line were less fit than isolates virulent on the *B. juncea* recombinant line. As yet there are not enough data to test either of these hypotheses.

A nation-wide survey of commercial crops in France over an 8-year period (1994–2001) showed a decline in the efficiency of resistance conferred by a major gene, *Rlm1* (Rouxel et al., 2003). Increases in the frequency of virulent *avrLm1* isolates in the *L. maculans* population corresponded to the increased adoption of cultivars harbouring *Rlm1*. By 1998/1999 this led to a decrease in the effectiveness of resistance conferred by *Rlm1*. Thereafter, the area sown to cultivars harbouring *Rlm1* decreased significantly. Further large-scale surveys of the French *L. maculans* population structure in 2000/2001 confirmed that *AvrLm1* isolates represented less than 20% of the national population (Balesdent et al., 2006). Apart from the large-scale cropping of *Rlm1* cultivars, other factors probably contributed to the speed of this shift in allele frequency. These include (i) increased areas of oilseed rape in France (an increase of more than 35% between 1996 and 1999) leading to a larger pathogen population size; (ii) high inoculum concentrations in the autumns of 1998 and 1999, promoting colonisation of plants by *avrLm1* bearing-isolates and subsequent sexual recombination; and (iii) changes in cultural practices including closer crop rotations and development of minimum tillage practices. Integrated management strategies can increase the longevity of resistance (Mundt et al., 2002), however, such strategies need to be clearly communicated to growers and farm advisers (Gladders et al., 2006).

The *Rlm1*-attacking isolates were present in French *L. maculans* populations before the commercial release of cultivars with the *Rlm1* resistance gene. For example, an isolate collected at Le Rheu in 1985 and deposited in the International Blackleg of Crucifers Network (IBCN) collection (Rouxel et al., 2003) and isolate H5 obtained in 1989 (Ansan-Melayah et al., 1995) have the *avrLm1* allele. Van der Plank (1968) has proposed that the presence of a virulence gene would decrease the fitness of a pathogen, therefore the frequency of isolates harbouring that virulence gene would decline when the corresponding resistance gene was removed. This theory suggests that an isolate with an unnecessary virulence gene would be less fit and occur at very low frequency within a population. This hypothesis was tested and is discussed by Huang et al. (2006) who compared symptoms caused by near-isogenic isolates of *L. maculans* differing at the *AvrLm4* locus. Many avirulence genes are probably not associated with a fitness function and therefore a mutation to virulence is not necessarily associated with loss of fitness (Leach et al., 2001). As discussed previously, isolates able to overcome the *sylvestris*-derived resistance and *Rlm1* were present in Australia and France, respectively, prior to the introduction of cultivars harbouring the corresponding resistance gene. However, these isolates did not appear to increase in frequency until the widespread use of cultivars containing the resistance genes, indicating that virulent isolates may be less fit.

At the Lower Eyre Peninsula in South Australia, the reduced efficiency of resistance in commercial crops occurred more rapidly and was associated with greater yield loss than in France. For instance, up to 90% yield loss was reported in some

fields in the Lower Eyre Peninsula but few areas in France recorded such high yield loss. Although phoma stem canker of *B. napus* is a world-wide problem, yield loss due to the disease is generally greater in Australia than in other countries. This may be because *B. napus* crops experience a milder winter with higher spring and summer temperatures in Australia compared to France. These conditions maximise the growth rate of the fungus within the plant and therefore increase the stem canker severity (Sun et al., 2001). An additional explanation for the large yield loss and rapid decline in efficiency of resistance in Australia is that the general level of background resistance of the spring oilseed rape cultivars into which the *sylvestris*-derived resistance was introduced may have been less effective than that of the European winter oilseed rape cultivars. Cultivar Surpass 400, with *sylvestris*-derived resistance has some background resistance. The severity of leaf lesions and stem canker was less on cv. Surpass 400 than on the highly susceptible cultivars Q2 and Westar when inoculated with isolates able to overcome *sylvestris*-derived resistance (Li et al., 2003b). Without specific molecular markers for resistance, the extent of polygenic resistance in a cultivar with an effective major gene resistance in field trials cannot be evaluated.

Similar changes in virulence of *L. maculans* populations leading to overcoming of resistance have not been documented in other countries. This may reflect the fact that major gene resistance in oilseed rape cultivars has only recently been described; hence any changes in field behaviour of cultivars (such as increasing disease severity) has not been attributed to a decline in efficiency of such resistance. However, major gene resistance becoming less efficient has been reported in other host–pathogen systems. *Mycosphaerella graminicola*, a foliar pathogen of wheat, and *Venturia inaequalis*, the cause of apple scab are pathogens with a similar lifecycle to *L. maculans*. These fungi undergo sexual recombination on infected debris, airborne dispersal of sexual spores and asexual multiplication within the crop, and are able to overcome major gene resistance of their hosts. Isolates of *M. graminicola* that could specifically overcome resistance of the *Stb4* major gene resistance in the wheat cv. Gene (Kronstad et al., 1994) were identified within 5 years of cultivar release in Oregon (Cowger et al., 2000). Although cv. Gene was highly resistant on release in 1992, some disease was evident (Mundt et al., 1995) suggesting that virulent isolates were already present in the population. Increased use of cv. Gene selected for virulent isolates, however, these isolates persisted in the population despite the reduction in acreage of cv. Gene (Cowger et al., 2000). In contrast to this situation with *M. graminicola*, and *L. maculans* in France and Australia, isolates of *V. inaequalis* able to overcome the *Vf* major gene resistance in apple apparently were not present in the population prior to the introduction of cultivars harbouring this resistance gene (Guerin and Le Cam, 2004). The authors propose that all virulent isolates arose from a single mutation event that occurred in the sampling year, as all virulent isolates collected could be assigned to a single clonal lineage. This hypothesis was supported by the high degree of genetic differentiation between virulent and avirulent populations, which indicated a lack of sexual recombination between the two populations (Guerin and Le Cam, 2004).

Although the oilseed rape genotype (winter vs. spring) and the environmental conditions under which crops are grown differ considerably between France and Australia, resistance conferred by major genes was overcome in a few years in both countries. The data presented for different resistance genes under diverse climatic environments highlight the need for better evaluation of the level of background resistance to *L. maculans* into which major resistance genes are introduced. Moreover, these results highlight the need to exploit the best combinations of different genetic factors (for example; by pyramiding effective major genes and/or by associating polygenic and major gene resistance) to develop cultivars with durable resistance. Delourme et al. (2006) discuss a range of resistance sources that are currently being tested for exploitation in oilseed rape breeding programs. The presence of the sexual stage of *L. maculans* on oilseed rape stubble with major resistance genes allows the recombination of virulence genes and is a source of primary inoculum for initiating epidemics. Integrated strategies that consider the genotype of the cultivars, agronomic practices to reduce fungal inoculum and the best strategy to deploy cultivars with the same major gene resistance (for example; minimum distance between fields) require further research to minimise the risk that resistance becomes inefficient in oilseed rape by changes in populations of *L. maculans*.

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