Analysis of Repeat Induced Point (RIP) Mutations in *Leptosphaeria maculans* Indicates Variability in the RIP Process Between Fungal Species

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ABSTRACT Gene duplication contributes to evolutionary potential, yet many duplications in a genome arise from the activity of “selfish” genetic elements such as transposable elements. Fungi have a number of mechanisms by which they limit the expansion of transposons, including Repeat Induced Point mutation (RIP). RIP has been best characterized in the Sordariomycete *Neurospora crassa*, wherein duplicated DNA regions are recognized after cell fusion, but before nuclear fusion during the sexual cycle, and then mutated. While “signatures” of RIP appear in the genome sequences of many fungi, the species most distant from *N. crassa* in which the process has been experimentally demonstrated to occur is the Dothideomycete *Leptosphaeria maculans*. In the current study, we show that similar to *N. crassa*, nonlinked duplications can trigger RIP; however, the frequency of the generated RIP mutations is extremely low in *L. maculans* (< 0.1%) and requires a large duplication to initiate RIP, and that multiple premeiotic mitoses are involved in the RIP process. However, a single sexual cycle leads to the generation of progeny with unique haplotypes, despite progeny pairs being generated from mitosis. We hypothesize that these different haplotypes may be the result of the deamination process occurring post karyogamy, leading to unique mutations within each of the progeny pairs. These findings indicate that the RIP process, while common to many fungi, differs between fungi and that this impacts on the fate of duplicated DNA.

KEYWORDS ascomycete; genome defense; RIP timing; repetitive DNA

ONE of the major mechanisms for gene and genome evolution involves DNA duplication, which allows the divergence in sequences between a pair of duplicated genes, potentially giving rise to genes with new functions (Ohno 1970; Zhang 2003). However, duplications within the genome can arise from the activity of mobile elements, such as transposable elements, with deleterious effects. Some fungi have evolved a genome defense mechanism, Repeat Induced Point mutation (RIP), which is predicted to protect against multicopy DNA elements such as transposons through the generation of mutations leading to gene inactivation (Kinsey *et al.* 1994). This process was first identified and is best characterized in the model filamentous fungus,

Neurospora crassa (Selker *et al.* 1987; Selker and Garrett 1988; Selker 1990). RIP generates G to A and C to T transitions, which often lead to the introduction of stop codons within genes. It also leads to changes in methylation patterns of the DNA in *N. crassa*, effectively further inhibiting the expansion of multicopy genes by preventing the expression of any remnant coding regions (Singer *et al.* 1995b).

RIP occurs during crossing, and affects DNA that can include genes or noncoding regions that are duplicated in the genome. In *N. crassa*, the duplicated DNA is mutated only during the sexual cycle, and analysis of octads, *i.e.*, a set of eight ascospores derived from a meiosis and one round of mitosis enclosed in an ascus, indicates that progeny emerge carrying no more than two different haplotypes of mutations (Watters *et al.* 1999). The observation of two rather than four progeny types indicates that the mutation event in *N. crassa* occurs after cell fusion but before nuclear fusion (Selker *et al.* 1987; Watters *et al.* 1999), hence the original name of “rearrangement induced premeiotically” for RIP. In *N. crassa*, RIP acts on duplications that are > 400 bp in length when

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in tandem or > 1 kb when duplications are unlinked (Selker and Garrett 1988; Watters *et al.* 1999). When the repeats are unlinked, a proportion of the sexual spores still show alterations in the unlinked duplications, although the frequency of RIP in the unlinked duplications can vary considerably (Selker 2002). Since RIP inactivates both copies of the duplicated genes, although at varying frequencies, some researchers used RIP as a strategy for mutating genes prior to the development of highly efficient methods for gene disruption (Selker 2002; Ninomiya *et al.* 2004).

Although in *N. crassa* RIP acts against the deleterious effects of multicopy DNA such as transposable elements (Kinsey *et al.* 1994), it appears also to have minimized the evolution of gene families, as genes with > 80% identity undergo RIP (Galagan and Selker 2004). Therefore, RIP has been proposed to also impede genome evolution because it restricts the potential for new gene evolution from duplications (Galagan and Selker 2004).

The RIP process has been experimentally shown to occur in only a few fungal species. These include ascomycetes *Fusarium graminearum*, *Nectria hematococca*, *Podospora anserina*, *Magnaporthe oryzae*, and *Leptosphaeria maculans* (Graña *et al.* 2001; Ikeda *et al.* 2002; Idnurm and Howlett 2003; Cuomo *et al.* 2007; Coleman *et al.* 2009; Pomraning *et al.* 2013). Although not shown experimentally, the hallmarks of RIP detected as RIP signatures have been reported in numerous fungi based on analyses of transposable elements or whole genomes (Clutterbuck 2011). Fungi with these signatures include other ascomycetes such as *Aspergillus* species, *F. oxysporum*, and *Cochliobolus heterostrophus*, and basidiomycete species like *Rhizoctonia solani* and members of the Pucciniomycotina subphylum (Clutterbuck 2011; Horns *et al.* 2012; Hane *et al.* 2014; Santana *et al.* 2014). However, although RIP signatures have been detected in fungi this does not automatically imply that it occurs, *e.g.*, in *Fusarium* species, RIP signatures have been detected although these species are generally considered to be predominantly asexual in reproduction (Waalwijk *et al.* 2017).

As previously mentioned, RIP has been experimentally demonstrated to occur in *L. maculans*, where tandem insertions of plasmid DNA were mutated during crossing (Idnurm and Howlett 2003). *L. maculans* is a plant pathogen that infects oilseed Brassicas worldwide (Fitt *et al.* 2006). In some countries, such as Australia, the life cycle includes a saprophytic stage of growth on the residual stubble from *Brassica napus* crops, during which the two mating types of this heterothallic fungus initiate the sexual cycle (Van De Wouw *et al.* 2016). In Australia, the start of the autumn rain triggers the release of ascospores that infect the newly planted crops. The sexual cycle produces ascospores within asci, that in laboratory crosses can be analyzed as octads; a set of eight progeny arising from meiosis and one round of mitosis, resulting in four sets of identical pairs (Gall *et al.* 1994). *L. maculans*, although less experimentally tractable than *N. crassa*, is a useful fungus in which to investigate RIP as it can be crossed *in vitro*, can be transformed, and has

genome-sequencing resources available. Furthermore, RIP is highly relevant to the evolution of pathogenicity of isolates within field populations of *L. maculans*. Analysis of the genome of this fungus revealed that many genes encoding known or putative avirulence and effector proteins, involved in disease, are embedded within or near highly repetitive DNA elements that appear to have been subject to RIP (Rouxel *et al.* 2011). In addition to RIP acting on multicopy regions in *L. maculans*, RIP can “leak” into nearby single-copy sequences, thereby mutating these avirulence genes (Fudal *et al.* 2009; Van de Wouw *et al.* 2010) and subsequently conferring on these isolates the ability to cause disease on formerly resistant cultivars of canola.

In this paper, we report that the RIP process in *L. maculans* has a number of differences to those characterized in *N. crassa* and other Sordariomycete species. This provides new insights into how RIP may not be as restrictive a force in fungal evolution as previously thought.

Materials and Methods

Isolates

L. maculans isolates are listed in Table 1. Wild-type isolates IBCN18, Lm691, D3, and D9 were transformed with constructs generated to analyze RIP patterns in progeny of crosses. Details of the constructs and fungal transformations are described in the following sections. The resulting transformants were used as parents in crosses (Table 2). All isolates were cultured on 10% Campbell's V8 juice agar.

In vitro crossing of *L. maculans*

The mating-type genotype of each isolate was determined by PCR as described in Cozijnsen and Howlett (2003) (primers listed in Supplemental Material, Table S1). Isolates of opposite mating type were set up for crossing as previously described (Cozijnsen *et al.* 2000). Briefly, agar plugs of each parent were placed 5-mm apart on mating media (20% V8 juice, 2% agar, and 0.2% CaCO₃) and allowed to grow under 12-hr dark/light cycles for 7 days. After 7 days, plates were overlaid with 1% Difco (Detroit, MI) water agar. Plates were then placed at 14°, with 12-hr dark/blue-black UV light cycles for 4–6 weeks. After this time, plates were examined under a dissecting microscope for pseudothecia (sexual fruiting bodies). Using a scalpel blade, pseudothecia were removed from the plate and placed in a drop of sterile water to allow asci to be ejected. Octads were then dissected by placing an individual ascus on 2% water agar in a droplet of sterile water. A glass coverslip was placed over the ascus and then, while visualizing under the dissecting microscope, pressure was gently applied to the coverslip to release the eight individual ascospores from the ascus. Each individual ascospore was then placed on a separate agar plate and allowed to germinate.

The procedure used for isolating each individual prevents any order of the ascospores within the ascus from being

Table 1 L. *maculans* isolates used in this study

Isolate	Mating type	Purpose	Relevant genotype information	Reference
IBCN18	<i>MAT1-2</i>	Transformation	Contains <i>AvrLm1</i> and <i>AvrLm4</i>	Marcroft <i>et al.</i> (2012)
Lm691	<i>MAT1-1</i>	Transformation and crosses	Contains <i>AvrLm1</i> and <i>AvrLm4</i>	Hayden <i>et al.</i> (2007)
D9	<i>MAT1-1</i>	Transformation and crosses		Marcroft <i>et al.</i> (2012)
D3	<i>MAT1-1</i>	Transformation and crosses		Marcroft <i>et al.</i> (2012)
D13	<i>MAT1-2</i>	Crosses	Contains <i>AvrLm4</i>	Marcroft <i>et al.</i> (2012)
IBCN18+ <i>AvrLm1</i>	<i>MAT1-2</i>	Crosses	Isolate IBCN18 transformed with <i>AvrLm1</i> resulting in nontandem duplication of <i>AvrLm1</i>	This study
IBCN18+ <i>AvrLm4</i> #8	<i>MAT1-2</i>	Crosses	Isolate IBCN18 transformed with <i>AvrLm4</i> resulting in tandem duplication of <i>AvrLm4</i>	This study
IBCN18+ <i>AvrLm4</i> #9	<i>MAT1-1</i>	Crosses	Isolate Lm691 transformed with <i>AvrLm4</i> resulting in nontandem duplication of <i>AvrLm4</i>	This study
IBCN18+ <i>Lema006030</i>	<i>MAT1-2</i>	Crosses	Isolate IBCN18 with an additional copy of <i>Lema006030</i> fused to a marker conferring nourseothricin resistance, resulting in a nontandem duplication of the <i>Lema006030</i> gene.	This study
D3- <i>Ip</i> ^R + <i>hos1</i>	<i>MAT1-1</i>	Crosses	Isolate was isolated as resistant to iprodione after <i>in vitro</i> selection and then transformed with wild-type <i>hos1</i> and a G418 selectable marker	Idnurm <i>et al.</i> (2017)
D9+double- <i>hph</i> #4	<i>MAT1-1</i>	Crosses	Isolate D9 transformed with a double- <i>hph</i> construct resulting in a nontandem insertion.	This study
D9+double- <i>hph</i> #7	<i>MAT1-1</i>	Crosses	Isolate D9 transformed with a double- <i>hph</i> construct resulting in a nontandem insertion.	This study
IBCN18+double- <i>hph</i> #8	<i>MAT1-2</i>	Crosses	Isolate IBCN18 transformed with a double- <i>hph</i> construct resulting in a nontandem insertion.	This study
IBCN18+double- <i>hph</i> #9	<i>MAT1-2</i>	Crosses	Isolate IBCN18 transformed with a double- <i>hph</i> construct resulting in a nontandem insertion.	This study
D3+double- <i>hph</i> #2	<i>MAT1-1</i>	Crosses	Isolate D3 transformed with a double- <i>hph</i> construct resulting in a nontandem insertion.	This study
D3+double- <i>hph</i> #5	<i>MAT1-1</i>	Crosses	Isolate D3 transformed with a double- <i>hph</i> construct resulting in a nontandem insertion.	This study
691+NPS10	<i>MAT1-1</i>	Crosses	Isolate 691 transformed with NPS10 hairpin construct.	This study
<i>sirP</i>	<i>MAT1-2</i>	Crosses	<i>sirP</i> gene-disruption strain	Gardiner <i>et al.</i> (2004)
LopP	<i>MAT1-2</i>	Crosses	Loss-of-pathogenicity mutant with tandem insertion of plasmid pUCATPH (<i>hph</i> gene for hygromycin resistance)	Idnurm and Howlett (2003)
LopC	<i>MAT1-2</i>	<i>in vitro</i> passaging	Loss-of-pathogenicity mutant with tandem insertion of plasmid pUCATPH (<i>hph</i> gene for hygromycin resistance)	Idnurm and Howlett (2003)

maintained. Hence, each of the resulting progeny from the octad was analyzed with molecular markers or by Southern blot analysis (see details below) to identify the progeny pairs from within a single octad. Specifically, primers were used to amplify between four and six independently segregating genes in the progeny whereby the parental isolates have different alleles. Pairs of progeny with the same genotype across each of the genes were determined as progeny pairs. The genes amplified were; the *MAT* locus, which has two

alternative forms, *MAT1-1* or *MAT1-2*, which are detected by a size polymorphism (Cozijnsen and Howlett 2003); *AvrLm1*, which is a presence/absence polymorphism detected by PCR (Gout *et al.* 2006); *AvrLm4*, which is a single-base pair change detected by PCR and then digest with restriction enzyme *HaeIII* (Van de Wouw and Howlett 2012); *AvrLm6*, which is a presence/absence polymorphism detected by PCR (Fudal *et al.* 2007); *AvrLm5*, which is a single-base pair change detectable by PCR and then digest with

Table 2 Crosses carried out to analyze RIP mutations in *L. maculans*

Cross number	Parent isolate 1	Parent isolate 2	Number of octads analyzed	Number of progeny collected	Number of progeny sequenced ^a	RIP detected in progeny
28	IBCN18+ <i>AvrLm1</i>	Lm691	5	21	14	No
27	IBCN18+ <i>AvrLm4</i> #8	Lm691	2	13	13	Yes
67	IBCN18+ <i>AvrLm4</i> #9	D9	2	71	7	No
66	IBCN18+ <i>Lema006030</i>	D9	1 ^b	71	7	No
63	D3-Ip ^R + <i>hos1</i>	D13	2 ^b	26	1	Yes
57	D9+double- <i>hph</i> #4	IBCN18+double- <i>hph</i> #8	6	35	20	Yes
58	D3+double- <i>hph</i> #2	D13	5	18	9	Yes
64	IBCN18+double- <i>hph</i> #9	D9+double- <i>hph</i> #7	5	33	22	Yes
65	IBCN18+double- <i>hph</i> #9	D3+double- <i>hph</i> #5	1	15	4	Yes
22	sirP	691+NPS10	7	35	26	Yes
38	LopP	D9	2	16	8	Yes

RIP, Repeat Induced Point.

^a All progeny were genotyped for the presence of the relevant construct (as indicated by the amplification of the *hph* gene). Only progeny harboring the relevant constructs were sequenced and analyzed for the presence of RIP mutations.

^b For crosses 66 and 63, in addition to progeny being collected from an octad, random progeny were also collected and analyzed.

restriction enzyme *AvaII* (Van de Wouw *et al.* 2014b, 2018); and primers specific for the selectable marker of the construct, *i.e.*, the hygromycin B phosphotransferase (*hph*) gene (selectable marker). All primers and markers were previously published, and are listed in Table S1.

Details of all crosses are in Table 2. The nomenclature used for the progeny is a cross number, followed by a letter to indicate an octad, followed by a number 1–8 for each of the members of the octad. For example, 22A1 refers to progeny one from octad A of cross number 22, while 22B1 refers to progeny one from a different octad, but still from cross number 22.

Construct development and fungal transformations

To test whether RIP could occur in nontandem duplicated sequences, two constructs that had previously been developed were used to transform wild-type isolates (Van de Wouw *et al.* 2014a,c). These constructs, pPZPHyg_*AvrLm1* containing *AvrLm1* and pPZPHyg_*AvrLm4* containing *AvrLm4*, were transformed into isolate IBCN18 or Lm691, which already contains wild-type copies of these genes. *Agrobacterium*-mediated transformation and selection with hygromycin was carried out as previously described (Gardiner and Howlett 2004). The resulting transformants, IBCN18+*AvrLm1*, IBCN18+*AvrLm4*, and 691+*AvrLm4* isolates, contained duplicate copies of the *AvrLm1* or *AvrLm4* genes (Figure S1A). The IBCN18+*AvrLm1* and IBCN18+*AvrLm4* isolates were crossed to Lm691, while 691+*AvrLm4* was crossed to isolate D13. Octad progeny, defined as the individual progeny collected from a single ascus, were collected and screened for the presence of RIP (further details below).

To trigger RIP by using a large fragment of DNA, two different constructs were generated, and used for transformation and crossing. For the first, a plasmid containing a 4596-bp fragment of the *Lema006030* gene encoding a putative transcription factor was generated. The gene was amplified with primers KCP014 and KCP015, and cloned

downstream of the *L. maculans* actin promoter in the pPZPNat vector. The resultant plasmid was transformed into isolate IBCN18 to create transformant IBCN18+*Lema006030* (Table 2). This transformant was then crossed with isolate D9, and progeny were collected and analyzed.

A second construct was generated previously, and harbors the wild-type copy of the *hos1* gene (encodes a histidine kinase) and the selectable marker for G418 resistance (Idnurm *et al.* 2017). This construct was transformed into isolate D3-Ip^R, which contains a spontaneous mutation in the *hos1* gene that confers resistance to the fungicide iprodione (Idnurm *et al.* 2017). As a consequence of this transformation, the resulting D3-Ip^R + *hos1* isolate contains a repeat region of 5736 bp (two copies of the *hos1* gene and surrounds). The construct was sequenced within isolate D3-Ip^R + *hos1* and shown to contain no truncations of the *hos1* gene from the transfer DNA (T-DNA) transformation (data not shown). This transformant was crossed to D13, and the resulting 19 progeny were screened for both G418 resistance and resistance to iprodione. For a progeny to be resistant to both these chemicals, the progeny must harbor the complementation construct but have undergone RIP to inactivate the construct copy of the *hos1* gene. A single progeny, 63R15, was detected with this phenotype, and the *hos1* gene was amplified and sequenced to determine whether mutations were present. Although the insertion site was not specifically determined for this construct, because all phenotypic categories were detected in the progeny of the cross, it shows that the construct was independently segregating and cannot be genetically linked, and therefore not in tandem, with the endogenous copy of the *hos1* gene.

A construct harboring two copies of the hygromycin (*hph*) resistance gene in tandem was generated and used in crosses as a means of reliably triggering RIP. The *hph* coding sequence was amplified from the *AvrLm1* construct mentioned above using the *hph*-CloningF and *hph*-CloningR primers,

which contained restriction sites on the ends. The 1026-bp fragment was cloned into plasmid pPZP-Hyg (Elliott and Howlett 2006), which contained restriction sites for *Acc65I* and *EcoRV* at the ends. The resultant plasmid, pPZPHygx2 (3816 bp, Figure S2A), was transformed into isolates D9, IBCN18, and D3 (Table 1). The resulting transformed isolates (Table 1) were crossed with isolates of opposite mating type, and progeny collected and analyzed (Table 2). To sequence across the entire construct and guarantee that the correct copy of the repeat was being amplified and sequenced, overlapping PCR products were amplified and sequenced. Primer pairs for each PCR were selected so that at least one primer bound to single-copy regions within the construct, for example the right border sequence, the *trpC* promoter, or the *trpC* terminator (Table S1). Alternatively, primer pairs with specific orientations were selected so that only the correct regions could be amplified, e.g., the 5' *hph* primer (CE245) used as a reverse primer with the 3' *trpCT* primer used as a forward primer. The resulting PCR products were then subjected to sequencing using multiple primers (not necessarily from the single-copy regions) so that the full length of the construct could be determined, while resolving each repeated DNA region.

A hairpin construct had previously been designed to silence a nonribosomal peptide synthetase (*NPS10*) gene of *L. maculans* using the pHYGGS vector, and was used to investigate the impact of this gene on virulence (Fox *et al.* 2008). A 620-bp region of the *NPS10* gene (GenBank accession CCT61194.1) was amplified from genomic DNA of *L. maculans* isolate IBCN18, using attB1- and attB2- tailed primers NPS10RNAiF and NPS10RNAiR, and cloned using Gateway recombination into pDONR207. The fragment was then moved from pDONRnps10 into the gene-silencing vector pHYGGS in two opposing orientations using LR Clonase (Invitrogen, Carlsbad, CA) to create the final *NPS10* gene silencing vector, pNPS10RNAi (Figure S1A). The vector was transformed into *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen). *L. maculans* isolate 691 was transformed using the *Agrobacterium* strain containing pNPS10RNAi as described in Gardiner and Howlett (2004). Isolates were assessed for silencing of the *NPS10* gene using quantitative reverse transcriptase PCR to compare the expression of *NPS10* relative to actin in wild-type and silenced isolates. The transformant with the lowest expression of *NPS10*, named 691+NPS10, was selected and crossed to a sirodesmin biosynthesis gene-disruption mutant, referred to as sirP since the *sirP* gene had been disrupted (Gardiner and Howlett 2004). This experiment was initially designed for the laboratory direction of creating double mutants affected in the synthesis of secondary metabolites, but both the construct used and parents of the cross served as a useful combination to detect RIP. The progeny of this cross were sequenced to detect RIP. As mentioned above, to sequence across the entire construct, a series of overlapping PCR fragments were generated using primer combinations designed to amplify specific fragments of the construct. The resulting PCR products

were then sequenced using primers with unique binding sites so that the entire length of the construct could be sequenced without confusing the identities of the different repeat regions.

Determining the copy number of T-DNA insertions

Southern blot analysis was carried out on the transgenic parents of crosses 27, 28, 57, 58, 64, 65, and 67 to determine the copy number of the plasmid within each parent isolate (Figures S1 and S2). Genomic DNA was digested with restriction enzymes as indicated in the figures and DNA fragments resolved on a 0.7% agarose Tris-acetate-EDTA gel. The gels were stained with ethidium bromide to visualize digested DNA before transfer. DNA was transferred to a nylon membrane using downward alkaline capillary transfer, as described in Sambrook and Russell (2001). Blots were hybridized with a digoxigenin-11-dUTP-labeled PCR fragment corresponding to 579 bp of the *hph* gene amplified with primers CE249 and CE250 (Table S1). The probe was generated using a PCR DIG Probe synthesis kit from Roche; blots were hybridized using conventional buffer [6× SSC, 0.1% SDS, and 1× blocking buffer (Roche kit)], 5× Denhardt's solution, and 40 µg salmon sperm DNA, and processed using DIG wash and a blocking buffer set followed by DIG luminescence detection. Signal detection was carried out using a Bio-Rad (Hercules, CA) Chemidoc imaging system equipped with Image Lab software using high-sensitivity detection with signal accumulation mode.

A single hybridizing band in lanes where *HindIII* was used to digest the DNA, and two hybridizing bands in lanes where *PstI* was used, indicate that the T-DNA had inserted in single copy (Figure S1, IBCN18+AvrLm1 and IBCN18+AvrLm4#9). Two or more bands in the *HindIII* lanes, and three or more bands in the *PstI* lanes, indicated multiple or tandem insertion of the T-DNA (IBCN18+AvrLm4#8 and 691+NPS10). For parents containing pPZPHygx2, one copy of the T-DNA was confirmed by digestion with two independent enzymes that showed two hybridizing bands corresponding to each of the hygromycin resistance genes (Figure S2).

Identification of T-DNA or plasmid insertion sites in the *L. maculans* genome

Two methods were used to identify where T-DNA or plasmids had inserted in the genome. For the T-DNA insertions, inverse PCR was used. Genomic DNA (2 µg) was digested with *Taq*[®]I restriction enzyme, the fragments circularized with T4 DNA ligase, and the ligation mix used for PCR with primers M13F-ai076. Amplicons were purified from agarose gels and used as a template for a nested PCR with primers M13F-MAI0324. Amplicons were sequenced and the sequences compared to the *L. maculans* genome sequence. The other side of the insertion was identified by amplification with an isolate-specific primer.

Genomes of isolates LopC and LopP, which contain tandem insertions of plasmid pUCATPH and were previously shown to

undergo RIP during mating (Idnurm and Howlett 2003), were sequenced using next-generation sequencing (100-bp paired end reads on an Illumina HiSeq 2500 instrument) at the Australian Genome Research Facility (AGRF) in Melbourne. The sequences (3.65 GB for LopC and 3.58 GB for LopP) were mapped with reiterations to the pUCATPH plasmid sequence using Geneious version 8.1.7, to provide coverage of the plasmid insertion and flanking regions.

Detection and analysis of RIP signatures

Genomic DNA of parental isolates and progeny was prepared as described previously (Gardiner *et al.* 2004). In progeny of the crosses, the genes and regions of interest were amplified using primers in Table S1, and then sequenced at the AGRF. Sequence chromatograms were visualized using Geneious version 9.1 (Kearse *et al.* 2012) or Sequencher version 5.4.1 (Gene Codes Corporation, Ann Arbor, MI) and G→A and C→T transitions identified. RIPCAL was used to determine CpA↔TpA dominance scores (Hane and Oliver 2008). For all analyses, the DNA sequence obtained from the parental isolate was used as the reference (non-RIP) sequence.

Culturing in the absence of hygromycin selection to trigger RIP

Two isolates, LopP and LopC, were subcultured into 10 starting cultures without hygromycin selection. Each week for 10 weeks, mycelial fragments were passaged on media without hygromycin. After the 10 weeks, spores from each culture were plated at low density to yield colonies. Of these, 20 were selected and grown on V8 media with or without hygromycin to detect whether RIP could be triggered during mitotic replication to inactivate the *hph* gene.

All strains and plasmids are available upon request. All primer information can be found in Table S1.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the manuscript are represented fully within the manuscript. All strains and plasmids are available on request. Supplemental material available at Figshare: <https://doi.org/10.25386/genetics.7221974>.

Results

The frequency of RIP in *L. maculans* when duplications are unlinked is extremely low and requires large repeated regions

In *N. crassa*, duplication of DNA can result in RIP mutations being generated in both copies of the DNA repeat, even if in unlinked parts of the genome. This has been used in *N. crassa* as a tool for mutating genes (Selker and Garrett 1988), as well as in other species such as *Trichoderma reesei*, where copies of genes in two different parts of the genome are both triggered for mutation during mating (Li *et al.* 2017). To test whether RIP mutations can be triggered and used as a way

of mutating genes in *L. maculans*, two constructs were designed, one harboring the *AvrLm1* gene (1805 bp) and the other the *AvrLm4* gene (1645 bp). An isolate of *L. maculans* that already harbored the *AvrLm1* and *AvrLm4* genes (IBCN18) was individually transformed with these constructs to create isolates, with either two copies of *AvrLm1* or two copies of *AvrLm4*. Transformants with a random insertion of the construct resulted in unlinked duplications of the avirulence genes (IBCN18+*AvrLm1* or IBCN18+*AvrLm4*) and were crossed to an isolate of opposite mating type, Lm691, also harboring these avirulence genes. Octad progeny were collected from the resulting crosses. The endogenous copy of the avirulence genes was sequenced from all progeny, while the copy of the avirulence gene within the construct and the *hph* selectable marker were sequenced in the progeny of the crosses that harbored the construct.

For the cross with isolate IBCN18+*AvrLm1* (cross 28), no mutations were detected in any progeny in the endogenous or construct copy of the *AvrLm1* gene, nor the *hph* gene of the construct (Table S2). Southern analysis and inverse PCR showed that there was a single insertion of the construct within the parental isolate (IBCN18+*AvrLm1*) and that this insertion site, located on supercontig 8, was unlinked to the endogenous *AvrLm1* gene located on supercontig 6 (Figures S1 and S3).

Testing the potential of RIP using a second gene, duplicated in isolate IBCN18+*AvrLm4*#8 (cross 27), RIP mutations were detected throughout the *hph* gene and a few RIP mutations within the construct copy of the *AvrLm4* gene, but not within the endogenous copy in any of the progeny (Table S2). Southern blot and PCR analysis showed a tandem insertion of the *AvrLm4* construct, which was probably triggering RIP in this situation (Figure S1). Therefore, a second isolate, IBCN18+*AvrLm4*#9, was generated, confirmed to have a single insertion using Southern analysis and inverse PCR, and crossed to isolate D9, also harboring *AvrLm4* (cross 67) (Figure S1). No mutations were detected in any progeny collected from this cross in either the endogenous or construct copy of the *AvrLm4* gene, nor the *hph* gene (Table S2).

Transforming a second copy of either *AvrLm4* or *AvrLm1* into *L. maculans* and crossing those isolates did not trigger RIP in endogenous genes. These genes and the amount of homologous DNA that is duplicated are small (repeat region of < 2 kb), which may not be big enough to trigger RIP in *L. maculans*. Therefore, additional crosses were assessed whereby larger constructs [transcription factor *Lema006030* = 4569 bp (cross 66) and fungicide resistance gene *hos1* (5736 bp)] had been introduced into one of the parents. First, for cross 66, the gene encoding the transcription factor was fused next to a selection marker conferring nourseothricin resistance. Of the 71 progeny, 53% were nourseothricin-resistant. A fragment of the gene from the endogenous and construct copy was amplified simultaneously from nourseothricin-resistant isolates and sequenced. No mutations were

Figure 1 Analysis of frequency of Repeat Induced Point (RIP) mutations in 55 progeny of *L. maculans* collected from crosses between isolates harboring a construct with two copies of the hygromycin (*hph*) resistance gene. (A) The frequency of RIP mutations increases markedly within the hygromycin repeat regions (*hph*) of the construct but decreases in the single-copy regions such as the *trpC* promoter, terminator, and spacer regions. (B) The pattern of RIP mutations in a subset of the tetrad progeny within a specific region of one of the *hph* regions. C → T transitions are represented in red while G → A transitions are represented in blue. The patterns of RIP mutations differ for each of the tetrad pairs. Repeat regions were amplified via PCR using primers binding in single-copy regions and the resulting products were sequenced with internal primers for the regions of interest.

observed in this region of the gene, suggesting that RIP had not occurred.

The second cross involved one parent harboring a spontaneous mutation within the *hos1* gene, which confers resistance to the fungicide iprodione when mutated, plus a complementation construct containing the wild-type *hos1* gene and the G418 antibiotic resistance marker. This isolate was crossed with a wild-type; 19 progeny were isolated, and screened for resistance to both G418 and iprodione, which is impossible unless the *hos1* complementation construct was mutated. A single progeny, 63R15, was identified with this phenotype, and the construct copy of the *hos1* gene was amplified and sequenced. The construct contained five RIP mutations across the entire 5736 bp region, suggesting that RIP can be triggered using nontandem repeats but at a frequency of < 0.1% (Figure S4). Furthermore, only one of the five substitutions is likely responsible for the iprodione resistance phenotype (of a conserved methionine to isoleucine at amino acid position 538). Taken together, these data suggest that targeted mutations of an endogenous gene using RIP may be difficult since the frequency of RIP is extremely low in nontandem repeats, which contrasts to other fungal species. Therefore, we were interested in determining what similarities and differences exist for RIP mechanisms in *L. maculans* compared to other species.

RIP mutations leak from repeat regions into single-copy sequences

To investigate the mechanisms leading to RIP, a construct harboring two copies of the *hph* gene (referred to as double-*hph*)

was generated, transformed into isolates, and then the resulting transgenic isolates used for crossing. Southern analysis showed that single copies of the construct had inserted into each isolate (Figure S2). Four different crosses (crosses 57, 58, 64, and 65; Table 2), using a combination of seven different parental isolates, were established. A total of 55 progeny, representing 17 different octads, were analyzed for the presence of RIP mutations. A 3342-bp region, spanning almost the entire construct, was sequenced from all progeny harboring the double-*hph* construct (Figure 1). The frequency of nucleotides that had undergone RIP mutations for the individual progeny ranged from 0.1–6.0%, with the GC content decreasing by up to 4.8% (Table S3). All sequences were also subjected to RIPCAL analysis, a software tool used to compare alignments and calculate RIP indexes, to determine which dinucleotide transitions were dominant. The CpA → TpA transitions were the most dominant RIP mutations, with an average RIP dominance score of 1.28, while CpG → TpG were the second most dominant with an average score of 0.81 (Figure S5 and Table S4).

The frequency of RIP mutations at each nucleotide position across the construct was determined in the 55 progeny (Figure 1A). The frequency of RIP was highest within the *hph* repeats; however, RIP was also detected within the single-copy regions of the *trpC* promoter, spacer regions, and *trpC* terminator (Table 3). Although RIP mutations were detected within these single-copy regions, the average number of RIP mutations for these regions was much lower (between 0 and 0.36%) than the repeated *hph* regions (4.45 and 4.88%) (Table 3).

Table 3 Total number and frequency of RIP mutations across specific regions of the double-*hph* construct when sequenced from 55 *L. maculans* progeny

Region ^a	Length of region (bp)	Total number of RIP mutations	Average number of RIP mutations per region
Left border	42	0	0
Spacer	58	11	0.36
<i>hph</i> (copy 1)	1026	2611	4.88
Spacer	49	3	0.14
TrpC promoter	362	350	1.89
Spacer	1	0	0
<i>hph</i> (copy 2)	1026	2388	4.45
TrpC terminator	724	105	0.31
Spacer	58	0	0

RIP, Repeat Induced Point.

^a A diagram of the double-*hph* construct is shown in Figure 1A.

RIP mutations differ in octad pairs in *L. maculans*

Analysis of the patterns of RIP in the octad progeny pairs from the multiple double-*hph* crosses showed that the RIP signatures differed for each progeny from a pair (Figure 1B), and furthermore, for many of the progeny, both G→A and C→T transitions were present (Table S3). To confirm that this was not an artifact of the double-*hph* construct, the repetitive regions of octad pairs derived from a variety of different crosses were also amplified and sequenced.

Crosses were set up between 691+NPS10 (containing a hairpin construct for silencing the *LmNPS10* gene) and the sirP isolate (containing a gene disruption of the *sirP* gene), originally to generate progeny that contained both the *sirP* gene-disruption mutation as well as the silencing of *NPS10* (cross 22), yet being informative for understanding the RIP mechanism in *L. maculans*. Southern analysis and targeted sequencing of the *NPS10* silencing construct in the 691+NPS10 parent isolate was used to determine the exact nature of the hairpin insertion (Figure S1). A partial duplication of the silencing construct was identified, whereby two copies of the *hph* gene, terminator sequence, and promoter sequence were integrated into the isolate (Figure 2A). Inverse PCR was used to identify the insertion site of the T-DNA into supercontig 2: it is unlinked to the endogenous copy of *NPS10*, which is located on supercontig 11 (Figure S3). Octad progeny were collected from seven individual octads and the pairs determined using PCR-based markers that differ between the two parents. Initially, three octads were analyzed (22A, 22B, and 22D). For each of these three octads, four progeny (two pairs) lacked the construct. The endogenous copy of the *NPS10* gene was sequenced from these progeny and no RIP was detected (data not shown). Since these four progeny did not harbor the construct, they were not analyzed further. For the remaining four progeny (two pairs) that did harbor the construct, a 2894-bp region of the construct encompassing the hairpin repeats and a 1474-bp region encompassing one of the *hph* repeats (Figure 2B) were sequenced. The number and position of

RIP mutations differed for each progeny, including between progeny pairs from the same octad (Figure 2A and Table 4). In all 12 progeny (representing six pairs from the three octads), there were both G→A and C→T transitions present across the two regions sequenced (Table 4). For some progeny, such as 22D3, the ratio of G→A to C→T transitions was 4:96, while other progeny had almost a 50:50 ratio (progeny 22A8 with 56.9% G→A and 43.1% C→T) (Table 4).

The RIP signatures for each pair within an octad, and all four progeny with duplicated genes from an octad, were analyzed to determine the number of mutations that are common between pairs, unique within pairs, the same within all four progeny from a single octad, or unique across all four progeny from a single octad (Table 5). For all three octads, there were more unique RIP mutations between pairs (16.5–60%) compared to common RIP mutations (1.7–29.4%). Furthermore, when all four progeny from a single octad were analyzed, the frequency of mutations unique across all four progeny was much significantly higher (3.1–25%) than the frequency of mutations common across all four progeny from an octad (0–5.3%) (Student's *t*-test $P < 0.001$). For 4 of the 12 progeny, both G→A and C→T unique transitions were observed across the sequenced regions.

The frequency of RIP occurring at each nucleotide position across the 2894-bp and 1474-bp regions sequenced from the cross 22A, 22B, and 22D tetrad progeny was determined (Figure S5). Similar to what was seen for the double-*hph* construct, the frequency of RIP increased within the hairpin repeat regions and was still present, although at a lower frequency, in the single-copy spacer region between the two hairpin repeats. The frequency of RIP remained constant across all other regions as these were in multiple copies due to the partial tandem insertion of the construct (Figure 2A). As seen for the double-*hph* progeny, the dominant RIP mutations were CpA→TpA (average RIP dominance score of 2.06) and CpG→TpG (average RIP dominance score of 0.5) (Figure S6 and Table S4).

A 347-bp region of the hairpin construct was also sequenced from the progeny from an additional five octads (Figure S7 and Table S3). As seen with the initial three octads of cross 22 (Figure 2B), the pattern and number of RIP mutations differed within all progeny pairs. The frequency of RIP mutations ranged from 1.15–4.02% across the 347-bp region and resulted in a decrease in overall GC content of up to 4.2% (Table S3).

For a subset of the progeny, the two repeat regions within a construct (*i.e.*, *hph* for the double-*hph* construct and the hairpin region of the *NPS10* silencing construct) were aligned to determine whether the repeats were being targeted by RIP in a similar manner. For cross 57, the *hph* repeats were aligned for each of the six progeny of the B octad, and mutations common and unique between each copy of the repeat were determined. The majority of RIP mutations (between 64 and 77%) differed between the two repeats for each of the six progeny, suggesting that the repeats are not subjected to the same RIP mutations (Table S4). Similarly, the hairpin repeat

Figure 2 Repeat Induced Point (RIP) mutations generated within a hairpin construct following crossing in *L. maculans*. (A) A partial tandem insertion of the hairpin construct integrated into *L. maculans* isolate 691+NPS10. This isolate was then crossed to a second isolate, sirP, to generate progeny for analysis of RIP. (B and C) Sequencing of a (B) 2894-bp region and (C) 1474-bp region in the subsequent progeny shows that RIP mutations (G→A shown in blue and C→T shown in red) differ within and between octad pairs. Repeat regions were amplified via PCR using primers binding in single-copy regions and the resulting products were sequenced with internal primers for the regions of interest.

regions were aligned for 12 of the cross 22 progeny, and between 74 and 100% of the mutations differed between the repeat regions (Table S4).

The RIP process was first identified in *L. maculans* when insertional mutants, such as LopC and LopP, which had been generated by restriction enzyme-mediated integration of plasmid DNA, were crossed to wild-type isolates and the hygromycin-resistant phenotype in these two isolates, brought about by the introduction of linearized plasmid pUCATPH, was lost. The plasmid insertions were in tandem, based on Southern blot analysis (Idnurm and Howlett 2003), and confirmed here when the integration sites of the plasmids into the genome were identified by sequencing genomes of the two isolates (GenBank accession SRP149958; Figure S3). We crossed the original LopC and LopP mutants to wild-type isolate D9, and collected octad progeny. As reported previously, all progeny were sensitive to hygromycin, rather than the expected 4:4 ratio of resistant:susceptible. The progeny from the LopP × D9 cross (cross 38) were examined in more detail. Regions of the pUCATPH plasmid

were amplified and sequenced to analyze the RIP signatures. For tetrad 38D, a 3977-bp region was sequenced, while for tetrad 38G, a 1024-bp region was sequenced. As seen for the double-*hph* and hairpin crosses described above, the RIP signatures differed both within and between all the octad pairs (Figure 3, Table 4, and Table 5). Also, as observed in the other crosses, both G→A and C→T transitions were seen in each progeny, and the frequency of unique mutations was much higher than the frequency of mutations that were common across all four progeny from a single octad (Table 5). The frequency of RIP within these progeny was higher than that seen in the other crosses, ranging from 3.87–8.11%, and as a consequence the GC content was also more dramatically decreased.

RIP mutations are linked to the sexual cycle

Since mutations differed within octad pairs, RIP may be occurring postmeiosis and could therefore be a mitotic process. To test if RIP occurs during vegetative growth, two isolates that undergo RIP during mating (LopP and LopC)

Table 4 Number and frequency of RIP mutations within progeny of crosses between *L. maculans* isolates harboring constructs designed to trigger RIP

Cross ^a	Octad	Pair	Progeny ^b	Size of region sequenced (bp)	RIP mutations (%)	G→A transitions (percentage of total RIP mutations)	C→T transitions (percentage of total RIP mutations)	Decrease in GC content (%)
22	1	1	22A3	2894	108 (3.7)	6 (5.6)	102 (94.4)	3.7
			22A8	2894	92 (3.2)	52 (56.5)	40 (43.5)	3.2
		2	22A5	2894	88 (3.0)	27 (30.7)	61 (69.3)	3.1
			22A6	2894	75 (2.6)	59 (78.7)	16 (21.3)	2.6
	2	1	22B2	2894	60 (2.1)	59 (98.3)	1 (1.7)	2.1
			22B4	2894	57 (2.0)	19 (33.3)	38 (66.7)	2.0
		2	22B3	2894	80 (2.7)	34 (42.5)	46 (57.5)	2.9
			22B5	2894	81 (2.8)	8 (9.9)	73 (90.1)	2.9
	3	1	22D1	2894	92 (3.1)	6 (6.5)	86 (93.5)	3.2
			22D6	2894	58 (2.0)	58 (100.0)	0 (0.0)	2.0
		2	22D3	2894	52 (1.8)	3 (5.8)	49 (94.2)	1.8
			22D7	2894	40 (1.4)	38 (95.0)	2 (5.0)	1.4
22	1	1	22A3	1474	39 (2.7)	2 (5.1)	37 (94.9)	2.7
			22A8	1474	52 (3.5)	30 (57.7)	22 (42.3)	3.5
		2	22A5	1474	51 (3.5)	17 (33.3)	34 (66.7)	3.5
			22A6	1474	54 (3.7)	46 (85.2)	8 (14.8)	3.7
	2	1	22B2	1474	40 (2.7)	37 (92.5)	3 (7.5)	2.7
			22B4	1474	38 (2.6)	20 (52.6)	18 (47.4)	2.6
		2	22B3	1474	36 (2.4)	19 (52.8)	17 (47.2)	4.2
			22B5	1474	33 (2.2)	0 (0.0)	33 (100.0)	1.8
	3	1	22D1	1474	66 (4.5)	8 (12.1)	58 (87.9)	4.5
			22D6	1474	38 (2.6)	35 (92.1)	3 (7.9)	2.6
		2	22D3	1474	29 (2.0)	0 (0.0)	29 (100.0)	2.0
			22D7	1474	31 (2.1)	30 (96.8)	1 (3.2)	2.1
38	1	1	38D2	3977	205 (5.2)	144 (70.2)	61 (29.8)	5.1
			38D5	3977	270 (6.8)	175 (64.8)	95 (35.2)	6.7
		2	38D3	3977	182 (4.6)	52 (28.6)	130 (71.4)	4.5
			38D7	3977	154 (3.9)	32 (20.8)	122 (79.2)	3.9
	2	1	38G1	1024	79 (7.7)	42 (53.2)	37 (46.8)	7.7
			38G4	1024	68 (6.6)	19 (27.9)	49 (72.1)	6.6
		2	38G3	1024	79 (7.7)	49 (62.0)	30 (38.0)	7.7
			38G5	1024	83 (8.1)	32 (38.6)	51 (61.4)	8.1

RIP, Repeat Induced Point.

^a For details of parental isolates used for crossing, see Table 2.

^b The remaining pairs of the octad, which are not provided in the table, do not harbor the construct and therefore cannot be analyzed for RIP signatures.

were passaged 10 times on media without hygromycin and then, after the 10th passage, spores were plated and colonies were cultured. All 400 remained hygromycin-resistant, suggesting that the induction of RIP is linked to the sexual cycle.

Discussion

The genome sequences of many fungal species carry signs of mutations that target repetitive DNA regions, especially transposable elements. One mechanism to create these mutations is RIP, with extensive evidence for this or a mechanism like RIP occurring in the fungi (Selker 1990; Clutterbuck 2011; Hane *et al.* 2015). However widespread these hallmarks are of a common mutation process, relatively little is known about the mechanisms behind RIP and how conserved they would be in the fungi. In the current study, analyses of a series of crosses and of DNA sequences in *L. maculans* that have or have not been affected by RIP indicate that there are both similarities and differences in how this process operates in

different fungi. This mutator phenomenon has consequences for the subsequent fate of DNA duplication in fungi.

In this study, we have shown that in *L. maculans* RIP occurs at CpA, CpG, and (to a lesser extent) CpT sites, with the CpA sites being the more predominant location. This is consistent with previous studies in *N. crassa* and *F. graminearum*, whereby CpA mutations are the predominant mutation, but CpG and CpT mutations are also targeted (Pomraning *et al.* 2013; Gladyshev 2017). Interestingly, a previous *in silico* study using bioinformatic approaches only to analyze repeat regions within the *L. maculans* genome sequence suggested that 90% of RIP mutations occur at CpA sites (Amselem *et al.* 2015). The differences between the two studies might reflect the fact that, in the latter study, transposable elements located within AT-rich regions of the genome were the focus of the study.

Two commonalities between *N. crassa* and *L. maculans* are that tandem repeats of only 1 kb are enough to trigger RIP, and that RIP leaks into single-copy regions (Selker and

Table 5 Analysis of common and unique RIP signatures within the octad progeny from crosses designed to trigger RIP

Cross	Octad	Progeny pairs	Progeny name	Length of region sequenced (bp)	Number of G→A		Number of C→T		Number of G→A		Number of C→T		Number of G→A		Number of C→T		Number of G→A		Number of C→T	
					unique transitions	within pairs (% of total mutations)	unique transitions	within pairs (% of total mutations)	unique transitions	within pairs (% of total mutations)	unique transitions	within pairs (% of total mutations)	unique transitions	within pairs (% of total mutations)	unique transitions	within pairs (% of total mutations)	unique transitions	within pairs (% of total mutations)	unique transitions	within pairs (% of total mutations)
22	1	1	22A3	2894	0 (0%)	66 (33.0%)	0 (0%)	27 (7.4%)	0 (0%)	41 (11.3%)	5 (2.5%)	39 (19.5%)	5 (2.5%)	39 (19.5%)	5 (2.5%)	14 (3.9%)	5 (2.5%)	39 (19.5%)	5 (2.5%)	14 (3.9%)
		2	22A8	2894	50 (25.0%)	0 (0%)	45 (27.6%)	0 (0%)	27 (7.4%)	0 (0%)	24 (14.7%)	17 (10.4%)	24 (14.7%)	17 (10.4%)	24 (14.7%)	17 (10.4%)	0 (0.0%)	17 (10.4%)	0 (0.0%)	17 (10.4%)
	2	1	22A5	2894	2 (1.2%)	0 (0%)	0 (0%)	24 (6.6%)	0 (0%)	0 (0%)	19 (5.2%)	0 (0.0%)	19 (5.2%)	0 (0.0%)	19 (5.2%)	0 (0.0%)	0 (0.0%)	19 (5.2%)	0 (0.0%)	19 (5.2%)
		2	22A6	2894	34 (20.8%)	0 (0%)	0 (0%)	30 (10.8%)	0 (0%)	0 (0%)	16 (5.8%)	3 (1.9%)	16 (5.8%)	3 (1.9%)	16 (5.8%)	3 (1.9%)	0 (0.0%)	16 (5.8%)	0 (0.0%)	16 (5.8%)
	3	1	22B2	2894	39 (33.3%)	0 (0%)	37 (31.6%)	0 (0%)	22 (7.9%)	0 (0%)	5 (1.8%)	2 (1.3%)	5 (1.8%)	2 (1.3%)	5 (1.8%)	2 (1.3%)	0 (0.0%)	5 (1.8%)	0 (0.0%)	5 (1.8%)
		2	22B5	2894	39 (24.1%)	0 (0%)	39 (24.1%)	0 (0%)	1 (0.4%)	0 (0%)	25 (9.0%)	3 (1.9%)	25 (9.0%)	3 (1.9%)	25 (9.0%)	3 (1.9%)	0 (0.0%)	25 (9.0%)	0 (0.0%)	25 (9.0%)
	2	1	22D1	2894	4 (2.7%)	86 (57.3%)	0 (0%)	4 (1.7%)	35 (14.5%)	0 (0%)	20 (8.3%)	2 (2.2%)	20 (8.3%)	2 (2.2%)	20 (8.3%)	2 (2.2%)	0 (0.0%)	20 (8.3%)	0 (0.0%)	20 (8.3%)
		2	22D6	2894	56 (37.3%)	0 (0%)	47 (51.1%)	0 (0%)	17 (7.0%)	0 (0%)	6 (3.1%)	2 (2.2%)	6 (3.1%)	2 (2.2%)	6 (3.1%)	2 (2.2%)	0 (0.0%)	6 (3.1%)	0 (0.0%)	6 (3.1%)
	2	1	22D3	2894	35 (23.3%)	0 (0%)	15 (16.5%)	0 (0%)	15 (7.7%)	0 (0%)	12 (6.1%)	17 (16.2%)	12 (6.1%)	17 (16.2%)	12 (6.1%)	17 (16.2%)	0 (0.0%)	12 (6.1%)	0 (0.0%)	12 (6.1%)
		2	22D7	2894	28 (30.8%)	0 (0%)	26 (24.8%)	0 (0%)	18 (9.2%)	0 (0%)	0 (0%)	20 (25.6%)	3 (3.8%)	20 (25.6%)	3 (3.8%)	20 (25.6%)	0 (0.0%)	3 (3.8%)	0 (0.0%)	3 (3.8%)
38	1	1	22A3	1474	0 (0%)	15 (19.2%)	0 (0%)	0 (0%)	0 (0%)	16 (10.9%)	0 (0%)	15 (21.7%)	0 (0%)	15 (21.7%)	0 (0%)	0 (0.6%)	15 (21.7%)	0 (0.6%)	15 (21.7%)	0 (0.6%)
		2	22A8	1474	0 (0%)	18 (26.0%)	0 (0%)	8 (5.4%)	0 (0%)	2 (1.4%)	4 (3.8%)	3 (2.9%)	4 (3.8%)	3 (2.9%)	4 (3.8%)	3 (2.9%)	0 (0.0%)	4 (3.8%)	0 (0.0%)	4 (3.8%)
	2	1	22B2	1474	19 (27.5%)	55 (52.9%)	0 (0%)	17 (10.4%)	0 (0%)	10 (6.1%)	0 (0.0%)	1 (1.7%)	0 (0.0%)	1 (1.7%)	0 (0.0%)	0 (0.6%)	1 (1.7%)	0 (0.6%)	1 (1.7%)	0 (0.6%)
		2	22B5	1474	31 (29.8%)	28 (40.0%)	0 (0%)	14 (8.5%)	0 (0%)	0 (0%)	101 (21.3%)	32 (6.7%)	101 (21.3%)	32 (6.7%)	101 (21.3%)	32 (6.7%)	25 (3.1%)	32 (6.7%)	25 (3.1%)	32 (6.7%)
	3	1	22D1	1474	42 (8.8%)	62 (6.3%)	0 (0%)	33 (4.1%)	0 (0%)	21 (2.6%)	32 (9.5%)	78 (23.2%)	32 (9.5%)	78 (23.2%)	32 (9.5%)	30 (9.7%)	32 (9.5%)	30 (9.7%)	32 (9.5%)	30 (9.7%)
		2	22D6	1474	75 (15.8%)	48 (14.3%)	0 (0%)	66 (8.1%)	0 (0%)	37 (4.6%)	21 (14.3%)	19 (6.1%)	21 (14.3%)	19 (6.1%)	21 (14.3%)	19 (6.1%)	0 (0.0%)	21 (14.3%)	0 (0.0%)	21 (14.3%)
	2	1	22D3	1474	22 (6.5%)	43 (12.8%)	0 (0%)	9 (2.9%)	0 (0%)	20 (2.5%)	34 (21.0%)	26 (16.1%)	34 (21.0%)	26 (16.1%)	34 (21.0%)	26 (16.1%)	0 (0.0%)	34 (21.0%)	0 (0.0%)	34 (21.0%)
		2	22D7	1474	1 (0.3%)	10 (6.8%)	0 (0%)	8 (2.6%)	0 (0%)	11 (3.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	2	1	22G1	1024	22 (14.9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
		2	22G4	1024	0 (0%)	17 (10.5%)	0 (0%)	8 (2.6%)	0 (0%)	0 (0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	2	1	22G3	1024	0 (0%)	21 (13.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
		2	22G5	1024	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

For details of parental isolates used for crossing, see Table 2.

The remaining pairs of the octad, which are not provided in the table, do not harbor the construct and therefore cannot be analyzed for RIP signatures.

Figure 3 Analysis of Repeat Induced Point (RIP) mutations in progeny of *L. maculans* isolates following a single sexual cycle. (A) Representation of the construct inserted into one of the parents, LopP, used to generate the tetrad progeny (38D and 38G). A 2953-bp region of the construct (underlined) was sequenced in the progeny. (B) The pattern of RIP mutations for the 38D tetrad progeny within the first 2953-bp region sequenced from a total of 3977 bp. (C) The pattern of RIP mutations within the remaining 1024-bp region sequenced in both the 38D and 38G tetrad progeny. C→T transitions are represented in red while G→A transitions are represented in blue. The patterns of RIP mutations differ for each of the tetrad pairs. Repeat regions were amplified via PCR using primers binding in single-copy regions and the resulting products were sequenced with internal primers for the regions of interest.

Garrett 1988; Fudal *et al.* 2009; Van de Wouw *et al.* 2010; Gladyshev and Kleckner 2014, 2017b). Furthermore, it appears that like *N. crassa*, both the RID-mediated and DIM-2-mediated RIP pathways are active in *L. maculans*. In *N. crassa*, it has been shown that the RID-mediated RIP pathway (involving RID) primarily acts on regions with shared homology, while the DIM-2-mediated RIP (involving DIM-5 and DIM-2) pathway leads to mutations that spread significantly into the linked, nonrepetitive regions (Gladyshev and Kleckner 2017a). In *N. crassa*, when key players in each of these pathways are mutated, the frequency of RIP-induced mutations changes in the homologous repeats or in the linked, nonrepeat regions. Although not experimentally defined to be active in *L. maculans*, all homologs of both RIP pathways have been identified in the genome (Rouxel *et al.* 2011), and the frequency of RIP-induced mutations reported in the current study are higher in the linked repeat regions compared to the single copy, nonrepeat regions, similar to that reported by Gladyshev and Kleckner (2017a) when both pathways are active. Further work involving mutations of some of the key players in these two RIP pathways would be needed to confirm their role in *L. maculans*. Unfortunately, the genera-

tion of knockout mutations in *L. maculans* is extremely difficult, with just 10 reported to date for the species. However, clustered regularly interspaced short palindromic repeat/Cas9 gene editing strategies are currently being developed and could potentially be used for such experiments in the future (Idnurm *et al.* 2017). Conversely, unlike in *N. crassa*, it appears that unlinked duplications in *L. maculans* only trigger RIP at a very low frequency. In a single cross whereby a ~5.7-kb repeat region was used, RIP was detected at a frequency of < 0.01% in one progeny from 34 screened (3%). Based on the fact that the frequency of RIP observed in linked duplications in this study (1.4–8.1%) was much lower than in both *N. crassa* (30%) and *F. graminearum* (10–39%) (Galagan and Selker 2004; Pomraning *et al.* 2013), it is not surprising that the frequency of RIP in unlinked duplications is also much lower in *L. maculans* than these other species. It should be noted that the current study on unlinked duplications has limitations. Not all progeny were sequenced, but instead phenotypic screening was used to increase the chances of identifying progeny harboring the RIP mutations. Therefore, the detection of RIP in only 3% of progeny will be biased. Regardless, the frequency of RIP mutations within

Figure 4 Comparison of Repeat Induced Point (RIP) mutation in *L. maculans* compared to the model organism *N. crassa*. (A) Representation of the replication cycle leading to the production of eight ascospores (octad) within an ascus. Black rectangles represent the heterokaryon formed after the fusion of nuclei from opposite mating types. Complementary DNA strands that represent a single repeat region are shown in black and will be targeted by RIP. Complementary DNA strands, shown in brown, represent the corresponding region from the other parent with no tandem repeat and therefore escape RIP. (B) In *N. crassa*, up to 15 rounds of premeiotic mitoses occur whereby RIP (indicated by red stars) is active. Initially, RIP generates C→T transitions (red dots) on a single strand and, after replication, complementary adenines (blue dots) are inserted on the complementary strand. Following karyogamy, meiosis I, meiosis II, and then mitosis, two RIP haplotypes result, with each progeny pair having identical haplotypes. (C) Similar to *N. crassa*, we propose that multiple rounds of premeiotic mitoses occur with RIP active. It is unknown how many rounds would be occurring in *L. maculans*. Differing to *N. crassa*, in *L. maculans* we propose that deamination (yellow stars) continues to be active resulting in C→T transitions. This continued deamination would result in the generation of unique mutations within each of the progeny pairs in the subsequent octad.

those progeny is still extremely low (5 bp within the 5736-bp region).

Perhaps the most significant difference between *L. maculans* and *N. crassa* is the generation of four different genotypes in the four pairs of progeny harboring the constructs within an octad, compared to only two for *N. crassa* (Watters *et al.* 1999). The progeny pairs, generated through a mitotic division, have different genotypes in *L. maculans*. In addition, across the pairs and across the octad, there are both common and unique G→A and C→T unique mutations on a single DNA strand. Several hypotheses could explain these findings and one is diagrammatically represented in Figure 4, with a simplified representation of the RIP process in *N. crassa* for comparison (Figure 4B). In *N. crassa*, duplications are likely to be detected during the G1 phase of the cell cycle, leading to multiple premeiotic rounds of RIP occurring whereby C→T transitions are generated. It has been hypothesized that up to 10–15 mitoses may occur and it has been shown that the frequency of RIP increases in older ascospores (Singer *et al.* 1995a). The occurrence of RIP during these premeiotic mitoses leads to the generation of two different haplotypes in the RIP-targeted progeny, one haplotype for each progeny pair. One possible hypothesis to explain the different haplotype number observed in *L. maculans* is that RIP is initially occurring as it does in *N. crassa*, but that the deamination step is continuing in *L. maculans* such that, during replication after meiosis II, the C→U deamination will be repaired to generate T→As (Figure 4C). The continuation of deamination would then result in unique C→T and G→A transitions on both strands of the four octad progeny. Much of the data generated in the current study supports this hypothesis. First, the occurrence of RIP during multiple rounds of premeiotic mitoses is supported by the presence of RIP mutations common across all four octad progeny and other mutations common across pairs of progeny. Mutations common across all four octad progeny would represent “older” mutations generated in the earliest rounds of RIP, while those common across pairs would represent RIP mutations generated in later mitoses. The continuation of deamination after karyogamy and the consequent generation of Ts→As is supported by the presence of unique RIP mutations in each of the progeny pairs. Furthermore, all the unique mutations are either G→A or C→T transitions on a single strand (Table 5), and not a combination of both, which supports this hypothesis. An alternative but unlikely hypothesis is that a mutation event as late as mitosis occurring postmeiosis is also feasible, although we show that recurrent mitotic passaging of strains was not able to trigger RIP. Further work is needed to fully understand differences and similarities in RIP between these species.

Other mechanisms could also contribute to the unusual patterns of RIP detected in *L. maculans*. These might include differences in mutation rates, methylation events, or the efficiency and timing of DNA repair between fungal species, which might account, at least in part, for some of the different levels of RIP or genetic haplotypes seen in the octad pairs in

L. maculans compared to *N. crassa*. However, currently very little is known about these processes in *L. maculans*, making it very difficult to speculate.

Although not explored in the current study, another noteworthy difference between *L. maculans* and *N. crassa* is the lack of association between RIP and cytosine methylation in *L. maculans*. In *N. crassa*, repeats that have been heavily mutated by RIP are then targets for DNA methylation (Selker 1990; Galagan and Selker 2004), and the frequency of RIP correlates with concentrations of S-adenosylmethionine in strains (Rosa *et al.* 2004). However, previous studies using methylation-sensitive restriction enzymes showed no differences in RIP-affected sequences, suggesting that RIP-associated methylation is not occurring in *L. maculans* (Idnurm and Howlett 2003). Similar studies have been performed in *F. graminearum*, with no methylation detected in association with RIP (Pomraning *et al.* 2013).

The consequences on gene and genome evolution due to the variability in RIP between fungal species remain to be fully established. It is curious that transposons at some point expanded throughout *L. maculans*, in which tandem duplications are required to ensure RIP occurs, before being brought under control by RIP (Rouxel *et al.* 2011), whereas in *N. crassa*, where the duplications can be on separate chromosomes to trigger RIP, transposons are rare (Galagan *et al.* 2003). Of note, the impact of unlinked duplications escaping RIP provides a mechanism by which duplicated DNA regions can undergo divergence in function.

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