Sterol Demethylation Inhibitor Fungicide Resistance in *Leptosphaeria maculans* is Caused by Modifications in the Regulatory Region of *ERG11*

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Abstract

Blackleg is a worldwide disease of canola (*Brassica napus*), caused by a complex of fungal species in the genus *Leptosphaeria*, that impacts canola production and seed quality. Demethylation inhibitor (DMI) fungicides that target sterol 14 α -demethylase are an integral part of disease control. Here, we report six DMI-resistant isolates of *Leptosphaeria maculans* and two different types of genetic modification related to the resistance. Analysis of the regulatory region of the DMI target gene *ERG11* (also known as *CYP51*) revealed a 275-bp insertion in two of the isolates and three long terminal repeat retrotransposons (5,263, 5,267, and 5,248 bp) inserted in the promoter region of three resistant isolates. Genetic approaches confirmed that these elements are responsible for DMI resistance in *L. maculans* and crosses show segregation consistent with a single locus. Reverse-transcription quantitative PCR assays

demonstrated that the 275-bp insertion increases *ERG11* gene expression, conferring DMI fungicide resistance both in vitro and in planta. Moreover, transformation of a susceptible isolate of *L. maculans* with *ERG11* driven by a promoter containing the 275-bp insertion increased resistance to tebuconazole. A minimal shift of the values of concentration whereby 50% of the mycelial growth is inhibited in vitro was observed in resistant isolates containing long terminal repeat retrotransposons; nevertheless, these isolates were able to develop significant lesions on cotyledons from fungicide-treated seedlings. This is the first report of genetic modifications in *L. maculans* relating to DMI fungicide resistance.

Keywords: DMI resistance, *ERG11*, insertion, *Leptosphaeria maculans*, overexpression

Leptosphaeria maculans Ces. & De Not. (anamorph = *Phoma lingam* (Tode) Desm.) is a major pathogen of oilseed rape (*Brassica napus* L.; canola or rapeseed), causing blackleg or Phoma stem canker that results in yield losses worldwide, especially in the canola-growing regions of Australia, Canada, and Europe (Fitt et al. 2006; Gugel and Petrie 1992; Van De Wouw et al. 2016; West et al. 2001; Zhang and Fernando 2018). Integrated measures such as farming strategies to reduce exposure to fungal spores, crop rotation, fungicide application, and sowing cultivars that harbor resistance genes are recommended to control the severity of the disease and maintain the production levels (Delourme et al. 2006; Kharbanda and Tewari 1996; Marcroft et al. 2004, 2012). Fungicides can play an integral part in the management of blackleg. For instance, in Australia since 2000, seed-dressing fungicides (active ingredient: fluquinconazole) and fertilizer-amended fungicides (active ingredient: flutriafol) have been widely used to minimize the effect of blackleg (Van De Wouw et al. 2016). Since 2011, in-crop foliar fungicides have also been used

to minimize blackleg, with applications of a tebuconazole/prothioconazole mixture at the 4- to 10-leaf stages. All three of these fungicides used in Australia are triazole fungicides belonging to the sterol demethylation inhibitors (DMI) fungicide class.

DMI fungicides target 14 α -demethylase (EC 1.14.13.70) coded by *ERG11* (also known as *CYP51*) to inhibit the synthesis of ergosterol, which is an essential component of fungal cell membranes (Becher and Wirsal 2012; Mair et al. 2016). However, continuing usage of DMI fungicides with the same site-specific mode of action can allow resistance to develop and shift the structure of the pathogen populations, as reported in a diverse array of plant pathogens, including *Zymoseptoria tritici* (Cools et al. 2012), *Pyrenophora teres* f. sp. *teres* (Mair et al. 2016), *Pyrenopeziza brassicae* (Carter et al. 2014), *Oculimacula yallundae*, *Mycosphaerella fijiensis* (Price et al. 2015), and *Sclerotinia homoeocarpa* (Sang et al. 2019). Resistance to fungicides is a major threat to disease control (Fisher et al. 2018). Agricultural practices such as multiple sprays in a single season can lead to strong selection pressure for resistance to evolve as well as potentially increasing fungicide residues in the soil and, therefore, environmental selection pressure. This can then extend into human and environmental security issues because these fungicides are often similar in structure to the azole fungicides used to treat human medical mycoses (Chowdhary and Meis 2018).

Three main mechanisms of resistance to the DMI fungicides are nonsynonymous mutations within the *CYP51* gene, *CYP51* gene overexpression, and overexpression of efflux transporters (Parker et al. 2014; Price et al. 2015). Point mutations or combinations of mutations encoding amino acid changes within 14 α -demethylase have been widely reported as responsible for increasing resistance to DMI fungicides in fungal species, including *Z. tritici*, *Parastagonospora nodorum*, and *Monilinia fruticola* (Fraaije et al. 2007; Leroux et al. 2007; Lichtemberg et al. 2017; McDonald et al. 2019; Pereira et al. 2017; Price et al. 2015). Similarly, succinate dehydrogenase (SDH) inhibitors (SDHI), targeting the mitochondrial SDH enzyme, are another fungicide class commonly used to control plant pathogens worldwide and have recently been approved for use as protection against

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blackleg (Aviator Xpro APVMA Product number 69361; Australian Pesticides and Veterinary Medicines Authority; <https://apvma.gov.au/>). However, target-site mutations have been reported as responsible for SDHI resistance in other plant pathogens such as *Pyrenophora teres*, *Alternaria alternata*, and *Botrytis cinerea* (Avenot et al. 2008; Rehfsu et al. 2016).

Modification in the *CYP51* regulatory region resulting in gene overexpression was first reported in filamentous fungi in 2000; the acquisition of DMI resistance was triggered by a tandem repeat of a 126-bp sequence upstream of the *CYP51* gene enhancing its expression in *Penicillium digitatum* (Hamamoto et al. 2000). Other modifications in the *CYP51* regulatory region resulting in DMI resistance were subsequently detected, as follows. High gene expression was correlated with the presence of a 553-bp insertion located upstream of *CYP51A1* in *Venturia inaequalis* (Schnabel and Jones 2001). A 126-bp insert in *Mycosphaerella graminicola* increased transcript levels between 10- and 40-fold (Cools et al. 2012). Three insertions (46, 151, and 233 bp) were reported in the predicted regulatory region of the *CYP51* gene in resistant isolates of *Pyrenopeziza brassicae*. Furthermore, the resistant isolate carrying a combination of an insertion (151 bp) and nonsynonymous mutation (S508T) was the least sensitive to azole fungicides tested (Carter et al. 2014). Last, a 65-bp transposable element located 113 bp upstream of the start codon was correlated to overexpression of the *MfCYP51* gene in *M. fructicola* (Chen et al. 2017; Luo et al. 2008). Fungicide resistance due to overexpression of efflux transporters has also been reported in plant pathogens. A 519-bp insertion was detected in the *MgMFS1* promoter and resulted in overexpression of an efflux pump of the major facilitator superfamily, conferring multidrug resistance in isolates of *Z. tritici* (Omrane et al. 2015). The overexpression of efflux transporter gene *ShatrD* in *S. homoeocarpa* genotypes was responsible for reduced DMI sensitivity (Hulvey et al. 2012).

Despite decades of fungicide use, few studies have investigated DMI fungicide resistance of *Leptosphaeria* pathogens (Van de Wouw et al. 2016). A DMI fungicide sensitivity comparison of *L. maculans* and *L. biglobosa* suggests that DMI fungicides maintain effectiveness in controlling Phoma stem canker in the United Kingdom (Sewell et al. 2017). These studies, as in many other pathogens, used the conventional system of in vitro growth assays, whereby media is spiked with fungicides at different concentrations to determine the concentration whereby 50% of the mycelial growth is inhibited (half maximal effective concentration [EC₅₀]) (Dekker 1988; Fraser et al. 2015). A high-throughput in planta assay was developed to detect fungicide resistance of *L. maculans*, whereby *B. napus* stubble (crop debris that harbors the sexual fruiting bodies) was used to inoculate plants treated with fluquinconazole (Van de Wouw et al. 2017). Unlike the conventional in vitro assay, whereby small numbers of isolates (usually only in the hundreds) are screened, the in planta assay allows several thousand isolates to be screened and, therefore, low frequencies of resistance potentially detected (Van de Wouw and Howlett in press). As highlighted by Dekker (1988), in planta assays are potentially more reflective of what the pathogens are exposed to in the field.

Using the in planta assay, resistance to a DMI fungicide, fluquinconazole, was reported in Australian *L. maculans* populations (Van de Wouw et al. 2017). From a survey of 200 populations collected around Australia in 2015, resistance to fluquinconazole was detected in 15%. When these fungicide-resistant isolates were analyzed at the molecular level, no mutations were detected within the *CYP51* gene sequence or promoter and no consistent changes in gene expression were detected. Furthermore, the resistance detected using the in planta assay resulted in minimal changes using in vitro plate assays (Van de Wouw et al. 2017).

In the present study, DMI-resistant isolates of *L. maculans* were identified from stubble assessed in 2018, showing altered sensitivity to fungicides both in vitro and in planta. No mutation was found in the *ERG11* coding region. Instead, insertions of various sizes were identified within the *ERG11* regulatory region, resulting in DMI resistance both in vitro and in planta.

Materials and Methods

Identification of resistant isolates using in planta assays. As previously described, Van de Wouw et al. (2017) established in planta assays for detecting fungicide resistance in *L. maculans*. Briefly, this involved treated seedlings of cultivars ATR-Stingray or Pioneer SturtTT containing the *Rlm3* major resistance gene with either Jockey Stayer containing fluquinconazole (seed-dressing), Verge containing flutriafol (as a foliar application), or Prosaro containing tebuconazole/prothioconazole (as a foliar application) at commercial field rates of 20 liters/ton, 400 ml/ha, and 450 ml/ha, respectively. The resistance to *Rlm3* has been overcome in Australian *L. maculans* populations and, therefore, all isolates are expected to be virulent toward ATR-Stingray and Pioneer SturtTT (Van de Wouw et al. 2017). These fungicide-treated seedlings were inoculated using the ascospore shower technique, whereby stubble (blackleg-infected crop debris) was placed over the seedlings and ascospores were allowed to naturally infect the underlying seedlings. In total, 196 stubbles were screened in 2018 using this method. At 21 days post-inoculation (dpi), lesions that developed on the fungicide-treated seedlings (lesion scale > 0) were cut out and single isolates were cultured from the lesions, as previously described (Van de Wouw et al. 2017). Approximately 100 isolates were cultured from these lesions and then reinoculated onto fungicide-treated plants to determine whether they were, indeed, truly resistant isolates. From these screens, six fungicide-resistant isolates were confirmed as resistant, named 18BL149 to 18BL154 (Table 1). These isolates were collected from fungicide-treated plants inoculated with stubble that was collected from Dookie (Victoria), Cosgrove (Victoria), and Gibson (Western Australia). These isolates were confirmed as having resistance to the various DMI fungicides by inoculating wounded cotyledons of eight *B. napus* 'Pioneer SturtTT' seedlings grown from fluquinconazole-treated seed or seedlings sprayed with flutriafol or tebuconazole/prothioconazole, as previously described (Van de Wouw et al. 2017). Symptoms were assessed at 21 dpi on a scale of 0 (no darkening around wounds) to 9 (large gray lesions with prolific sporulation) (Elliott et al. 2016). Differences in disease severity caused by the isolates were determined by one-way analysis of variance (ANOVA) after square root transformation on the data using GenStat 16th edition. *P* values < 0.05 were considered statistically significance. Least significant differences were used to determine significant differences in disease severity for the various isolates.

All isolates were maintained on 10% Campbell V8 juice agar (2%) in a 22°C incubator with 12 h of light and 12 h of darkness. Fungicide-sensitive isolates D3 and D16 were used as control isolates in the in planta assays. Isolates D3 and D17 were used for genetic crosses, and D5 was used for as the recipient for transformation of *ERG11* alleles. The fungicide-resistant and -sensitive isolates used in this study are listed in Table 1.

Triazole fungicide sensitivity test in vitro. Fungicide sensitivity tests to technical grade triazoles (Bayer Crop Science) were carried out based on the active ingredients of commercial fungicides to evaluate the resistance level in vitro using a radial growth assay (Eckert et al. 2010; Fraser et al. 2015). Plugs (approximately 4 mm in size)

Table 1. Details and fungicide sensitivity phenotype (Type) of isolates used in this study

Isolate	Year	Type ^a	Location
D3 (IBCN16)	1988	S	Mt. Barker, Western Australia
D5 (IBCN18)	1988	S	Penshurst, Victoria
D16	2013	S	Wangary, South Australia
D17	2013	S	Wangary, South Australia
18BL149	2018	R	Dookie, Victoria
18BL150	2018	R	Dookie, Victoria
18BL151	2018	R	Dookie, Victoria
18BL152	2018	R	Gibson, Western Australia
18BL153	2018	R	Cosgrove, Victoria
18BL154	2018	R	Cosgrove, Victoria

^a S = sensitive and R = resistant.

from the edge of 7-day-old cultures of each isolate were inoculated onto the center of Campbell V8 medium petri dishes (9 cm) amended with a range of concentrations of fungicides based on the different sensitivity of isolates to different fungicides: tebuconazole (0, 0.25, 0.5, 1.0, 2.0, and 4.0 $\mu\text{g/ml}$), fluquinconazole (0, 0.0625, 0.125, 0.25, 0.5, and 1.0 $\mu\text{g/ml}$), prothioconazole (0, 0.025, 0.05, 0.1, 0.2, and 0.4 $\mu\text{g/ml}$), and flutriafol (0, 0.078, 0.156, 0.3125, 0.625, 1.25 and 2.5 $\mu\text{g/ml}$). The fungicides available as powders were dissolved in 100% dimethyl sulfoxide (DMSO) and added to V8 medium cooled to 50°C. The colony diameters of two replicates at each concentration were measured in two perpendicular directions at 14 dpi and EC_{50} values were calculated as previously described (Van de Wouw et al. 2017). Statistical analysis was carried out by GenStat 16th edition. Data were square root transformed prior to analysis. The resistance factor (RF) of each resistant isolate was calculated as the fold change in EC_{50} value divided by the mean EC_{50} of sensitive isolates (D3, D5, D16, and D17). For the remaining experiments, tebuconazole was used as the representative DMI fungicide for screening progeny and transformants in vitro assays.

DNA extraction, PCR, and sequencing of the *ERG11* promoter and coding region. Isolates were cultured in 10% cleared Campbell V8 liquid medium (pH 6.0) at 22°C for 4 to 5 days. Mycelium was harvested, snap frozen, then freeze dried overnight. DNA extraction followed the cetyltrimethylammonium bromide buffer method, as previously described (Pitkin et al. 1996). The *ERG11* coding and promoter regions were amplified by primer pairs EC1/EPS5 and EPS1/EPS6, respectively, based on the *L. maculans* *ERG11* gene sequences (Griffiths and Howlett 2002), and additional genome sequences from the Joint Genome Institute (lm_SuperContig_17_v2: 1070108-1073242). Primer sequences used in this study are shown in Supplementary Table S1. PCR assays were carried out under the following conditions: 95°C for 2 min; 35 cycles of 94°C for 40 s, 58°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. PCR conditions were modified for amplifying promoter regions in isolates 18BL149, 18BL150, and 18BL153 to 95°C for 2 min; 35 cycles of 94°C for 40 s, 62°C for 30 s, and 72°C for 2 min; and 72°C for 5 min. Sensitive isolates D3 and D5 were included for comparison. The amplicons were Sanger sequenced at the Australian Genome Research Facility, Melbourne, Australia. Sequences were analyzed using Geneious version R11.

Quantification of the *ERG11* expression in vitro and in planta. Resistant isolates 18BL151 and 18BL153 and sensitive isolates D3 and D5 were cultured in 15 ml of 10% cleared Campbell V8 liquid medium (pH 6.0) at room temperature in darkness for 72 h, then treated with tebuconazole at 22°C for 72 h, with shaking at 150 rpm. The final treatment concentration for each isolate was equal to its own EC_{50} value, with three biological replicates. Untreated groups were treated with an equivalent volume of DMSO as a control. Mycelium was harvested by centrifuging for 10 min at 4°C, snap frozen, and freeze dried overnight. RNA was extracted from ground mycelia with 4 ml of TRIzol reagent following the manufacturer's protocol (Invitrogen). For analysis of *ERG11* expression level in planta, wounded cotyledons of 10-day-old *B. napus* 'Westar' plants were inoculated with a 10- μl pycnidiospore suspension of isolates D3, D5, 18BL151, and 18BL153 at 10^6 spores/ml. Eight 12-mm samples around the wound site from eight cotyledons were collected at 7 dpi, snap frozen, and freeze dried overnight for RNA extraction using the TRIzol reagent.

First-strand cDNA was produced using AMV reverse transcription, following the manufacturer's protocol (New England Biolabs); that is, the RNA sample and oligo dT primer were denatured at 65°C for 5 min, then reverse transcription reaction mix was added following the manufacturer's protocol. The 20- μl cDNA synthesis reaction was incubated at 42°C for 1 h, then 80°C for 5 min. Quantitative reverse-transcription (qRT)-PCR was performed in 20- μl reactions (10 μl of 2 $\times$ KAPA SYBR FAST master mix, 0.4 μl of 10 μM primer set Erg11-qPCR-F and Erg11-qPCR-R [Supplementary Table S1], 2 μl of cDNA, and 7.2 μl of H_2O) using the Bio-Rad CFX384 qPCR system. Housekeeping gene *Actin* was amplified as an endogenous control using primers MAI0247 and MAI0248 (Supplementary Table S1).

The qPCR protocol was 95°C for 3 min, followed by 39 cycles of 94°C for 20 s, 58°C for 20 s, and 72°C for 20 s. For each sample, three biological and two technical replicates were examined. Relative transcript abundances were calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001). Statistical analysis was performed in GenStat 16th edition, with relative expression levels compared using one-way ANOVA. Data were square root transformed prior to analysis.

Genetic segregation analyses. Crosses between resistant and sensitive isolates were set up and progeny collected as previously described (Van de Wouw et al. 2009). In total, 107 progeny were collected from cross 18BL151 $\times$ D3 (Supplementary Table S2), 12 progeny were collected from cross 18BL152 $\times$ D3 (Supplementary Table S3), and 42 progeny were collected from cross 18BL153 $\times$ D17 (Supplementary Table S4). A subset of progeny from each cross was screened for the segregation of resistance using both in vitro and in planta assays. For in vitro assays, all progeny were screened on 10% Campbell V8 plates amended with tebuconazole at 0, 1.0, and 2.0 $\mu\text{g/ml}$ to test the inheritance of the fungicide-resistant phenotype. For in planta assays, a subset of progeny of each cross was inoculated onto Pioneer SturtTT seedlings either untreated or treated with fungicide (fluquinconazole, flutriafol, or tebuconazole/prothioconazole), as described above. To correlate the fungicide response with the presence or absence of the *ERG11* insertions, PCR was performed on the progeny and primers EPS1 and EPS6 (Supplementary Table S1) were used for crosses 18BL151 $\times$ D3 and 18BL152 $\times$ D3 and primers BIS1, EPS1, and EPS6 were used for 18BL153 $\times$ D17. The mating type locus was used to demonstrate the independent assortment of genetic markers in the cross with the two idiomorphs scored using mating type PCR as described (Cozijnsen and Howlett 2003).

Construct for green fluorescent protein gene and *ERG11* gene expression plasmids and transformation of sensitive isolate D5. The *ERG11* promoter regions were amplified using primers EPC1 and RG-2 (Supplementary Table S1) from genomic DNA from isolates D5, 18BL151, and 18BL153, named as D5-Pro, 151-Pro, and 153-Pro, respectively. The *green fluorescent protein* (*GFP*) gene and *trpC* terminator were amplified by primers RG-3 and RG-4 (Supplementary Table S1) from plasmid pLAU17 (Idnurm et al. 2017). The recombinant plasmids pMYQ1, pMYQ2, and pMYQ3 were formed by assembling the promoters and GFP fragment into plasmid pMAI6, which were used to transform isolate D5. Q5 high-fidelity DNA polymerase was used in all PCR assays (New England Biolabs).

The *ERG11* gene expression transformations were carried out by introducing a second copy of the *ERG11* into sensitive isolate D5, driven by the actin promoter and *ERG11* native promoters from isolates D5, 18BL151, or 18BL153, named as actin-Pro, D5-Pro, 151-Pro, and 153-Pro, respectively, to test the sensitivity change to tebuconazole. The *ERG11* promoter, coding region, and terminator from isolates D5 and 18BL151 were amplified using primers EPC1 and EPC2 and cloned (Supplementary Table S1). Two primer sets, EPC1/EPC3 and EPC2/EPC4 (Supplementary Table S1), were used to amplify promoter, coding region, and terminator separately from isolate 18BL153 because the large insertion occurred in the promoter region. The *ERG11* alleles driven by promoters (actin-Pro, D5-Pro, 151-Pro, and 153-Pro) were cloned into pMAI6 to form recombinant plasmids pMYQ4, pMYQ5, pMYQ6, and pMYQ7. All plasmids were confirmed by digestion with restriction enzyme *EcoRI* and sequencing.

***Agrobacterium tumefaciens*-mediated transformation and characterization of transformants.** *Agrobacterium tumefaciens* EHA105 was transformed with plasmids by electroporation and cultured on Luria-Bertani selection plates (kanamycin at 50 $\mu\text{g/ml}$). Then, transformations mediated by *A. tumefaciens* were carried out on fungicide-sensitive isolate D5, as previously described (Idnurm et al. 2017). GFP transformants were cultured in 10% cleared Campbell V8 juice and observed in vitro 3 dpi. Furthermore, 10 μl of pycnidiospore suspension of GFP transformants was inoculated onto *B. napus* Westar cotyledons and examined after 14 days using a Leica DM6000 microscope. Tebuconazole was used as a preferred

fungicide to test the fungicide sensitivity of transformants because of the greater difference between wild-type D5 and isolate 18BL151. The EC_{50} values to tebuconazole of 10 transformants were measured and analyzed by one-way ANOVA using GenStat 16th edition. Data were square root transformed prior to analyses.

The representative transformants with the *ERG11* allele driven by promoters actin-Pro, D5-Pro, 151-Pro, and 153-Pro were cultured in 10% cleared Campbell V8 juice for 72 h, then cultured with and without tebuconazole for 72 h. The transcript levels of *ERG11* were measured using RNA extraction and qRT-PCR, and relative transcript abundances were calculated as previously described. The data were statistically compared using one-way ANOVA (Genstat 16th edition). Data were square root transformed prior to analyses.

Results

Identification of *L. maculans* isolates with triazole fungicide resistance. Lesions developed on *B. napus* seedlings treated with fungicide after being showered with ascospores from three stubble samples; suggesting that these stubbles harbored resistant populations of *L. maculans*. From these lesions, six isolates, named 18BL149 to 18BL154, were obtained into culture. To confirm that these isolates harbor fungicide resistance, they were inoculated onto seedlings treated with fluquinconazole, flutriafol, or tebuconazole/prothioconazole regimes and compared with the fungicide-sensitive isolates D3 and D16 (Fig. 1). Isolates 18BL149, 18BL150, 18BL151, and 18BL152 caused significantly more disease than the control isolates on all three fungicide regimes. Isolate 18BL153 caused significantly more disease on plants treated with tebuconazole/prothioconazole but not flutriafol or fluquinconazole. Isolate 18BL154 caused significantly more disease on plants treated with flutriafol and tebuconazole/prothioconazole but not with fluquinconazole. All isolates were equally virulent on an untreated control (data not shown).

As previously reported, the resistant isolates of *L. maculans* can cause significantly more disease than sensitive isolates on plants treated with fungicides but may have minimal changes in EC_{50} values in vitro (Van de Wouw et al. 2017). To determine whether the isolates identified in the current study showed in vitro characteristics similar to or different from those previously identified, sensitivity

tests of resistant isolates in vitro were performed based on the active ingredients of commercial fungicides used in the canola industry in Australia. Comparison of colony sizes on fungicide selection plates illustrated that resistant isolates grew on the tebuconazole-spiked plates while sensitive isolates D3, D5, and D17 were inhibited, especially on plates with tebuconazole at 1.0 and 2.0 $\mu\text{g/ml}$ (Fig. 2). Moreover, isolates 18BL151 and 18BL152 had limited inhibition with tebuconazole at 4.0 $\mu\text{g/ml}$, demonstrating a higher tolerance level toward tebuconazole than the other isolates, which is consistent with the results in planta (Fig. 1). The EC_{50} values were calculated for the fungicide-sensitive and -resistant isolates. For tebuconazole, the mean EC_{50} for the sensitive isolates was 0.54 $\mu\text{g/ml}$, compared with 2.15 $\mu\text{g/ml}$ for the resistant isolates, with 18BL151 and 18BL152 having the highest values of 3.96 and 4.08 $\mu\text{g/ml}$, respectively. The RFs for tebuconazole ranged from 1.82 to 7.56. For fluquinconazole, the difference in EC_{50} values was lower (sensitive = 0.10 compared with resistant = 0.34), albeit still significantly different. Compared with the sensitive isolates, significant differences in resistance toward prothioconazole were also observed in isolates 18BL150 to 18BL154 but not 18BL149, with a mean RF of 2.36. For flutriafol, the resistance was increased in isolates 18BL150, 18BL151, and 18BL152 compared with all sensitive isolates, resulting in the highest mean RF at 5.12, whereas the EC_{50} values of the other three resistant isolates were not significantly different from the sensitive isolates D16 and D17 (Table 2). Isolates 18BL151 and 18BL152 were consistently resistant to the three DMI fungicides tested, both in in vitro and in planta, whereas the resistance varied between fungicide active ingredients in the other four isolates.

Identification of insertions within the promoter region of the *ERG11* gene. Amplification of the *ERG11* coding region in sensitive isolates D3 and D5 and resistant isolates 18BL149 to 18BL154 produced 1,781-bp amplicons, and sequencing revealed no modification in the *ERG11* coding region (submitted to GenBank as accessions MN331773 to MN331776). Amplification of the *ERG11* promoter region (to 1,000 bp upstream of the start codon) yielded the expected 1,099-bp fragment from isolates D3, D5, and 18BL154. Larger PCR products between 1.0 to 1.5 kb were amplified from isolates 18BL151 and 18BL152, while approximately 6-kb fragments were

Fig. 1. Average disease severity of fungicide-sensitive and fungicide-resistant *Leptosphaeria maculans* isolates. Isolates were inoculated onto plants treated with three different fungicide regimes: flutriafol (black bars), fluquinconazole (white bars), or tebuconazole/prothioconazole (hashed bars). Average disease severity was determined after 21 days. All six fungicide-resistant isolates (18BL149 to 18BL154) caused significantly higher levels of disease on one or more of the fungicide regimes compared with the fungicide-sensitive control isolates, D3 and D16. The same letter above the bar of the graph indicates a lack of significant difference at $P < 0.05$, as determined by analysis of variation of the square root transformed data.

amplified from isolates 18BL149, 18BL150, and 18BL153 (Fig. 3A), indicating that modifications occur in the promoter regions of those resistant isolates.

Sequencing revealed that two insertions with 275 bp of the same sequence and location (Fig. 3B) occurred upstream of *ERG11* in isolates 18BL151 and 18BL152 (represented by GenBank accession

MN331775). NCBI BLAST of this insertion revealed there are multiple (>400) copies in the genome of *L. maculans*. BLAST comparison with a dataset of consensus sequences of the *L. maculans* transposable elements (Grandaubert et al. 2014) revealed that this element is related to the retrotransposon *Pholy*, with the 275 bp matching the long terminal repeat (LTR) region. Sequencing of the PCR

Fig. 2. *Leptosphaeria maculans* isolates growing on 10% Campbell V8 agar amended with tebuconazole at A, 0.0; B, 1.0; C, 2.0; or D, 4.0 µg/ml.

Table 2. Half maximal effective concentration (EC₅₀) measurements of fungicide for isolates of *Leptosphaeria maculans*^u

Isolate	Size (bp) ^v	Tebuconazole		Fluquinconazole		Prothioconazole		Flutriafol	
		EC ₅₀ (µg/ml)	RF	EC ₅₀ (µg/ml)	RF	EC ₅₀ (µg/ml)	RF	EC ₅₀ (µg/ml)	RF
D3	N/A	0.67 (± 0.02) d	...	0.13 (± 0.06) e	...	0.08 (± 0.002) de	...	0.29 (± 0.004) de	...
D5	N/A	0.59 (± 0.04) d	...	0.12 (± 0.04) e	...	0.07 (± 0.002) e	...	0.35 (± 0.03) de	...
D17	N/A	0.38 (± 0.07) e	...	0.06 (± 0.02) f	...	0.09 (± 0.004) d	...	0.17 (± 0.01) e	...
D16	N/A	0.52 (± 0.02) d	...	0.098 (± 0.02) ef	...	0.05 (± 0.0002) e	...	0.21 (± 0.02) e	...
FungS ^w	...	0.54	...	0.10	...	0.07	...	0.26	...
18BL149	5,267	1.08 (± 0.04) c	2.00	0.18 (± 0.012) d	1.80	0.08 (± 0.004) de	1.14	0.45 (± 0.006) d	1.73
18BL150	5,248	1.77 (± 0.02) b	3.27	0.21 (± 0.06) cd	2.10	0.13 (± 0.006) b	1.86	0.78 (± 0.05) c	3.00
18BL151	275	3.96 (± 0.25) a	7.33	0.69 (± 0.02) a	6.90	0.31 (± 0.01) a	4.43	2.23 (± 0.6) b	8.58
18BL152	275	4.08 (± 0.19) a	7.56	0.57 (± 0.01) b	5.70	0.27 (± 0.02) a	3.86	3.76 (± 0.05) a	14.46
18BL153	5,263	0.98 (± 0.04) c	1.82	0.22 (± 0.002) c	2.20	0.10 (± 0.02) c	1.43	0.33 (± 0.002) d	1.27
18BL154	N/A	1.05 (± 0.04) c	1.94	0.19 (± 0.02) d	1.90	0.10 (± 0.03) c	1.43	0.44 (± 0.007) d	1.69
FungR ^x	...	2.15	...	0.34	...	0.17	...	1.33	...
Mean RF ^y	...	...	3.99	...	3.43	...	2.36	...	5.12
P value	...	<0.001	...	<0.001	...	<0.001	...	<0.001	...
LSD ^z	...	0.1245	...	0.02218	...	0.01989	...	0.1939	...

^u RF = resistance factor, the EC₅₀ value of a resistant isolate divided by the mean EC₅₀ value of the sensitive isolates. Numbers followed by a different lowercase letter indicate significant differences (*P* < 0.05).

^v Insertion size. N/A = not available.

^w Mean FungS EC₅₀ values are the average EC₅₀ values of sensitive isolates (D3, D5, D16, and D17).

^x Mean FungR EC₅₀ values are the average EC₅₀ values of resistant isolates (18BL149 to 18BL154).

^y Mean RF is the average of RF values of resistant isolates to the same fungicide.

^z Least significant difference.

products from isolates 18BL149, 18BL150, and 18BL153 demonstrated that the insertions were different in size and location within the region upstream of the *ERG11* open reading frame (Table 3). The insertions in 18BL149 and 18BL153 are most similar to the retrotransposon *zolly-2* and, in 18BL150, a form intermediary between *zolly-1* and *zolly-2* (Grandaubert et al. 2014). The insertions in 18BL149 and 18BL153 were closely related, with similar LTRs, whereas there were deletions in the LTRs in isolate 18BL150. Furthermore, comparison of the three insertions found the presence of single nucleotide polymorphisms following a mainly C-to-T and G-to-A change pattern, which corresponds to the action of repeat induced point (RIP) mutation acting on repetitive elements in the *L. maculans* genome (Rouxel et al. 2011). No open reading frames were predicted for the *zolly* copies.

The presence of *ERG11* promoter insertions was examined in 30 fungicide-sensitive isolates by PCR using primers EPS1 and EPS6, with no insertions present in any of the sensitive isolates (data not shown).

***ERG11* expression is influenced in planta and in vitro by the promoter insertions.** The *ERG11* expression levels in resistant isolate 18BL151 were significantly higher than in the sensitive isolates (D3 and D5) and 18BL153 ($P < 0.05$) in both the absence and presence of tebuconazole (Fig. 4A), indicating that the 275-bp insertion within the promoter region increased the expression level of *ERG11* and is associated with DMI fungicide resistance. Specifically, the relative *ERG11* transcript level to the *Actin* gene of isolate 18BL151 increased dramatically ($P < 0.05$) to 35-fold higher with tebuconazole treatment than without (Fig. 4A), demonstrating that the 275-bp insertion is correlated with the constitutive *ERG11* expression level as well as the azole-induced *ERG11* expression level.

The *ERG11* transcript level was measured in planta, showing that the level was markedly higher in the RNA sample from isolate 18BL151-infected *B. napus* cotyledons, which is significantly different from that in samples from cotyledons infected by isolates D5 or 18BL153 (Fig. 4B). Therefore, the *ERG11* expression levels both in vitro and in planta were increased in isolate 18BL151 containing the 275-bp insertion in the promoter region the *ERG11*.

The expression level of the *ERG11* in isolate 18BL153 treated with tebuconazole was significantly different from sensitive isolates but not for its constitutive expression level in the absence of DMI fungicide. The *ERG11* expression levels in isolate 18BL153 were not increased as dramatically as 18BL151 either in vitro and in planta but were statistically different from sensitive isolate D5 (Fig. 4), indicating that the 5,263-bp insertion in the *ERG11* promoter region is related to its resistance in vitro and in planta, further explaining the minimal increase of the EC_{50} values to the fungicides tested.

To explore the role of the insertion elements further, the *ERG11* promoter regions were fused to the open reading frame for GFP, the constructs transformed into *L. maculans*, and fluorescence examined qualitatively. The transformant with GFP driven by the promoter

with the 275-bp insertion (151-Pro) expressed more and brighter green fluorescence in vitro and in planta while the transformants driven by D5-Pro and 153-Pro showed a lower fluorescence intensity (Fig. 5). This difference in fluorescence intensity demonstrates that the 275-bp insertion in the promoter region upregulated the GFP expression level compared with the other promoters. This characteristic indicates that the promoter carrying the 275-bp insertion is able to drive overexpression of a gene other than the *ERG11*.

Genetic segregation analysis links insertion events to increased resistance. To determine whether the *ERG11* promoter insertion segregated with the fungicide-resistant phenotype, progeny from three different crosses were analyzed using in planta or in vitro assays. For cross 18BL151 × D3, 107 progeny were screened by PCR for their version of the *ERG11* promoter, resulting in 46 with the insertion and 61 without (Fig. 6A), not significantly deviating from the expected 1:1 ratio ($\chi^2 = 2.10$, $0.5 > P > 0.1$). In all, 107 progeny were also screened for resistance to tebuconazole using the in vitro assay, with 46 progeny being resistant and 61 being susceptible, showing 100% correlation between the *ERG11* insertion and the in vitro phenotype (Fig. 6B; Supplementary Table S2). A subset of 30 progeny was also screened for fungicide resistance in planta, with 100% correlation between the in vitro assays and with the presence or absence of the insertion (Supplementary Table S2). Similarly, all 12 progeny from cross 18BL152 × D3 were screened for the *ERG11* insertion, resulting in 6 progeny with the insertion and 6 without, again not significantly deviating from the expected 1:1 ratio ($\chi^2 = 0$, $P > 0.995$). All progeny were also screened for fungicide resistance in vitro and in planta, with 100% correlation between the in vitro and in planta fungicide-resistant phenotypes and the presence of the insertion in the *ERG11* promoter (Supplementary Table S3). The 41 progeny from the 18BL153 × D17 cross were screened for the presence of the *ERG11* insertion promoter, resulting in 15 isolates with the insertion and 26 without, not significantly deviating from the expected 1:1 ratio ($\chi^2 = 2.95$, $0.1 > P > 0.05$). Because 18BL153 and its progeny from the cross with isolate D17 have a limited in vitro phenotype (Fig. 6C), progeny were screened using the in planta assay only. However, when the progeny were examined, a sporulation and virulence defect was identified that segregated in the progeny, whereby the progeny were not fully virulent on the untreated control (average

Table 3. Details of retrotransposon-like elements inserted into the *ERG11* promoter region of fungicide-resistant *Leptosphaeria maculans* isolates

Isolate	Insertion size (bp)	Long terminal repeat size (bp)	GC content (%)	GenBank accession
18BL149	5,267	205	41.8	MN331773
18BL150	5,248	182	42.8	MN331774
18BL153	5,263	207	37.6	MN331776

Fig. 3. Analysis of the *ERG11* promoter region in fungicide-resistant *Leptosphaeria maculans* isolates. **A**, Amplification of the *ERG11* promoter in isolates of *L. maculans* using primers EPS1 and EPS6. DNA from isolates D3 (lane 1), D5 (lane 2), 18BL149 (lane 3), 18BL150 (lane 4), 18BL151 (lane 5), 18BL152 (lane 6), 18BL153 (lane 7), and 18BL154 (lane 8). D3 and D5 are two sensitive isolates. Lane M = 1-kb Plus DNA ladder. **B**, Diagram of the *ERG11* promoter region (1,000 bp upstream of the translation start codon) and the sites of the insertions in isolates. Numbers indicate positions relative to the start codon. Arrows a and b indicate the primer-binding sites (primers EPS1-F and EPS6-R) for amplification of the region.

disease severity scores < 5.0) (Supplementary Table S3). As a consequence, the in planta fungicide resistance phenotype could not be determined in the 25 progeny displaying the growth defects. Within these 25 progeny, 13 harbored the insertion and 12 did not. Of the 13 progeny that did not display the growth defect and were fully

virulent on the untreated control, 12 were characterized as fungicide susceptible and lacked the *ERG11* insertion while 2 were characterized as fungicide resistant and contained the insertion, consistent with the other crosses, whereby there was 100% correlation between insertion and fungicide resistance.

Fig. 4. Transcript levels of the *ERG11* gene relative to the housekeeping *Actin* gene in fungicide-sensitive isolates (D3 and D5) and fungicide-resistant *Leptosphaeria maculans* isolates (18BL151 and 18BL153) in vitro and in planta. **A**, White and black bars indicate cultures untreated or treated, respectively, with tebuconazole at a concentration equaled to half maximal effective concentration value. **B**, The *ERG11* transcript level was measured at 7 days after inoculating onto *Brassica napus* 'Westar' cotyledons. Values in vitro and in planta are mean of three biological and two technical replicate experiments, with standard deviations shown as error bars. Different letters above the bars indicate a significant difference ($P < 0.05$).

Fig. 5. Microscopy of *Leptosphaeria maculans* transformants with green fluorescent protein driven by *ERG11* promoters from isolates D5 (D5-Pro), 18BL151 (151-Pro), and 18BL153 (153-Pro). Images were captured using the same exposure time and settings.

As a control in the genetic segregation analysis, the *MAT* genotype was determined for each progeny by PCR and shown to segregate independently from the *ERG11* insertion and fungicide phenotypes (supporting material).

Transformations of a sensitive isolate with *ERG11* promoter insertion alleles confer fungicide resistance. *ERG11* alleles driven by the actin promoter (actin-Pro) and *ERG11* native promoters from isolates D5 (D5-Pro), 18BL151 (151-Pro), and 18BL153 (153-Pro) were individually transformed into the triazole-sensitive isolate D5. The fungicide resistance levels of transformants from the four constructs were increased substantially, although their EC₅₀ values were variable (Fig. 7A). The EC₅₀ values of 151-Pro transformants were significantly different from wild-type D5 and transformants driven

by actin-Pro and D5-Pro, demonstrating that the *ERG11* native promoter from isolate 18BL151 maintains its function to increase the resistance level in sensitive isolate D5. Furthermore, the EC₅₀ values of 153-Pro transformants were significantly different from D5 and actin-Pro but not D5-Pro, which is consistent with the results of GFP-expression transformation. Although the EC₅₀ values of 151-Pro transformants were increased differently, ranging from 1.2 to 4.5 µg/ml, the average value was not as high as that in the 18BL151 wild type.

Comparison of the *ERG11* relative expression levels among transformants containing the four type promoters (actin-Pro, D5-Pro, 151-Pro, and 153-Pro) demonstrated that 151-Pro transformants expressed significantly higher levels of *ERG11* than others in the

Fig. 6. Analysis of tebuconazole resistance in progeny from the cross D3 × 18BL151. **A**, Amplification of the *ERG11* promoter region (to 1,000 bp upstream of the start codon) by primer EPS1/ESP6. Lane M = the Invitrogen 1-kb+ ladder. Lanes D3 and 18BL151 = two parents for crossing and lanes R1 to R16 = progeny from the cross D3 × 18BL151 (representative from 107 in total). **B**, Growth of two parents and the 16 progeny on 10% Campbell V8 agar without and with tebuconazole (at 1.0 and 2.0 µg/ml). Progeny R5, R6, R9, R10, R13, and R15 that inherited the 275-bp insertion allele of *ERG11* grew on V8 agar plates with tebuconazole at 2.0 µg/ml while other progeny were inhibited. **C**, Growth of two parents and the progeny from the D17 × 18BL153 cross, illustrating the lack of clear in vitro response to tebuconazole.

absence of tebuconazole. In the presence of the fungicide, the expression levels of native promoter transformants were dramatically increased due to the stronger transcriptional response, showing a significant difference from the transformant driven by actin-Pro. The statistically significant differences were also observed among D5-Pro, 151-Pro, and 153-Pro transformants (Fig. 7B).

Discussion

Resistance to antimicrobial agents is a growing concern for the long-term ability to combat diseases in both agriculture and medicine. In terms of agriculture, the vast majority of the world's population is dependent upon grain crops (especially wheat and rice) for their calorific intake; hence, fungicide resistance in pathogens that infect grain crops is a threat to global food security. To maximize grain production, many farming systems also employ the use of crop rotations with other plant species, such as the use of canola as a rotation crop with wheat. Therefore, a rise in fungicide resistance in pathogens affecting these nongrain crops can have similar deleterious impacts on food security. This is the first report to define the basis for DMI-resistant phenotypes in *L. maculans*, the species causing the most common canola disease worldwide, as due to insertions of remnants of transposable elements into the *ERG11* promoter region.

DMI fungicide application contributes to maintaining the production of the canola industry and has become integral to controlling blackleg disease, particularly in Australia, where DMI fungicides are mainly employed, compared with the United Kingdom, where azoles, SDHIs, and strobilurins are all applied, or Canada, which does not use fungicides extensively. DMI fungicides can be applied up to four times on the same crop but most crops receive only one or two applications, depending on the severity of disease. Along with pathogen evolution and selection pressure on cultivar genetics, fungicide resistance might be a potential challenge to disease control.

In the current study, six resistant isolates of *L. maculans* were identified and characterized. A 275-bp insertion corresponding to an LTR of the *Pholy* retrotransposon family in the *ERG11* promoter region increased the gene expression level that confers triazole fungicide resistance and consistently changed the fungicide response both in vitro and in planta. The fluquinconazole-resistant isolates previously identified by Van de Wouw et al. (2017) showed minimal shifts in sensitivity in vitro. However, these isolates also lacked any changes in the promoter region, consistent with the finding that the 275-bp insertion is responsible for conferring the in vitro phenotype. Three

related LTR retrotransposons were identified in the *ERG11* promoter region of three resistant isolates and affected triazole fungicide resistance differently. Sensitivity tests in vitro showed that the EC₅₀ values of isolates with these *Zolly* retrotransposons were less increased than in isolates containing the 275-bp insertion, so much so that they could not be detected by using the regular in vitro assay. The finding of these retrotransposon insertions is not unique to *L. maculans*, with a non-LTR retrotransposon (5,585 bp) being found inserted 181 bp upstream of the *CYP51* homolog that resulted in the overexpression of *CYP51* in a DMI-resistant isolate of *Blumeriella jaapii*, the agent of cherry leaf spot (Ma et al. 2006). The RFs identified in the current study were lower than many of those reported in other species. For example, isolates of *Z. tritici* have been identified with an RF of 320 when screened on tebuconazole (Cools et al. 2012) and isolates of *P. teres* f. sp. *teres* have been identified with an RF of 16.2 (Mair et al. 2016). In this first instance, the *Z. tritici* isolates carry both point mutations in the *ERG11* coding region and a 120-bp insertion (Cools et al. 2012) while, in the latter example, the *P. teres* f. sp. *teres* isolates contain a F489L mutation (Mair et al. 2016). These different types of mutations suggest that there are multiple mechanisms possible for resistance, and the level of resistance will depend on the modification. It is possible that, if the *L. maculans*-resistant isolates identified here gained mutations within the coding region, in addition to the promoter insertion, the RF might increase dramatically. With low RF, it does mean that, from a disease management point of view, control of the disease could still be achieved, potentially by increasing concentrations or numbers of applications (if permitted), choosing more active products within the class, or ensuring preventative rather than curative spray timings.

The genome of *L. maculans* 'brassicae' has numerous transposable elements, representing 32% of the total DNA content (Grandaubert et al. 2014), which explains the presence of repetitive elements that might move to upregulate the specific gene for adapting the selection pressure. However, *L. maculans* has an active RIP mutation process that targets duplicated DNA such as retrotransposons for mutation during the sexual cycle (Idnurm and Howlett 2003; Van de Wouw et al. 2019). No active transposon has been identified to date because of mutations within them, which is further illustrated for the insertions of *zolly-2* in the *ERG11* promoter, none of which encode an open reading frame. The effect of RIP is the increase of AT richness in blocks in the *L. maculans* genome. Notably, these regions are under chromatin repression in vitro, which is released when the fungus causes plant

Fig. 7. Analysis of sensitivity to tebuconazole of transformants and *ERG11* expression of transformants introduced *ERG11* with different promoters. **A**, Half maximal effective concentration (EC₅₀) values of 10 strains where the *ERG11* genes were transformed into sensitive isolate D5 (driven by the *Actin* promoter), *ERG11* promoter from isolate D5, *ERG11* promoter carrying the 275-bp insertion from isolate 18BL151, and *ERG11* promoter carrying the 5,263-bp insertion from isolate 18BL153 for actin-Pro, D5-Pro, 151-Pro, and 153-Pro, respectively. The EC₅₀ values of wild-type D5 were measured by five independent experiments. The EC₅₀ values of 151-Pro and 153-Pro transformants are significantly different from wild-type D5. Different letters above the bars indicate a significant difference ($P < 0.05$). **B**, Expression of the *ERG11* gene in representative transformants from actin-Pro, D5-Pro, 151-Pro, and 153-Pro constructs compared with the untreated isolate D5. Values are the mean of three biological and two technical replicate experiments, with standard deviations shown as error bars. Different letters above the bars indicate a significant difference ($P < 0.05$).

disease (Soyer et al. 2014). Although one hypothesis that could be proposed is that the insertions of these large AT-rich regions into the *ERG11* promoter would trigger a similar type of regulation, our analysis of gene expression in planta does not support it. This research has shown that the context of the large element in the genome can alter gene expression; for example, contrast levels of *ERG11* transcript in the native context in Figure 1A with when in a different location (Fig. 7B). An alternative hypothesis is that the large repeated elements impact the overall folding of the DNA to drive expression. A role for such repeats has been proposed for the grass endophytic fungus *Epichloë festucae* (Winter et al. 2018). The mechanism by which such elements change in planta responses remains to be established but is particularly relevant given how many plant pathogens have genome structures similar to that of *L. maculans* and that in vitro assays for fungicide resistance cannot capture what occurs in the plant. Nevertheless, identifying this mechanism should allow molecular diagnostics to be developed to test for DMI resistance in *L. maculans* in other genomic regions, based on the modifications discovered in the *ERG11* promoter region.

A resistant isolate 18BL154 without any modification in *ERG11* coding and promoter region was identified, implying that there are other mechanisms to confer altered azole sensitivity (e.g., overexpression of efflux transporters), which will be addressed in future studies.

Isolates 18BL151 and 18BL152 were collected from stubble from locations separated by more than 2,000 km and yet contained the same insertion in the *ERG11* promoter. This suggests the independent emergence of resistance, and its frequency in field isolates of *L. maculans* should be monitored in Australia. A digital PCR assay has been developed for detecting and monitoring DMI fungicide resistance of *Blumeria graminis* f. sp. *hordei* in the barley industry in Australia (Zulak et al. 2018). Based on the data in the present study, a rapid molecular method could be developed for large-scale detection of DMI resistance frequency of *L. maculans* in fields, at least as based on the currently identified insertions in *ERG11*.

In summary, in the current study, fungicide-resistant isolates were obtained and the genetic mechanism accounting for their resistance was described as four insertion events upstream of the *ERG11* gene to result in DMI fungicide resistance.

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