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Determining frequencies of avirulent alleles in airborne *Leptosphaeria maculans* inoculum using quantitative PCR

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ABSTRACT

Phoma stem canker (blackleg disease) of *Brassica napus* (oilseed rape, canola) is caused by the fungus *Leptosphaeria maculans*. Frequencies of avirulent alleles for loci where virulence can be associated with gene deletion (*AvrLm1* and *AvrLm6*) were determined in samples of *L. maculans* airborne inoculum using quantitative PCR. The accuracy, reproducibility and limitations of detection were determined. Changes in the frequency of avirulent alleles were determined for the 2006/2007, 2007/2008 and 2008/2009 growing seasons for winter oilseed rape in the UK. The frequency of *AvrLm1* remained small (between 9 and 16%), whilst the frequency of *AvrLm6* fluctuated between 66 and 35%. Estimation of frequencies of avirulent alleles in airborne pathogen inoculum gives an efficient and unbiased method to assess the potential that crop cultivars with corresponding resistance genes are at risk of disease.

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Key Words: airborne inoculum, effector genes, *Avr* genes, *Leptosphaeria maculans*, air sampling, disease forecasting.

INTRODUCTION

Leptosphaeria maculans, the causal agent of phoma stem canker (blackleg disease), is responsible for major yield losses of oilseed rape (*Brassica napus*) worldwide, costing the industry more than $1000M per season at current prices (Fitt *et al.,* 2008). This disease is controlled by fungicide applications, crop management strategies and breeding for resistance (Aubertot *et al.,* 2006; Fitt *et al.,* 2006). Numerous genes conferring resistance to *L. maculans* have been identified in oilseed rape and other *Brassica* species (Delourme *et al.,* 2006). The interaction between *L. maculans* and oilseed rape is a typical gene-for-gene relationship

(Balesdent *et al.,* 2005). Numerous effector genes are involved in the interaction between the pathogen and its host, but a subset of these, termed *Avr* genes, render the pathogen avirulent on host genotypes with the corresponding resistance (*R*) genes (Ellis *et al.,* 2009). At least twelve *Avr* (*AvrLm*) genes that are involved in recognition of *L. maculans* by *Brassica* species have been identified (Balesdent *et al.,* 2005; Yu *et al.,* 2005; Van de Wouw *et al.,* 2009a). Seven of these genes map to two gene clusters with three of them, *AvrLm1, AvrLm6* and *AvrLm4-7,* having been cloned (Gout *et al.,* 2006; Fudal *et al.,* 2007; Parlange *et al.,* 2009). *AvrLm1* and *AvrLm6* confer avirulence towards the genes *Rlm1* and *Rlm6,* respectively (Gout *et al.,* 2006; Fudal *et al.,* 2007). *AvrLm4-7* differs from *AvrLm1* and *AvrLm6* in that it confers avirulence towards two different *R* genes, *Rlm4* and *Rlm7* (Parlange *et al.,* 2009).

Like *Avr* genes in other fungi, these genes encode small proteins with N-terminal signal peptides (secretion signals), and have no homology to other fungal sequences and no conserved domains. Additionally, *AvrLm6* and *AvrLm4-7* are cysteine-rich (6 and 8 cysteines, respectively) (Fudal *et al.,* 2007; Parlange *et al.,* 2009). Mutations affecting the coding regions of these *Avr* genes can lead to virulence towards the corresponding resistance genes. Virulence towards *Rlm1* and *Rlm6* can be associated with deletion of the entire open reading frames of *AvrLm1* and *AvrLm6*, respectively (Gout *et al.,* 2006; Fudal *et al.,* 2007; Fudal *et al.,* 2009). Deletion of *AvrLm1* was responsible for virulence in more than 90% of 460 virulent isolates genotyped from France, Mexico and Australia and the break-points of this deletion were highly conserved (Gout *et al.,* 2007). Additionally, PCR screens using molecular markers showed that absence of an amplified product was associated with virulence towards *Rlm1* in 98.3% of isolates (Gout *et al.,* 2007). Although the major mechanism, deletion of *AvrLm6* was responsible for virulence in only 66% of 70 virulent isolates genotyped from France (Fudal *et al.,* 2009). Unlike *Avrlm1*, deletion break points were not conserved in all *avrLm6* isolates (Gout *et al.,* 2007; Fudal *et al.,* 2009). Due to this,

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association between the absence of PCR amplification and virulence towards *Rlm6* ranged between 93 and 66%, depending on the location of the primers within the *AvrLm6* locus (Fudal *et al.*, 2009). The frequency of virulent isolates within populations can change rapidly in response to selection resulting from widespread use of cultivars with corresponding *R* genes. When the frequency of virulent isolates reaches a particular threshold, resistance can be rendered ineffective. Such circumstances have occurred in France and Australia when newly introduced *R* genes were rendered ineffective by changes in *L. maculans* populations within three to five years of the introduction of commercial cultivars carrying them (Rouxel *et al.*, 2003a; Sprague *et al.*, 2006a; 2006b). An epidemic in south eastern Australia in 2003 where major gene resistance derived from *Brassica rapa* subsp. *sylvestris* became ineffective is estimated to have cost the Australian canola industry between $5-10 million AUD (P.A. Salisbury, Victorian Department of Primary Industries, Australia, personal communication). Accordingly, monitoring the frequency of avirulent and virulent isolates in fungal populations can play an important role in predicting and managing the risk of disease epidemics. Currently frequencies of avirulent alleles in fungal populations are determined by genotyping single isolates cultured from diseased plants at various locations and times by pathogenicity testing on cultivars with corresponding *R* genes and/or using molecular markers for avirulent alleles (Balesdent *et al.*, 2005; Stachowiak *et al.*, 2006; Gout *et al.*, 2007).

Another potential method for estimating avirulent allele frequencies in fungal populations is molecular analysis of airborne spore samples. Spore-based forecasting systems are being used successfully to predict the occurrence and spread of diseases such as blue mould (*Peronospora tabacina*) on tobacco crops (www.ces.ncsu.edu/depts/pp/bluemold/), soya bean rust (*Phakospora pachyrhizi*) (www.ces.ncsu.edu/depts/pp/soybeanrust/howtoreadforecast.php) and cereal rusts (*Puccinia spp.*) (www.ars.usda.gov/Main/docs.htm?docid=14574) in the USA and phoma stem canker

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on oilseed rape in Poland (www.spec.edu.pl). Disease risk predictions are based on the quantity of pathogen inoculum present in airborne samples, whereby the amount of inoculum is estimated by amplification of DNA using species-specific primers (West *et al.,* 2008; 2009). However, these sampling methods do not provide data about changes in frequencies of alleles at particular loci, such as *Avr* loci. The purpose of this study was to develop molecular techniques, specifically quantitative PCR, to determine frequencies of avirulent alleles in airborne inoculum of *L. maculans*.

MATERIALS AND METHODS

Preparation of isolate mixtures with known frequencies of avirulent alleles

Genomic DNA from two individual isolates of known avirulent *AvrLm1* or *AvrLm6* genotypes were mixed together with DNA of two virulent *avrLm1* or *avrLm6* isolates in different proportions to generate a series of DNA isolate mixtures. These virulent isolates had deletion alleles of the *AvrLm1* or *AvrLm6* loci, as shown by a PCR based screen. These DNA mixtures were used to assess whether quantitative PCR amplification could discriminate different frequencies of avirulent alleles. The DNA mixtures were created to represent populations with known frequencies of avirulent alleles of 100, 80, 60, 40, 20 or 0%. An isolate mixture representing a population with 100% of the avirulent allele consisted of 500 pg μL^{-1} of DNA from an isolate with genotype *AvrLm1* or *AvrLm6*. Mixtures indicative of a population with 80% avirulent and 20% virulent isolates consisted of 400 pg μL^{-1} of DNA from an *AvrLm1* or *AvrLm6* isolate combined with 100 pg μL^{-1} of DNA from an *avrLm1* or *avrLm6* isolate. The final DNA concentration of all isolate mixtures was 500 pg μL^{-1}. An internal control for amplification efficiency, consisting of 1250 pg of DNA from an isolate with a genotype of *AvrLm1-AvrLm6,* was included in all quantitative PCR runs. All primer

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Single *L. maculans* isolates were cultured from pycnidia in phoma leaf spot lesions from oilseed rape cv. Drakkar (no known *R* genes) collected in autumn in October/November 2006 at Rothamsted, Harpenden, Hertfordshire, UK, as previously described (West *et al.,* 2002). The crop had been sown in late August and was at the seedling stage of growth when lesions were sampled. All cultured isolates were maintained on Campbell's V8 juice agar media. The genotypes at the *AvrLm1* and *AvrLm6* loci of all cultured isolates were determined by assessing their ability to cause lesions on cultivars with known *R* genes (Balesdent *et al.,* 2006). The oilseed rape cultivars used were Westar (no *R* gene), Darmor-MX (*Rlm6*) and Columbus (*Rlm1*, *Rlm3*). Interaction phenotypes were assessed following inoculation of 15-day-old cotyledons with a 10 µL droplet of 10^6 spores mL^{-1} of conidial suspension (Balesdent *et al.,* 2006). Symptoms were scored on 10-12 plants per isolate-cultivar interaction, at 14 and 18 or 19 days after inoculation, on a 0-6 scale. Isolates producing scores of zero to three were designated as avirulent whilst isolates producing scores of four to six were virulent (Van de Wouw *et al.,* 2009). ¶
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sets tested generated a product with this template, thus enabling different amplification efficiencies to be compared.

Sampling of airborne inoculum

Airborne ascospores of *L. maculans* were collected using a Burkard seven day volumetric spore sampler (Burkard Manufacturing Company Ltd, UK). Two different methods were used to collect samples of airborne inoculum. Firstly, artificial samples of airborne inoculum were generated by ejecting ascospores from individual pieces of diseased oilseed rape stems. Wax-coated Melinex plastic tape (14 x 48 mm) (Burkard Manufacturing Company Ltd, UK) was placed on microscope slides in an up-turned lid of a Petri dish (9 cm diameter). Diseased stem debris of cv. NK Bravour (carries *Rlm1*) was attached to the inside of the Petri dish using Vaseline and misted with sterile water to stimulate ejection of ascospores above the slides. After 30 to 60 min, the tape with ascospores attached was removed and cut longitudinally into two pieces (each 7 x 48 mm). Using a light microscope, the number of ascospores on the entire surface of each piece of tape was counted before it was transferred into a 2 mL screw-top tube (Alpha Laboratories, UK) for extraction of DNA.

Secondly, field samples of airborne inoculum were collected at Rothamsted, Harpenden, Hertfordshire, UK during the 2006/2007, 2007/2008 and 2008/2009 winter oilseed rape growing seasons using a Burkard seven day volumetric spore sampler. Airborne inoculum was collected each day between the end of August and the following March each season. Diseased stem debris of infected oilseed rape was located within 5 m of the Burkard spore sampler, which was operating according to standard methods described in Lacey & West (2006). Weekly strips of Melinex tape were divided into daily pieces (14 x 48 mm) (Kaczmarek *et al.,* 2008). These were then cut longitudinally, with one piece used for extraction of DNA whilst the other was mounted on a microscope slide and stained with 1 g

L^{-1} trypan blue in lactophenol. The number of ascospores on this stained piece of tape was determined by counting two 200 µm-wide longitudinal transverses (Rogers *et al.,* 2009). The date of maximum ascospore release in each season was determined by counting the number of ascospores collected each day during sampling. In each growing season, maximum ascospore release occurred in autumn between mid-October and mid-December (Stonard, 2008). To estimate frequencies of avirulent alleles, DNA was extracted from spores collected on four selected days within the period of maximum ascospore release each season. These days were 9 Oct, 10 Oct, 29 Nov, 30 Nov for the 2006/2007 season, 25 Oct, 27 Oct, 11 Nov, 12 Nov for the 2007/2008 season and 1 Nov, 2 Nov, 7 Nov, 8 Nov for the 2008/2009 season. A minimum of 1000 ascospores were collected on each of these selected days (Stonard, 2008).

DNA extraction

DNA was extracted from either individual isolates grown in culture or from ascospores deposited on pieces of wax-coated Melinex tape. The CTAB extraction protocol (Rogers *et al.,* 2009) was used with the following minor adaptations. Tape pieces were not dissected further but kept as single 7 x 48 mm pieces when extracting DNA from ascospores. DNA was extracted from freeze dried mycelium of individual isolates. All DNA was archived at -20°C.

Quantitative PCR

Primers specific for amplification of alleles for the *Avr* genes *AvrLm1* and *AvrLm6* were designed using the web-based program Primer3 (Rozen and Skaletsky, 2000). For *AvrLm1*, the primers amplify a 198 bp region between positions 261 and 458 of the 667 bp *AvrLm1* open reading frame. For *AvrLm6*, the primers amplify a 211 bp region between positions 270

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and 480 of the 581 bp *AvrLm6* open reading frame. Production of an amplicon following PCR with these primers represented the occurrence of an avirulent allele, whilst lack of amplification indicated the occurrence of a virulent allele. Additional primers specific for amplification of the internal transcribed spacer (ITS) region of ribosomal DNA of *L. maculans* (Liu *et al.,* 2006) and actin (Van de Wouw *et al.*, 2009b) were also used (Table 1). All quantitative PCR reactions were done in triplicate and consisted of 2.5 µL of DNA, 10 µL of SYBR® Green Jump Start Ready Mix (Sigma, UK), 0.08 µL of Internal Reference Dye (Sigma), 0.6 µL(forward) and 0.6 µL (reverse) of 10 pmol μL^{-1} primers and 6.22 µL of sterile deionised water. DNA was replaced with nuclease-free water (Sigma) in the no-template controls. All reactions were done in Bioplastic 96 x 0.2 mL PCR plates (ABgene, UK) capped with optical, thin-wall eight-cap strips, using a Stratagene Mx3000P PCR machine. The *AvrLm1*, *AvrLm6* and ITS regions were amplified as follows: 95°C for 2 min; 40 cycles of 95°C for 15 sec, annealing for 30 sec, 72°C extension time, and fluorescence detection for 15 sec. The specific temperatures and times for each primer set are listed in Table 1. To ascertain the specificity of the PCR, a dissociation (melting) curve was done after the final amplification cycle. Data were analysed using the Stratagene MxPro-Mx3000p v3.20 software.

Table 1 near here

In each quantitative PCR run, a standard curve was generated. DNA was amplified from serially diluted samples consisting of 2500, 1250, 125, 12.5 and 1.25 pg of DNA from an isolate with genotype *AvrLm1-AvrLm6.* Quantities of DNA were plotted against cycle threshold (C_t) values. The resulting regression equations were then used to quantify the amount of DNA in 'unknown' samples. The R^2 values, where values near 1.0 imply a direct relationship between X and Y variables, generated from all standard curves were greater than 0.91.

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When comparing amounts of DNA amplified with individual primer sets, differences in amplification efficiency were considered. To correct for these differences an adjustment value (V) was determined

$$V = A/B \quad \text{eqn (1)}$$

where A is the average amount of the avirulent allele amplified using primers specific for *Avr* genes and B is the average amount of *L. maculans* ITS amplified from the 1250 pg internal control. The adjustment value was then applied to all 'unknown samples' to estimate adjusted amounts of DNA (X) that could be used to determine estimated allele frequencies.

$$X = C/V \quad \text{eqn (2)}$$

where C is the average amount of avirulent allele amplified in 'unknown samples' using primers specific for *Avr* genes and V is the adjustment value determined from equation (1). Allele frequencies were estimated by comparing differences in the amount of DNA. In experiments where only avirulent alleles were amplified, the estimated percentage of avirulent allele was determined by comparing the amount of DNA amplified from the 80, 60, 40, 20 and 0% isolate mixtures to the amount of DNA amplified in the sample representing a population where all isolates were avirulent. Alternatively, in experiments where both ITS and avirulent alleles were amplified from the same sample, estimated allele frequencies were determined by comparing the amount of avirulent allele DNA (after adjustment for amplification efficiency) with the amount of ITS DNA.

Pathogenicity testing of *Leptosphaeria maculans* isolates

Single *L. maculans* isolates were cultured from pycnidia in phoma leaf spot lesions from oilseed rape cv. Drakkar (no known *R* genes) collected in autumn in October/November 2006 at Rothamsted, Harpenden, Hertfordshire, UK, as previously described (West *et al.*, 2002). The crop had been sown in late August and was at the seedling stage of growth when lesions

were sampled. All cultured isolates were maintained on Campbell's V8 juice agar media. The genotypes at the *AvrLm1* and *AvrLm6* loci of all cultured isolates were determined by assessing their ability to cause lesions on cultivars with known *R* genes (Balesdent *et al.*, 2006). The oilseed rape cultivars used were Westar (no *R* gene), Darmor-MX (*Rlm6, Rlm9*) and Columbus (*Rlm1*, *Rlm3*) and lines 02-22-2-1 (*Rlm3*) and 01-190-1-1 (*Rlm9*). Interaction phenotypes were assessed following inoculation of 15-day-old cotyledons with a 10 µL droplet of 10^6 spores mL^{-1} of conidial suspension (Balesdent *et al.*, 2006). Symptoms were scored on 10-12 plants per isolate-cultivar interaction, at 14 and 18 or 19 days after inoculation, on a 0-6 scale. Isolates producing average scores of <3.0 were designated as avirulent whilst isolates producing average scores of ≥3.0 were virulent (Van de Wouw *et al.*, 2009a).

Statistical analysis

To determine whether estimated frequencies (%) of avirulent alleles were significantly different from the known frequencies of avirulent alleles in mixtures of avirulent and virulent isolates, the mean squared error of prediction (MSEP) was compared to the residual mean square (RMS) from regression analysis. The MSEP compares the predicted y-values (estimated percentage of avirulent allele) against the known x-values (known percentage of avirulent allele) (Wallach & Goffinet 1987). The ratio of the MSEP and RMS then considers whether or not the residual variation from two different models is the same. For this analysis, 100 and 0% values were not included since they are essentially fixed values, giving 12 degrees of freedom for MSEP and ten for RMS. Values less than 3.62 for $F_{12,10}$ were considered not significantly different for a 5% two-tailed test.

RESULTS

Quantitative PCR detection of differences in *AvrLm1* and *AvrLm6* avirulent allele frequencies

The avirulent alleles for both *AvrLm1* and *AvrLm6* genes were amplified from samples of mixed DNA from avirulent and virulent isolates representing populations of known avirulent allele frequencies. Increasing amounts of the target sequence were amplified from each of the samples of mixed DNA from avirulent and virulent isolates; this correlated with increases in the frequency of avirulent alleles (Fig. 1a and b). The estimated frequency of the avirulent allele (based on the measured amount of the DNA) was plotted against the known frequency of the avirulent allele for each sample (Fig. 1c and d). There were direct linear relationships between estimated and known frequencies as indicated by R^2 values near 1.0. These estimated and known frequencies of the avirulent allele were not significantly different (*AvrLm1* $F_{12,10} = 0.88$, *AvrLm6* $F_{12,10} = 1.08$). Similar results were obtained for samples of mixed DNA from avirulent and virulent isolates at three additional concentrations (125, 12.5 and 1,25 pg of total DNA) (data not shown). However, at extremely low concentrations of DNA, the estimated frequencies were greater than 100%, suggesting that this assay is inappropriate at these low concentrations.

Fig 1 near here

Allele frequency estimation by comparing DNA quantities amplified from *Avr* genes and the ITS region.

Since the mechanism of virulence in *avrLm1* and *avrLm6* isolates that we are detecting is deletion, both the ITS region (present in all isolates) and the avirulent allele were amplified. Amounts of DNA amplified using primers for both *Avr* genes and ITS region were used to

Deleted: **Pathogenicity testing of *Leptosphaeria maculans* isolates**¶ Twenty-four single *L. maculans* isolates collected in 2006 were genotyped for *AvrLm1* and *AvrLm6* by pathogenicity testing on cultivars Columbus (*Rlm1, Rlm3*) and Darmor-MX (*Rlm6*) (Balesdent *et al.*, 2005; 2006). Two of the 24 isolates did not produce lesions on cv. Columbus and were designated avirulent towards *Rlm1*. Conversely, 11 of the 24 isolates did not produce lesions on cv Darmor-MX and were designated avirulent towards *Rlm6*. All 24 isolates were virulent towards the susceptible control, Westar (no *R* gene). ¶

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estimate frequencies of avirulent alleles. The ITS region was amplified from samples of artificial airborne inoculum using *L. maculans*-specific primers. There was a direct relationship (R^2=0.78) between amount of ITS DNA amplified and number of ascospores in each sample (Fig. 2). Additionally, quantitative PCR was used to analyse four isolates with known variation in the number of rDNA repeats (ranging from 56 to 225 repeats (Howlett *et al.*, 1997)). As expected, the amount of ITS amplification varied for each isolate (Fig. 3). *AvrLm1*, *AvrLm6* and ITS were amplified from samples of mixed DNA from avirulent and virulent isolates representing artificial populations. Amounts of *AvrLm1* and *AvrLm6* DNA were adjusted to account for differences in amplification efficiency before comparison with amounts of ITS DNA. The estimated frequencies of avirulent alleles were not significantly different from the known frequencies (*AvrLm1* $F_{12,10} = 0.84$, *AvrLm6* $F_{12,10} = 1.08$) (data not shown).

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Comparison of avirulent allele frequencies estimated from airborne inoculum and plant-derived isolates.

The *AvrLm1*, *AvrLm6* and ITS regions were amplified from artificial samples of airborne inoculum. Amounts of *AvrLm1* or *AvrLm6* DNA were adjusted for amplification efficiency and then compared to amounts of ITS DNA (Table 2). For all samples, the frequency of the *AvrLm1* allele was extremely small (8% or less). Conversely, frequencies of the *AvrLm6* allele ranged between 71 and 93% in all except two artificial airborne samples. In these two samples, frequencies were greater than 100%. These two samples each contained less than 50 ascospores, suggesting that this assay is inappropriate for spore samples with small numbers of ascospores.

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Frequencies of *AvrLm1* and *AvrLm6* alleles were determined from field samples of airborne ascospores collected during the 2006/2007 growing season. Airborne inoculum samples from four selected days (9 Oct, 10 Oct, 29 Nov, 30 Nov 2006) were used; these dates were selected because they were days when large numbers of ascospore were released. The frequencies of *AvrLm1* and *AvrLm6* alleles were 9 and 66% respectively (Fig. 4). Twenty-four single *L. maculans* isolates collected at Rothamsted during the 2006/2007 growing season were genotyped for *AvrLm1* and *AvrLm6* by pathogenicity testing on cultivars Columbus (*Rlm1, Rlm3*) and Darmor-MX (*Rlm6, Rlm9*) (Balesdent *et al.*, 2005; 2006). Two out of the 24 isolates (8%) did not produce lesions on cv. Columbus and were accordingly designated avirulent towards *Rlm1*. This frequency of *AvrLm1* isolates is similar to that determined with field samples of airborne inoculum (9%). Conversely, 11 out of the 24 isolates (46%) did not produce lesions on cv Darmor-MX and were designated avirulent towards *Rlm6*, less than that determined using field samples of airborne inoculum (66%) (Fig. 4). All 24 isolates were virulent towards the susceptible control, Westar (no *R* gene) and lines 02-22-2-1 (*Rlm3*) and 01-190-1-1 (*Rlm9*) suggesting all avirulent interactions on Columbus and Darmor-MX are due to *AvrLm1* and *Avrlm6*, respectively. Similar results were obtained for an additional 96 isolates collected from four other regions in England (data not shown).

Isolates collected from five different locations across England, including Rothamsted, were tested with the *AvrLm1* and *AvrLm6* primer sets used in the quantitative PCR screens to assess the correlation between deletion alleles and virulence. Of 53 isolates virulent towards *Rlm1*, 51 (96%) did not produce an amplicon. All 11 isolates avirulent towards *Rlm1* produced an amplicon of correct size. Conversely, of 25 isolates virulent towards *Rlm6*, 10 (40%) did not produce an amplicon. All 39 isolates avirulent towards *Rlm6* produced an amplicon of the correct size.

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***AvrLm1* and *AvrLm6* allele frequencies within three UK oilseed rape growing seasons**

Frequencies of *AvrLm1* or *AvrLm6* avirulent alleles were determined from field samples of airborne ascospores collected during three consecutive seasons, 2006/2007, 2007/2008 and 2008/2009. Airborne inoculum samples from four selected days were used to determine the average frequency of avirulent alleles in each season. Although the specific days changed each year, the samples used for this analysis were all collected between mid-October and mid-December each season with a minimum of 1000 ascospores collected on each of the four days (Stonard, 2008). Within the three seasons, the frequency of *AvrLm6* was greater than that of *AvrLm1*. The frequency of *AvrLm1* remained consistently small, between 9 and 16%, over all years (Fig. 5). Conversely, the frequency of *AvrLm6* fluctuated from 66% in 2006/2007 to 35% in 2007/2008 and then 49% in 2008/2009.

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DISCUSSION

We have demonstrated that quantitative PCR can rapidly estimate frequencies of avirulent alleles in airborne *L. maculans* inoculum. The accuracy of this method was tested by comparing the avirulent allele frequencies estimated using quantitative PCR analysis of airborne inoculum with frequencies determined by pathogenicity testing of 24 single *L. maculans* isolates. Using the two methods, similar values were obtained for frequencies of *AvrLm1* but a 20% difference was observed for frequencies of *AvrLm6*. This difference may reflect the size of the population being sampled. Using the quantitative PCR method, between 7000 and 9000 ascospores (essentially single isolates) were analysed, compared with only 24 isolates analysed by the pathogenicity testing. Thus the allele frequency estimated using

airborne inoculum should be a more accurate estimation since a greater proportion of the population is sampled. In a recent survey of 40 *L. maculans* isolates cultured from cultivars containing the *Rlm1* resistance gene in Australia, no *AvrLm1* isolates were detected (Van de Wouw *et al.,* 2009a). Similarly in this UK study, the frequency of *AvrLm1* isolates was less than 3% in the artificial samples of airborne inoculum generated using cultivars with *Rlm1* resistance, highlighting the accuracy of the quantitative PCR assay. However, the accuracy of the quantitative PCR assay was limited when ascospore numbers were small, whereby implausible allele frequencies, greater than 100%, were estimated from samples containing less than 50 ascospores. Similar problems with accuracy were encountered when quantities of *L. maculans* and *L. biglobosa* ascospores in airborne samples in Poland were determined (Kaczmarek *et al.,* 2008). In airborne samples with small numbers of ascospores, there was a poor correlation between amount of DNA and number of ascospores. Based on this information, to avoid errors in estimating avirulence allele frequencies when using the quantitative PCR analysis, it is advisable to assay only samples that have large numbers of ascospores.

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We have used the ITS region of the rDNA as a control for estimating avirulent allele frequencies. However, one potential problem with using this region as a control is that the number of copies varies (up to three times) between isolates (Howlett *et al.,* 1997). The finding that the quantity of ITS DNA amplified was directly proportional to the number of ascospores suggests that variation in rDNA copy number did not significantly influence the results of the assay. In addition, the adjustment of amplification efficiency would have decreased the effect of multiple copies of ITS sequences. As expected, we showed that the number of rDNA repeats indeed influences the amplification of ITS for individual isolates, however, the impact of isolates with extremely high or low number of repeats is reduced when this is averaged over a large population. Since the ITS region is already used for

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quantifying inoculum for a number of systems, including *L. maculans, L. biglobosa* and *Sclerotinia sclerotiorum* (Kaczmarek *et al.,* 2008; Rogers *et al.,* 2009), using ITS DNA as a control for estimating frequencies of avirulent alleles will minimise the number of extra assays needed to assess frequency of avirulent alleles from airborne inoculum.

There are numerous benefits of determining avirulent allele frequencies using airborne inoculum. Firstly, the size of the population being analysed can be extremely large, as in this study where over 7000 ascospores were analysed each season. Secondly, the sampling of the population is less biased than that used for estimating allele frequencies by culturing single isolates. When individual isolates are cultured from single pieces of diseased tissue of specific cultivars, the *R* genes present in these cultivars will influence the frequency of *Avr* alleles. Van de Wouw *et al.* (2009a) showed that the frequency of *AvrLm1* was approximately 85% in isolates cultured from diseased stem debris of cultivars that lacked the *Rlm1* gene. Conversely, no *AvrLm1* isolates were cultured from diseased stem debris of cultivars that harboured the *Rlm1* gene. Although the population being sampled by spore trapping will still be influenced by the cultivars being grown in fields adjacent to the spore trap, the ascospores will come from multiple stubble sources, including wild hosts, reducing the bias due to specific *R* genes in cultivars. Spore trapping also removes the potential for selecting against isolates with a fitness cost. Fitness costs have been associated with two *L. maculans Avr* genes, *AvrLm1* and *AvrLm4* (Huang *et al.,* 2006; Huang *et al.,* 2009). When single isolates are cultured from cultivars without *Rlm1* or *Rlm4*, virulent isolates would be less fit and therefore may not be detected in the population when determining allele frequencies from single cultured isolates. When determining *AvrLm1* and *AvrLm4* allele frequencies in airborne inoculum, fitness costs would be irrelevant since the isolates are not required to infect any cultivars. Lastly, estimating frequencies of avirulent alleles from airborne

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inoculum is less time-consuming and more cost-efficient than genotyping single plant-derived isolates in pathogenicity screening tests.

One potential problem with the quantitative PCR assay described is that it detects specific mutations that lead to virulence, namely deletion of the *AvrLm1* or *AvrLm6* loci. As shown by Gout *et al.* (2007) and in the current study, deletion of the *AvrLm1* locus is the major mechanism conferring virulence towards *Rlm1* in populations in continental Europe (> 90%) and England (96%) and PCR based molecular markers are extremely accurate in detecting these alleles. These data suggest that the quantitative PCR assay described in the current study will be reliable for estimating the frequency of isolates avirulent towards *Rlm1*. However, deletion of the *AvrLm6* locus is associated with virulence towards *Rlm6* at a lower frequency, i.e. 66% in Europe (Fudal *et al.*, 2009) and only 40% in England (this study), and the detection of these deletion alleles depends on the region of the *AvrLm6* gene being amplified (Fudal *et al.*, 2009). Therefore, frequencies of isolates avirulent towards *Rlm6* will be overestimated when using the method described. This over estimation may explain why in 2006 the frequency of isolates avirulent towards *Rlm6* was estimated as 66% using the spore trap method and only 46% when single isolates were screened for virulence towards a *Rlm6* containing cultivar. Estimations of these avirulent alleles using the spore trap method rely on knowledge of the mechanisms of virulence in the population. Whilst deletion of *AvrLm6* was correlated with virulence in only 40-66% of isolates in European populations, this mutation is correlated with virulence in 77% of 87 virulent isolates tested in Australia (Van de Wouw pers. comm.). The differences in frequency of deletion alleles associated with virulence towards *Rlm6* suggest that the spore trap assay may not be suitable for monitoring all populations. Conversely, the association between deletion and virulence towards *Rlm1* is more consistent, suggesting that the spore trap assay would be suitable for all populations.

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Therefore, it is recommended that a set of virulent isolates be screened each year to determine whether virulence is still associated with deletion of the *Avr* gene.

For avirulent alleles where deletion is not the major mechanism conferring virulence then different molecular techniques will be required. For example, the primary mutation leading to virulence towards *Rlm4* is a non-synonymous nucleotide substitution leading to a glycine to arginine transition within the *AvrLm4-7* coding sequence (Parlange *et al.,* 2009). In this case, the DNA from the airborne inoculum could be analysed using technologies such as pyrosequencing that can determine ratios of single nucleotide polymorphisms within mixed DNA samples (Metzker 2005).

The observation that the frequency of *AvrLm1* alleles was less than 20% in all three seasons analysed is consistent with extensive cultivation of *Rlm1*-containing cultivars throughout Europe (Rouxel *et al.,* 2003b), which would exert extensive selection towards virulent isolates. Similarly, in 2002 the frequency of *AvrLm1* was estimated to be 18% following pathogenicity testing of 100 isolates collected at Rothamsted, UK (Stachowiak *et al.,* 2006). These data suggest that constant selection exerted on the population has resulted in the frequency of *AvrLm1* isolates remaining small. Conversely, when the same 100 isolates from Rothamsted were screened for virulence towards *Rlm6* in 2002, all were avirulent (Stachowiak *et al.,* 2006). However, in the current study frequencies of avirulent isolates fluctuated between 35 and 66%. Additionally, these frequencies are likely to be overestimated due to the low correlation between deletion mutations and virulence, as described above. In France, *Rlm6* resistance has been rendered ineffective within three years due to the rapid increase in the frequency of *avrLm6* isolates after selection from sowing *Rlm6*-containing cultivars into *L. maculans*-infected stubble in a field trial (Brun *et al.,* 2000; Brun *et al.,* 2010). Cultivars with *Rlm6* (Eurol MX, DarmorMX) were grown in field trials at

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Rothamsted and Boxworth after 2002 (West and Fitt, pers comm.) and as seen in France, this may have led to rapid changes in the frequency of *avrLm6* isolates at Rothamsted since 2002.

The results of this study demonstrate how molecular techniques can be used to monitor the changes in frequency of specific alleles in pathogen populations. As additional *Avr* genes are cloned and molecular markers to distinguish avirulent and virulent alleles are developed, quantitative PCR assays of airborne inoculum can play a critical role in disease management strategies. These assays can be used to assess the potential risk of severe epidemics for crop cultivars with corresponding *R* genes. This technology is transferable to other plant pathogen systems where effector genes with avirulence activity have been cloned. In addition to monitoring of frequencies of alleles of *Avr* genes, the level of resistance to triazole fungicides, for example, can also be assessed from air-sample DNA if a mutation in a target gene has been identified. The *CYP51* gene has been sequenced and the point mutations producing resistance towards triazole fungicides identified (Cools *et al.,* 2006). Similarly, for *Fusarium graminearum* and *F. culmorum,* the proportion of spores with genes for mycotoxin production can now be assessed (Stepien *et al.,* 2008; Wang *et al.,* 2008). The combination of spore sampling and molecular diagnostics provides new opportunities to predict severe disease epidemics, which will contribute to the protection of national food security.

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Table 1 PCR primers and conditions used to quantify allele frequencies of *Leptosphaeria maculans Avr* genes.

Gene amplified	Primer name	Sequence (5' to 3')	PCR conditions			
			Annealing temp (°C)	Extension time (sec)	Fluorescence detection (°C)	Number of cycles
AvrLm1	AvrLm1 qF	GGGTGTTTACTTCGCCTCAC	58	30	81	40
	AvrLm1 qR	ACGTTGTAATGAGCGGAACC				
AvrLm6	AvrLm6 qF	TATTGGACAAAAGCCGAAGG	58	30	81	40
	AvrLm6 qR	GCGAGAAGCAAGTGGAATGT				
L. maculans ITS[a]	LmacF	CTTGCCCACCAATTGGATCCCCTA	60	45	83	40
	LmacR	GCAAAATGTGCTGCGCTCCAGG				
L. maculans Actin[b]	ActinF	TTGGTCTTGAAAGCGGTGGTAT	59	30	81	40
	ActinR	CATCACTGTCCCACGAATTG				

[a] Liu *et al.*, 2006 [b] Van de Wouw *et al.*, 2009b

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Table 2 Allele frequencies of *Leptosphaeria maculans Avr* genes, *AvrLm1* and *AvrLm6*, in DNA extracted from artificial samples of airborne inoculum.

Number of ascospores[a]	Quantity of DNA[b] (pg)			Frequency (%) of avirulent allele[c]	
	ITS	*AvrLm1*	*AvrLm6*	*AvrLm1*	*AvrLm6*
1700	243.3	7.0	195.6	3	80
1114	165.3	0.1	149.8	0	91
1081	400.3	0.3	371.2	0	93
758	299.7	0.4	237.5	0	79
495	157.3	0.1	112.3	0	71
169	39.2	0.1	32.9	0	84
101	19.5	0.5	16.1	0	82
32	9.0	0.2	7.9	3	88
20	1.8	0.1	5.5	3	301[d]
18	2.6	0.2	5.6	8	218[d]

[a] Artificial samples of airborne inoculum were produced by ejecting ascospores from individual pieces of diseased oilseed rape stem onto sections of Melinex tape. Ascospore numbers were counted from subsections of tape, prior to DNA extraction.

[b] The amount of DNA in each sample was quantified using regression equations determined from standard curves.

[c] The frequencies of avirulent alleles were estimated by comparing the quantity of *Avrlm1* or *AvrLm6* DNA with the quantity of ITS DNA amplified in each sample.

[d] This assay has reduced accuracy at low numbers of ascospores (less than 50), leading to unreliable predictions of allele frequencies.

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Figure 1 Relationships, assessed by linear regression, between amount of DNA (a and b) or estimated allele frequencies (c and d) and the known frequency of *AvrLm1* (a and c) or *AvrLm6* (b and d) avirulent alleles of *Leptosphaeria maculans* in mixtures of DNA extracted from avirulent and virulent (*avrLm1* or *avrLm6*) isolates ((a) *AvrLm1* R^2=0.97, (b) *AvrLm6* R^2=0.97, (c) *AvrLm1* R^2=0.99, (d) *AvrLm6* R^2=0.99). Amounts of DNA were estimated using regression equations determined from standard quantitative PCR curves. Allele frequencies were estimated by comparing the amounts of amplified *Avr* DNA from each artificial population sample, to the amount of amplified *Avr* DNA from the sample representing a population where all isolates were avirulent. Vertical error bars represent standard errors and points are the means of three biological experiments, each with three technical replicates.

Figure 2 Relationship between amount of DNA amplified with primers specific for the internal transcribed spacer (ITS) region of the ribosomal DNA of *Leptosphaeria maculans* and number of ascospores in artificial samples of airborne inoculum ejected onto Melinex plastic tape, assessed by linear regression (R^2=0.78). Amounts of DNA were estimated using regression equations from standard quantitative PCR curves. Data points represent the average quantity of ITS amplified. Vertical error bars represent the standard errors and points are the means of three technical replicates. When the single outlier is removed from this analysis the R^2 value increases to 0.98.

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¶
Figure 2 Relationships between estimated frequency of *AvrLm1* (a) or *AvrLm6* (b), determined by comparison of *Avr* gene amplification, and known frequency of avirulent alleles of *Leptosphaeria maculans* in mixtures of avirulent and virulent isolates (*AvrLm1* R^2=0.99, *AvrLm6* R^2=0.99). Allele frequencies were estimated by comparing the amounts of amplified *Avr* DNA from each artificial population sample, to the amount of amplified *Avr* DNA from the sample representing a population where all isolates were avirulent. Amounts of DNA were estimated using regression equations from standard quantitative PCR curves. Vertical error bars represent standard errors and points are the means of three biological experiments, each with three technical replicates.¶

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Figure 3 Relationship between amount of amplified ITS (white columns) and number of rDNA repeat units (black circles) of four *Leptosphaeria maculans* isolates. The average amount of ITS amplified for the four isolates is represented by the dotted line. Amounts of ITS amplified are relative to the amplification of actin amplified in each sample. Vertical error bars represent the standard errors of three technical replicates.

Figure 4 Comparison of estimated avirulent allele frequencies for the two *Avr* genes (*AvrLm1* and *AvrLm6*) of *Leptosphaeria maculans* using molecular analysis of field samples of airborne inoculum (white) or pathogenicity tests of single isolates (black). Genomic DNA was extracted from ascospores trapped on tape on four selected days in the 2006/2007 growing season. Histograms (white) represent the average frequency of *AvrLm1* or *AvrLm6* in spore populations for these four days. Additionally, 24 single isolates were cultured from leaf spots on cv. Drakkar collected during the 2006/2007 season. Each of these isolates was tested for virulence towards cultivars with *Rlm1* or *Rlm6* resistance genes. Histograms (black) represent the frequency of avirulent isolates from these 24 isolates.

Figure 5 Frequencies of avirulent alleles of two *Avr* genes (*AvrLm1* and *AvrLm6*) of *Leptosphaeria maculans* within three UK winter oilseed rape growing seasons, 2006/2007 (black), 2007/2008 (white) and 2008/2009 (grey). Genomic DNA was extracted from ascospores collected on four selected days each season. Histograms represent the average frequency of *AvrLm1* or *AvrLm6* from these four days within each season.

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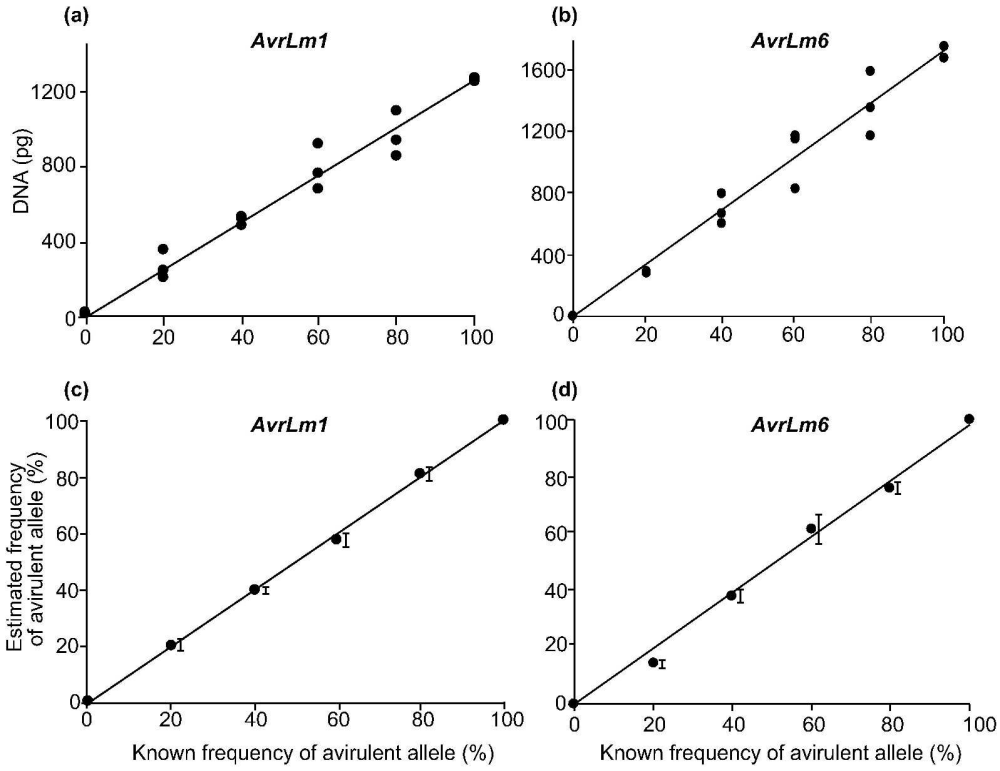

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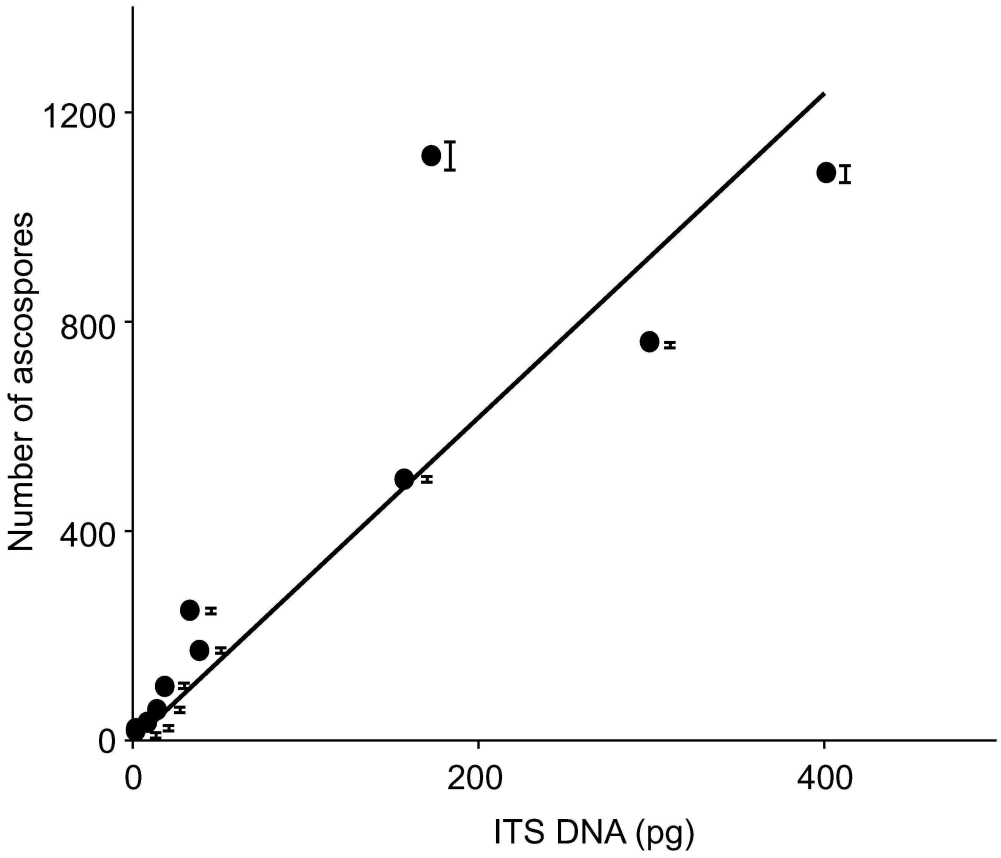
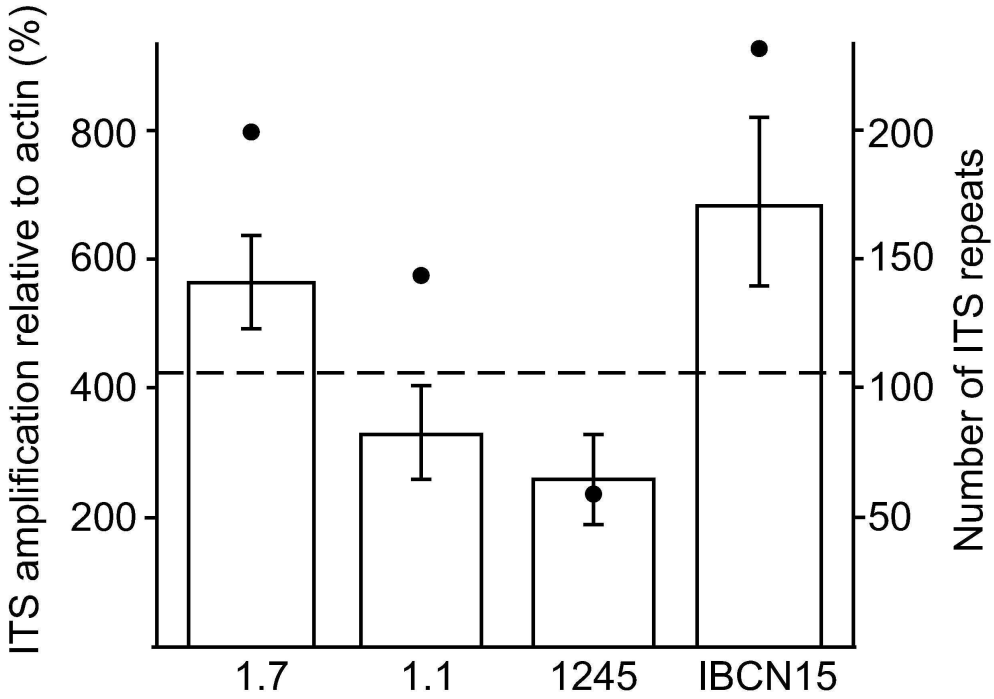
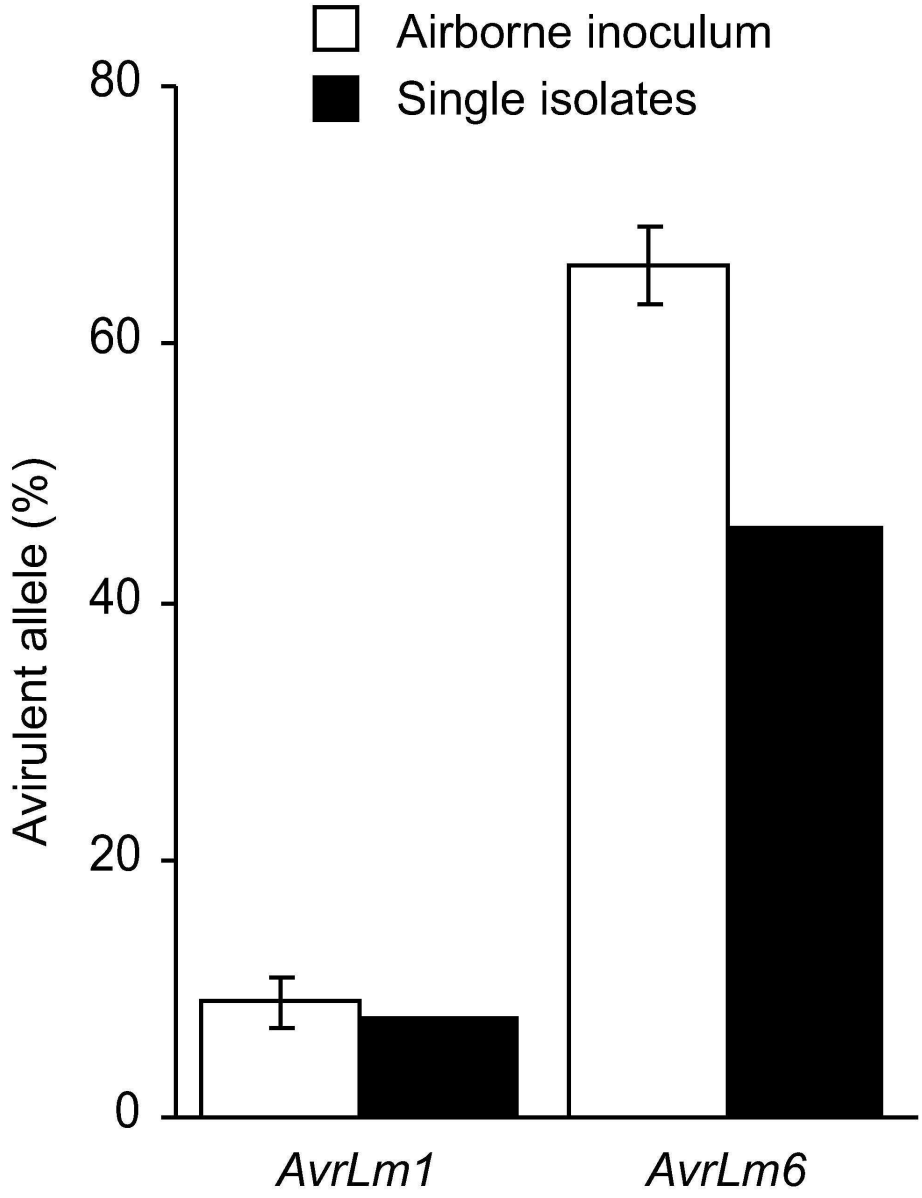


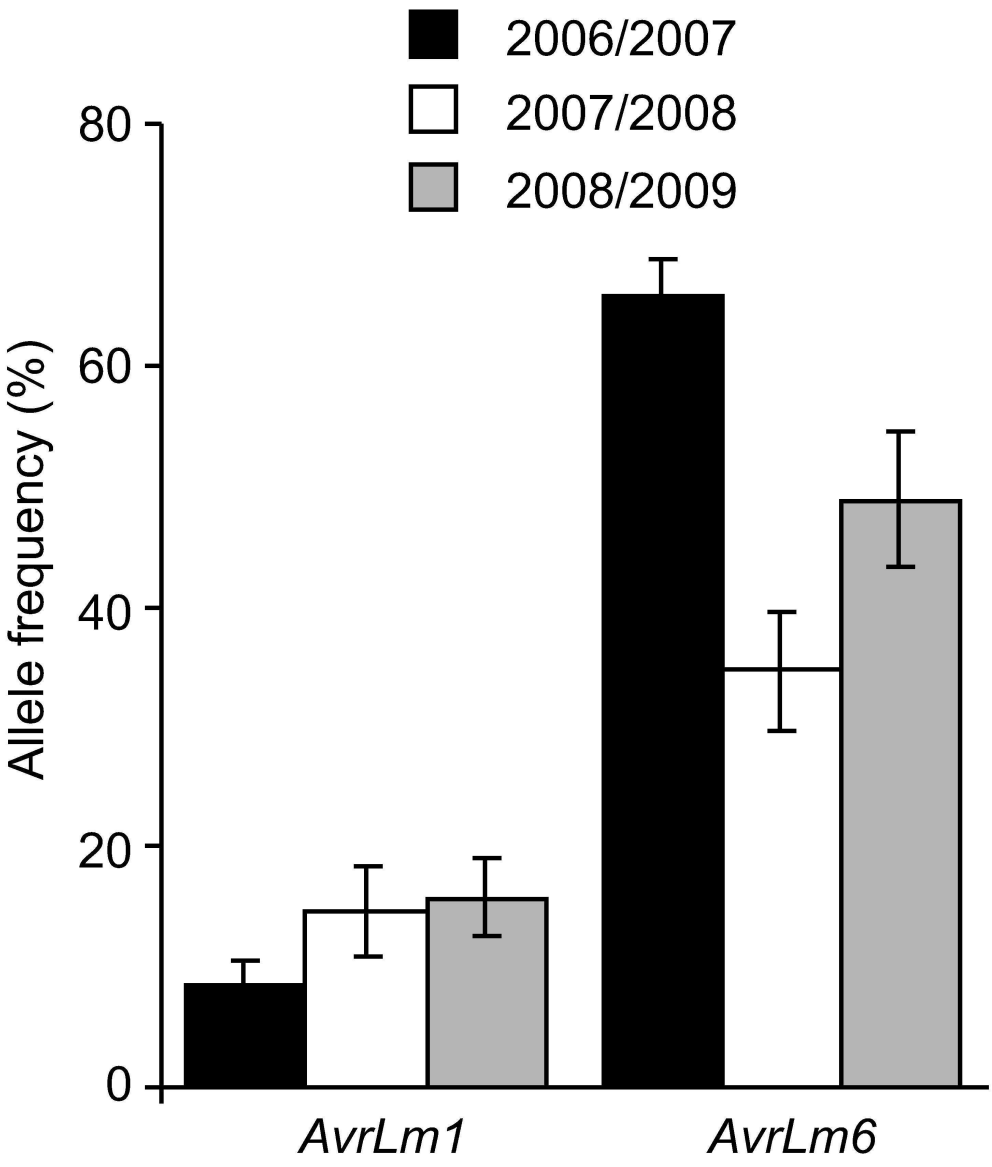
124x108mm (600 x 600 DPI)



105x74mm (600 x 600 DPI)



83x108mm (600 x 600 DPI)



124x144mm (600 x 600 DPI)