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Gene drive by *Fusarium SKC1* is dependent on its competing allele

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ABSTRACT

The *Fusarium verticillioides SKC1* gene driver is transmitted to offspring in a biased manner through spore killing. The mechanism that allows *SKC1* to kill non-*SKC1* offspring while sparing others is poorly understood. Here we report that gene drive by *SKC1* is dependent on *SKC1*'s competing allele. We propose that *SKC1*'s competing allele influences the ability of a genome defense process to detect *SKC1*, and we provide evidence that this genome defense process is meiotic silencing by unpaired DNA (MSUD). Our findings suggest that the successful deployment of gene drivers to control pathogenic fungi will require researchers to consider how competing alleles influence the ability of gene drivers to be detected by genome defense processes.

1. Introduction

Gene drive elements, or gene drivers, use various mechanisms to propagate themselves through sexual reproduction to the next generation in a biased manner. In fungi, gene drivers that achieve biased transmission through spore killing are called spore killers. Spore killers have been identified in several genera, including *Bipolaris, Fusarium, Neurospora*, *Podospora*, and *Schizosaccharomyces* (Raju, 1994; Zanders and Johannesson, 2021). For the most part, the mechanisms by which spore killers kill spores and achieve drive are poorly understood.

The filamentous fungus *Fusarium verticillioides* is an endophyte and pathogen of maize that harbors a spore killer called *SKC1* (Kathariou and Spieth, 1982; Lohmar et al., 2022; Pyle et al., 2016; Sidhu, 1984; Xu and Leslie, 1996). *SKC1* demonstrates high levels of gene drive in the laboratory. For example, nearly all viable offspring (>99 %) from a cross between *F. verticillioides* reference strains Fv999-*SKC1* and Fv149-*SkS*, where *SkS* denotes sensitivity to *SKC1,* possess the *SKC1* genotype (Pyle et al., 2016). The mechanism by which *SKC1* achieves this high level of gene drive is mostly unknown. *SKC1* is associated with at least four molecularly distinct transcripts, two protein-coding sense transcripts and two antisense transcripts of unknown coding potential (Lohmar et al., 2022). Upon translation, the sense transcripts produce either a 71 amino acid (aa) protein called Skc1a or an 84 aa protein called Skc1b. While the longer Skc1b is essential for spore killing, spore killing does not occur when Skc1a is expressed without Skc1b.

While seeking to understand how the known *SKC1* transcripts contribute to spore killing, we discovered that strain Fv149-*SkS* can be made resistant to spore killing by inserting DNA into the locus that directly competes with *SKC1* for existence in the next generation of sexual offspring. Below, we describe the properties of this "*SKC1*-resistance-inducing DNA", as well as results from spore killing and gene drive assays. We propose and provide evidence that this novel form of resistance to *F. verticillioides SKC1* is achieved through a small RNA-mediated defense process called meiotic silencing by unpaired DNA (MSUD).

2. Materials and methods

2.1. Strains and culture methods

The *F. verticillioides* reference strains used in this study are Fv149 (FGSC 7600) (*SkS*, *MAT1-1*) and Fv999 (FGSC 7603) (*SKC1*, *MAT1-2*). For clarity, Fv149 is referred to as Fv149-*SkS* and Fv999 is referred to as Fv999-*SKC1* hereafter. In addition, Fv149-*SkS* was transformed to construct strains TJML20.45-*GENR*$^{3165\text{-}64}$ and TJML68.2-*GENR*^{ect}. Care and handing of *F. verticillioides* was performed as described previously (Lohmar et al., 2022) unless otherwise indicated.

2.2. SKC1 vs SkS locus comparison

The Fv999-*SKC1* sequence (GenBank KU963213.1, positions 95,939–100,297, inclusive) and Fv149-*SkS* sequence (GenBank CM000582.1, positions 761,716–765,601, inclusive) were aligned with

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EMBOSS Needle (Madeira et al., 2022). Aligned sequences were annotated with BioEdit (Hall, 1999) and MsWord (Fig. S1). The borders of two intervals of high identity ("left" and "right") were visually demarcated, and percent identities were calculated with custom Python scripts (Fig. S2).

2.3. Plasmid construction

The sequences of the primers used to amplify DNA with the polymerase chain reaction (PCR) are listed in Table S1. Plasmid pJB6.3 was constructed by fusion PCR with genomic DNA from strain Fv149-*SkS* serving as the template. Primer sets I1471/I1477, I1478/I1479, and I1480/I1481 were used for the left fragment, right fragment, and fusion product, respectively. The fusion product was cloned to pJet1.2 with ThermoFisher's CloneJET PCR Cloning Kit to produce pJB6.3. Plasmid pJML18.1 was constructed by ligating a 2.5 kb geneticin resistance cassette from pGENAscI (Brown et al., 2007) into the *Asc*I site of pJB6.3.

2.4. F. verticillioides transformations

The procedure used to transform *F. verticillioides* was described previously (Lohmar et al., 2022). Transformant TJML20.45-*GENR*$^{3165\text{-}64}$ was obtained by transformation of Fv149-*SkS* with a 4.8 kb PCR product (template pJML18.1 and primer set 2581/2582) and selection for geneticin resistance. Transformant TJML68.2-*GENR*^{ect} was obtained by transformation of Fv149-*SkS* with a 2.5 kb PCR product (template pGENAscI and primer set 739/740) and selection for geneticin resistance.

2.5. Spore killing and gene drive assays

Spore killing assays were performed by dissecting perithecia and imaging asci as previously described (Lohmar et al., 2022). For the *SKC1* gene drive assay, crosses were performed, cirri were collected from perithecia and dispersed in sterile 0.2 % Tween® 60, ascospores were plated on VM agar (Rhoades et al., 2020) in Petri dishes, germlings were transferred to VM agar in culture tubes, vegetative tissue for genomic DNA was cultured in VM liquid, and genomic DNA was isolated from lyophilized tissue with IBI Scientific's Genomic DNA Kit for plants. Offspring were genotyped with primer set I1079/I1080, which produces a 767 bp amplicon from *SKC1* genotypes and a 287 bp amplicon from *SkS* and *GENR*$^{3165\text{-}64}$ genotypes (Fig. S1).

2.6. RNA isolation and sequencing

Vegetative tissue for RNA isolation was collected from point-inoculated, four-day old cultures of Fv149-*SkS* or Fv999-*SKC1* on 100-mm diameter Petri dishes containing 30 ml of carrot agar. Vegetative tissue was collected from the surface of the culture plates into 5.0 ml of water with a spatula. The resulting tissue-water mixture was filtered with Miracloth, blotted dry with paper towels, lyophilized, and stored frozen at −80 °C before RNA isolation. Perithecia for RNA isolation were collected from crosses ten days post fertilization (10 dpf). Total RNA was isolated from vegetative tissue and perithecia as previously reported (Lohmar et al., 2022), except that the manufacturer's recommendations for the retention of low molecular weight RNA molecules with Invitrogen's PureLink RNA Mini Kit was followed. Small RNA library preparation (New England Biolabs NEBNext® Small RNA library prep kit) and sequencing were performed by Novogene (Sacramento, CA) on the Illumina Novoseq 6000 platform. Read filtering to remove low quality reads was also performed by Novogene and the filtered datasets were uploaded to the National Center for Biotechnology Information's Sequence Read Archive (NCBI-SRA; Leinonen et al., 2011).

2.7. Small RNA dataset analysis

RNA sequences (reads) in FASTA format were aligned to an aggregate reference sequence consisting of the Fv149-*SkS* genome (release 54, FungiDB) and *SKC1* (GenBank KU963213.1, positions 97591–98257, inclusive) with Bowtie2 (version 2.3.5.1, default settings; Langmead and Salzberg, 2012). A custom Python script (Fig.S3) was used to calculate RPKM (reads per kilobase reference sequence per million mapped reads; Mortazavi et al., 2008) values for *SKC1* and two reference gene sets (Set U and Set P). Only reads between 18 and 35 bases in length (inclusive) that aligned to the reference sequence without mismatches and without secondary alignments were used for the calculations. Read mapping position was defined by the left most mapping position of each read relative to the reference sequence.

2.8. Reference gene sets.

Genome sequencing reads for Fv999-*SKC1* were downloaded from the SRA database (SRR3271586) and assembled with MEGAHIT (v.1.2.9, default settings for paired end reads; Li et al., 2015). Protein sequences from an Fv149-*SkS* genome annotation (FungiDB, release 54) were used as queries with TBLASTN (version 2.11.0; Camacho et al., 2009) to search the MEGAHIT Fv999-*SKC1* genome assembly. Genes for ten Fv149 protein sequences that failed to match sequences (no hits with E-value < 0.01) were selected for Set U (Table 1). Genes for ten Fv149 proteins that matched sequences (E-value $= 0.00$) were selected for Set P (Table 1). Unusually short or long genes were avoided for both Set U and Set P, as a result, the shortest and longest genes in both sets are 2124 bp and 4815 bp, respectively (Table 1).

2.8. Identification of MSUD orthologs

Candidate *Fusarium* orthologs of *Neurospora* MSUD proteins were identified with BLASTP (Camacho et al., 2009). BLAST databases were compiled from FASTA files of *F. verticillioides* Fv149-*SkS* (FungiDB, release 54) and *F. graminearum* PH1 (FungiDB, release 54) proteins, and the databases were searched with *Neurospora* MSUD protein sequences

Table 1
Reference gene sets used to test for the existence of MSUD in *F. verticillioides*. Set U genes are present in Fv149-*SkS* and *GENR*$^{3165\text{-}64}$ genomes, but they could not be identified in the Fv999-*SKC1* genome. Set P genes are present in the genomes of all three strains. Chr: GenBank accession numbers for chromosomes are as follows: 2, CM000579.1; 3, CM000580.1; 4, CM000581.1; 5, CM000582.1; 6, CM000583.1; 7, CM000584.1; 11, CM000588.1. um, unmapped contig DS022267.1. Left and right: gene border position. Dir: the coding strand is found on the minus strand (M) or plus strand (P). Len: the length of the gene.

	Chr	Left	Right	Dir	Len
Set U					
FVEG_01569	6	3,759,985	3,762,544	M	2560
FVEG_06982	7	1,396,803	1,399,049	M	2247
FVEG_06984	7	1,399,509	1,401,769	M	2261
FVEG_06992	7	1,421,427	1,424,870	P	3444
FVEG_15979	7	1,429,220	1,432,040	M	2821
FVEG_15986	7	1,446,915	1,449,365	P	2451
FVEG_15996	7	1,467,588	1,471,492	M	3905
FVEG_16316	11	1,932,520	1,934,700	M	2181
FVEG_08400	11	1,958,649	1,960,845	M	2197
FVEG_14075	um	64,307	67,037	P	2731
Set P					
FVEG_06405	2	1,810,225	1,813,586	P	3362
FVEG_08131	3	2,027,171	2,031,985	P	4815
FVEG_04999	4	2,007,851	2,011,857	P	4007
FVEG_04854	4	1,579,603	1,582,273	P	2671
FVEG_03415	5	37,279	40,326	M	3048
FVEG_09182	5	3,477,878	3,480,777	P	2900
FVEG_03378	5	127,740	129,863	M	2124
FVEG_13299	6	74,115	76,748	M	2634
FVEG_06732	7	634,910	637,567	P	2658
FVEG_12924	11	280,756	283,746	P	2991

as queries (Table 2).

2.9. Whole genome sequencing

Genome sequencing was performed for strain TJML20.45-*GENR*$^{3165\text{-}64}$ as described previously (Lohmar et al., 2022). Genome sequence analysis and PCR assays were used to confirm the correct integration of *GENR* in strain TJML20.45-*GENR*$^{3165\text{-}64}$ (Fig. S4).

3. Results

3.1. Spore killing by SKC1 is dependent on the structure of SKC1′s competing allele

In *F. verticillioides*, *SKC1* is located on Chromosome V between the hypothetical protein coding genes *03165* and *03164* (Lohmar et al., 2022). These two genes are also present in the equivalent genomic location in *SkS* strains (Pyle et al., 2016). Comparison of the *03165–03164* intergenic region in strains Fv999-*SKC1* and Fv149-*SkS* revealed long intervals of high nucleotide identity (94.2–93.9 %) that flank shorter intervals of unique sequence (Fig. 1A). In Fv999-*SKC1,* the unique region is 667 bp and spans the *SKC1* coding region. In contrast, the unique region in Fv149-*SkS* is 189 bp . Herein, we define the *SKC1* allele as the entire interval between the coding sequences of *03165* and *03164* in Fv999-*SKC1*. Likewise, we define an *SKC1*-competing allele to be the entire interval between the coding sequences of *03165* and *03164* in an Fv999-*SKC1* mating partner.

Crosses between strains of *F. verticillioides* that lack spore killer genes typically produce eight viable ascospores (offspring) per ascus (offspring sac) (Leslie and Summerell, 2006). In contrast, *SKC1*-based spore killing causes Fv149-*SkS* × Fv999-*SKC1* crosses to produce asci with only four viable ascospores (Raju, 1994). To determine if *SKC1*′s competing allele influences *SKC1*′s ability to kill spores, we inserted a 2551 bp geneticin resistance cassette (*GENR*) into the *03165–03164* intergenic region in Fv149-*SkS* to create strain TJML20.45-*GENR*$^{3165\text{-}64}$, herein abbreviated as strain *GENR*$^{3165\text{-}64}$ (Fig. 1B and S2). While spore killing occurred in our control crosses of Fv149-*SkS* × Fv999-*SKC1* (Fig. 1B, left), it did not occur in crosses of *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* (Fig. 1B, middle), indicating that *SKC1*′s competing allele influences spore killing by *SKC1*. To eliminate the possibility that *GENR* itself is responsible for *SKC1* resistance, rather than the structure (e.g., sequence and/or length) of *SKC1*′s competing allele, we inserted *GENR* into a location other than the *03165–03164* intergenic region (i.e., an ectopic insertion) in Fv149-*SkS* to create strain TJML68.2-*GENR*^{ect}, herein abbreviated as strain *GENR*^{ect}. As expected, spore killing occurred in crosses of *GENR*^{ect} × Fv999-*SKC1* (Fig. 1B, right), demonstrating that *GENR* does not disrupt spore killing when present at genomic locations other than the *03165–03164* intergenic region.

3.2. SKC1 gene drive is dependent on spore killing

It has long been assumed that *SKC1* achieves gene drive through spore killing. The finding that *GENR*$^{3165\text{-}64}$ prevents spore killing by *SKC1* allowed us to directly test this assumption by isolating and genotyping offspring from three crosses: 1) Fv149-*SkS* × Fv999-*SKC1*, 2) *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1,* and 3) *GENR*^{ect} × Fv999-*SKC1*. We found that *SKC1* demonstrated complete gene drive (100 %) in the two crosses where spore killing occurred (Fv149-*SkS* × Fv999-*SKC1* and *GENR*^{ect} × Fv999-*SKC1*) and no gene drive in the one cross where spore killing was absent (*GENR*$^{3165\text{-}64}$ × Fv999-*SKC1*; Fig. 2). In the spore killing-deficient cross, *SKC1* was transmitted to only 37.5 % of the offspring, a transmission value that is consistent with Mendelian inheritance ($n = 24$, $\chi^2 = 1.5$, $p > 0.05$). These findings support a mechanistic link between *SKC1* spore killing and *SKC1* gene drive, and they demonstrate that *GENR*$^{3165\text{-}64}$ disrupts both spore killing and gene drive by *SKC1*.

3.3. SKC1-specific small RNAs are present in GENR$^{3165\text{-}64}$ × Fv999-SKC1 crosses

The genome defense process of meiotic silencing by unpaired DNA (MSUD) defends the genomes of *Neurospora* fungi from selfish genetic elements (Raju et al., 2007; Rhoades et al., 2019; Svedberg et al., 2021; Wang et al., 2015). Specifically, MSUD scans homologous chromosomes for segments of "unpaired DNA" during meiosis. Unpaired DNA is defined as a gene or other DNA element that is present in one chromosome while absent from the allelic location in the chromosome's meiotic pairing partner (i.e., its homolog) (Aramayo and Selker, 2013; Hammond, 2017). Although the molecular mechanism used by MSUD to identify unpaired DNA is poorly understood, detection of unpaired DNA triggers the silencing of transcripts from the unpaired DNA through a mechanism involving MSUD-associated small interfering RNAs (masiRNAs). In *N. crassa*, masiRNAs are characterized by a terminal 5' U bias (5' U-small RNAs) and a length range that peaks at 25 nucleotides (Hammond et al., 2013a; Wang et al., 2015).

To investigate the possibility that MSUD is behind *GENR*$^{3165\text{-}64}$′s ability to disrupt spore killing and gene drive by *SKC1*, we isolated and sequenced small RNAs from *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* perithecia. We also isolated and sequenced small RNAs from Fv149-*SkS* × Fv999-*SKC1* perithecia, as well as Fv149-*SkS* vegetative tissue and Fv999-*SKC1* vegetative tissue. We then mapped the resulting sequences (reads) to a reference sequence containing the Fv149-*SkS* genome and *SKC1*. We detected considerably more *SKC1*-specific reads in *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* perithecia (Fig. 3A, 80 reads, 47.5 RPKM) than in Fv149-*SkS* × Fv999-*SKC1* perithecia (Fig. 3A, 4 reads, 5.3 RPKM).

Table 2
Candidate *Fusarium* orthologs of *Neurospora* MSUD proteins. *N. crassa* MSUD protein sequences were used as queries to search *F. verticillioides* and *F. graminearum* protein databases for orthologs. The E-value returned by BLASTP for each candidate ortholog is shown. n.d., no candidate detected. The lowest E-values for the best matches to SAD-2, SAD-4, and SAD-5 are shown.

N. crassa protein	Reference	*F. verticillioides* candidate	E-value	*F. graminearum* candidate	E-value
SAD-1 (NCU02178)	Shiu et al., 2001	FVEG_01945	0	FGRAMPH1_01T10647	0
SAD-2 (NCU04294)	Shiu et al., 2006	n.d.	0.31	n.d.	0.4
SAD-3 (NCU09211)	Hammond et al., 2011	FVEG_13112	0	FGRAMPH1_01T11343	0
SAD-4 (NCU01591)	Hammond et al., 2013b	n.d.	0.87	n.d.	0.01
SAD-5 (NCU06147)	Hammond et al., 2013b	n.d.	0.027	n.d.	1.6
SAD-6 (NCU06190)	Samarajeewa et al., 2014	FVEG_05313	0	FGRAMPH1_01T24381	0
SAD-7 (NCU01917)	Samarajeewa et al., 2017	FVEG_15078	8e-126	FGRAMPH1_01T11179	4e-131
SAD-8 (NCU01310)	Boone et al., 2020	FVEG_07359	3e-78	FGRAMPH1_01T04579	1e-82
DCL-1 (NCU08270)	Alexander et al., 2008	FVEG_06696	0	FGRAMPH1_01T28097	0
QIP (NCU00076)	Lee et al., 2010; Xiao et al., 2010	FVEG_05385	1e-58	FGRAMPH1_01T23003	1e-59
SMS-2 (NCU09434)	Lee et al., 2003	FVEG_00803	0	FGRAMPH1_01T00923	0
CBP80 (NCU04187)	Decker et al., 2017	FVEG_07937	0	FGRAMPH1_01T24533	0
CBP20 (NCU00210)	Decker et al., 2017	FVEG_07036	7e-92	FGRAMPH1_01T18045	9e-92

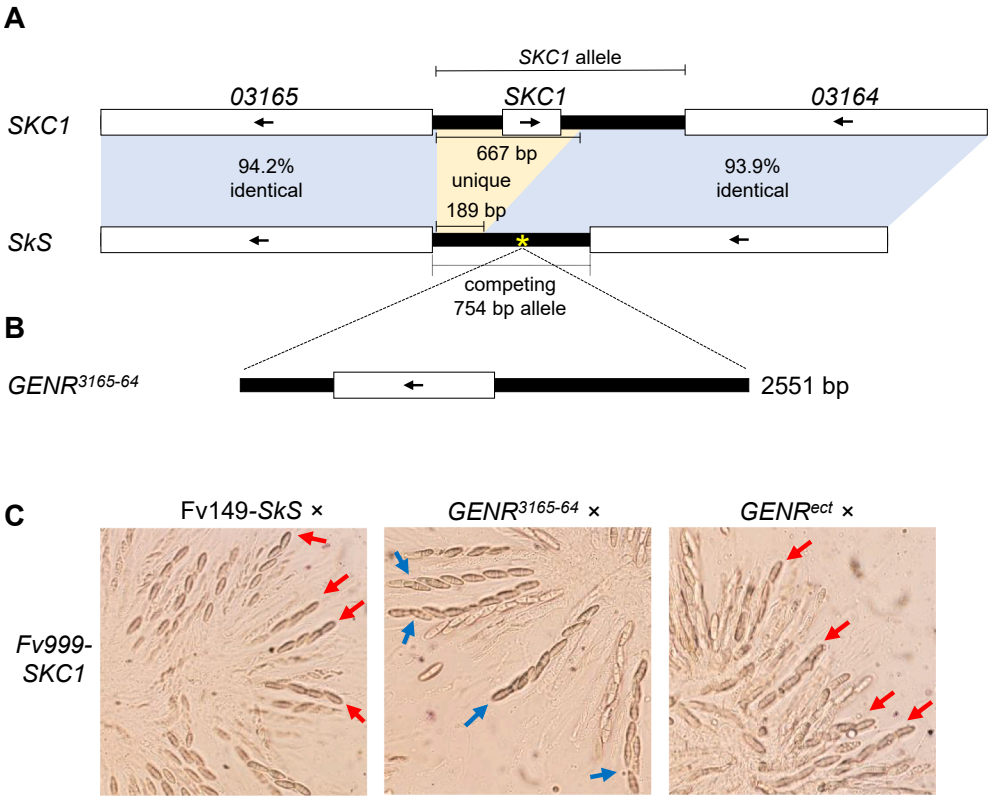

Fig. 1. *SKC1*-based spore killing is prevented by *GENR*$^{3165\text{-}64}$. (A) A diagram of the *SKC1* locus in Fv999-*SKC1* is shown above the allelic location in Fv149-*SkS*. Flanking genes *03165* and *03164* are indicated. Coding regions are shown as open rectangles and black arrows indicate direction of transcription. Intervals with high identity (94.2 % and 93.9%) and no identity (unique) are indicated in blue and yellow, respectively. The red asterisk marks the location in Fv149-*SkS* that was used to insert *GENR*, thereby creating *GENR*$^{3165\text{-}64}$. (B) A diagram of the geneticin-resistance cassette (*GENR*) in *GENR*$^{3165\text{-}64}$. (C) *GENR*$^{3165\text{-}64}$ disrupts spore killing but *GENR*^{ect} does not. Asci from crosses between Fv999-*SKC1* and either Fv149-*SkS* (left), *GENR*$^{3165\text{-}64}$ (middle), or *GENR*^{ect} (right) are shown. Red and blue arrows highlight asci that have and have not undergone spore killing, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

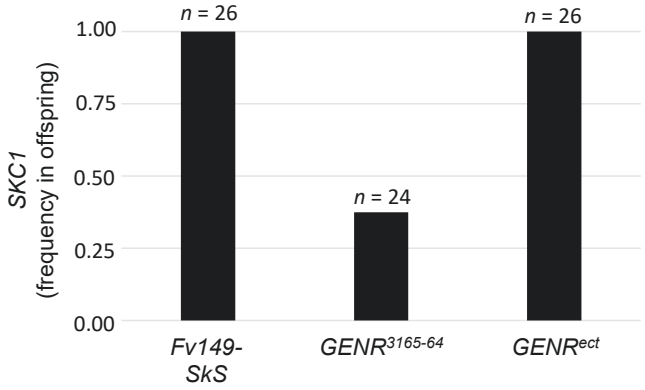

Fig. 2. Gene drive by *SKC1* is disrupted by *GENR*$^{3165\text{-}64}$. Offspring were collected from crosses of Fv999-*SKC1* with three mating partners: Fv149-*SkS*, *GENR*$^{3165\text{-}64}$, and *GENR*^{ect}. Offspring were genotyped by PCR. *SKC1* demonstrates complete drive in Fv149-*SkS* × Fv999-*SKC1* and *GENR*^{ect} × Fv999-*SKC1*. The frequency of *SKC1* in offspring of *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* is consistent with Mendelian inheritance. *n* = number of offspring examined.

Furthermore, while 85 % of *SKC1*-specific reads in the *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* perithecial dataset possess a terminal 5' U, none of the *SKC1*-specific reads in the Fv149-*SkS* × Fv999-*SKC1* perithecial dataset possess a uridine at the 5' terminus. Additionally, the length range of *SKC1*-specific reads in the *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* perithecial dataset is similar to the length range of *Neurospora* masiRNAs (Fig. 3B; Hammond et al., 2013a; Wang et al., 2015). These findings suggest that the *SKC1*-specific small RNAs in the *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* perithecial dataset are a result of MSUD, and that MSUD is responsible for *GENR*$^{3165\text{-}64}$-based disruption of *SKC1*-induced spore killing.

3.4. Unpaired genes are associated with elevated 5' U-small RNA levels in *F. verticillioides* perithecia

MSUD activation and masiRNA generation occur when DNA is unpaired during meiosis. While our detection of *SKC1*-specific small RNAs in perithecia of *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* crosses is consistent with the existence of MSUD in *F. verticillioides*, MSUD has yet to be demonstrated in this species. Therefore, to provide additional evidence that MSUD exists in *F. verticillioides*, we examined small RNA levels in perithecia and vegetative tissue for ten genes (Set U) that are present in Fv149-*SkS* and *GENR*$^{3165\text{-}64}$ but absent from Fv999-*SKC1* (Table 1, see methods). We predicted that if MSUD exists in *F. verticillioides,* 5' U-small RNAs with sequences matching Set U genes should be present at higher levels in perithecia than in vegetative tissue. Indeed, we found that 5' U-small RNAs are present at considerably higher levels in perithecia (1888–3988 RPKM) than in vegetative tissue (8–36 RPKM; Fig. 3, C and E) for genes in Set U. These findings are consistent with the existence of MSUD in *F. verticillioides*. As a control, we also examined 5' U-small RNA levels for a set of ten genes that are present in Fv149-*SkS*, *GENR*$^{3165\text{-}64}$, and Fv999-*SKC1* genomes (Table 1, Set P). Because these genes are expected to be paired during meiosis, we predicted that MSUD should have no effect on Set P genes, and 5' U-small RNA levels should be similar in perithecia and vegetative tissue for genes in this set. Accordingly, we found that 5' U-small RNAs with sequences matching Set P genes are present at similar levels in perithecia (18–43 RPKM) and vegetative tissue (23–32 RPKM; Fig. 3, D and F). Overall, our analysis of Set U and Set P genes suggests that MSUD exists in *F. verticillioides* and that *F. verticillioides* MSUD triggers the production of masiRNAs when it detects unpaired DNA.

3.5. MSUD genes in *F. verticillioides*

Over a dozen MSUD proteins have been functionally characterized in *N. crassa*. To examine the possibility that *F. verticillioides* possesses orthologs of these proteins, we searched an *F. verticillioides* protein

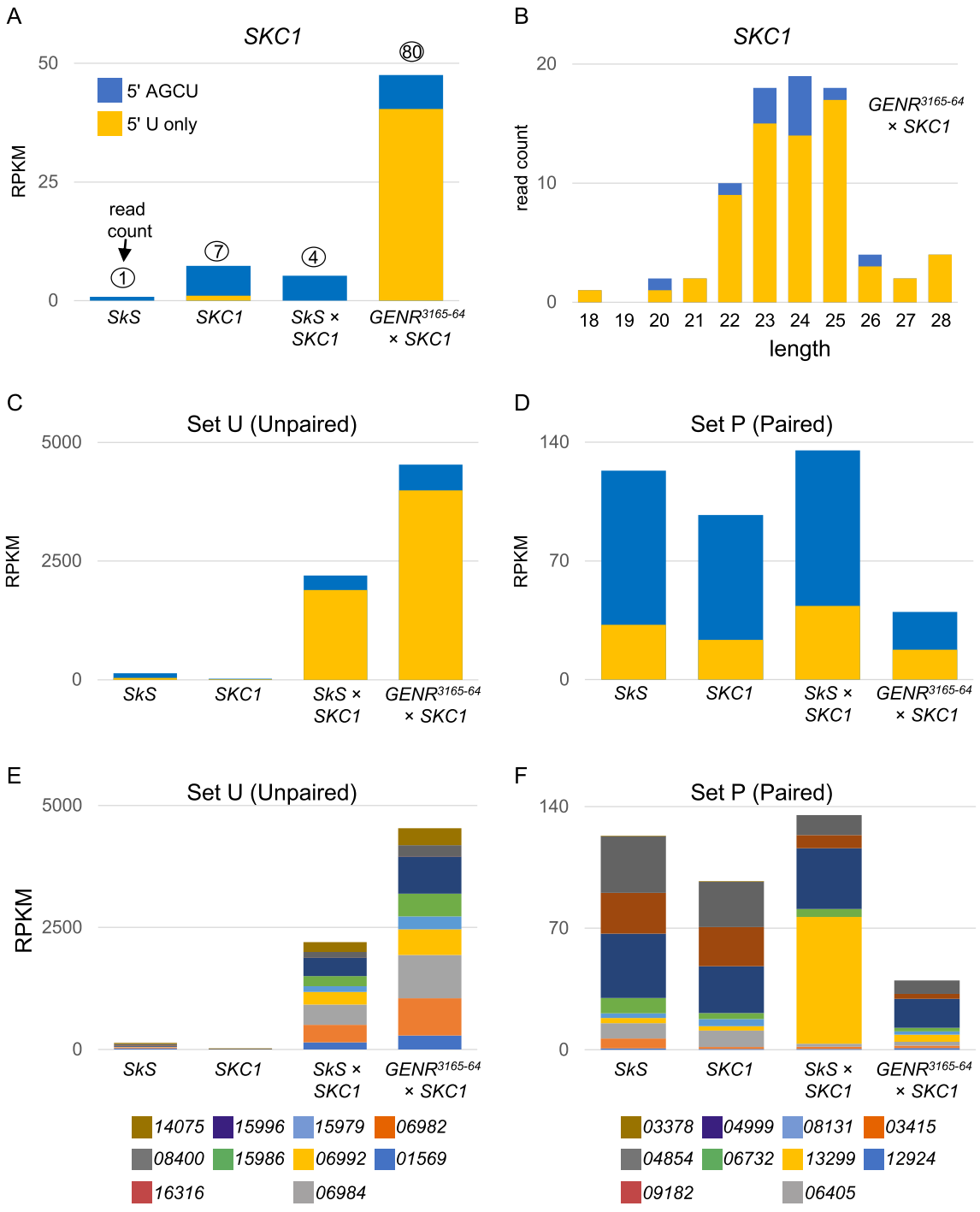

Fig. 3. *SKC1* small RNAs are present in *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* perithecia. (A) The levels of *SKC1*-specific reads in four RNA sequencing datasets are shown. The Y axis is RPKM. For reference purposes, raw read counts are shown with a circled number above each bar. Blue, total *SKC1*-specific reads; orange, 5' U *SKC1*-specific reads; *SkS,* Fv149-*SkS* vegetative tissue; *SKC1,* Fv999-*SKC1* vegetative tissue; *SkS* × *SKC1,* perithecia from Fv149-*SkS* × Fv999-*SKC1*; *GENR*$^{3165\text{-}64}$ × *SKC1,* perithecia from *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1*. (B) The length profile of *SKC1*-specific reads in *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* perithecia. (C) Read levels for ten genes that are predicted to be unpaired in Fv149-*SkS* × Fv999-*SKC1* and *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* crosses (Set U genes). (D) Read levels for ten genes that are predicted to be paired in Fv149-*SkS* × Fv999-*SKC1* and *GENR*$^{3165\text{-}64}$ × Fv999-*SKC1* crosses (Set P genes). (E) Similar to panel C except read levels for each gene in Set U are indicated without 5' U bias information. (F) Similar to panel D except read levels for each gene in Set P are indicated without 5' U bias information. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

database with *Neurospora* MSUD protein sequences. While we identified an *F. verticillioides* ortholog for most *Neurospora* MSUD proteins, we were unable to identify *F. verticillioides* orthologs for three proteins: SAD-2, SAD-4, and SAD-5 (Table 2). To determine if the lack of SAD-2, SAD-4, and SAD-5 orthologs was a feature of *Fusarium* in general, we searched an *F. graminearum* protein database for MSUD orthologs. Consistent with our *F. verticillioides* findings, we identified a candidate *F. graminearum* ortholog for all MSUD proteins except SAD-2, SAD-4, and SAD-5 (Table 2). It thus seems the apparent lack of SAD-2, SAD-4, and SAD-5 orthologs is a general feature of *Fusarium* fungi.

4. Discussion

Fusarium verticillioides is a pathogen of agricultural significance due to its ability to cause ear and stalk rot of maize and to contaminate maize kernels with fumonisin mycotoxins (Alberts et al., 2016). There is a growing interest in using gene drivers to eliminate or reduce the ability of populations of fungi, including *Fusarium* species, to cause crop disease and mycotoxin contamination problems (Gardiner et al., 2020). However, the mechanisms by which fungal gene drivers function are poorly understood, and such mechanistic understanding is likely necessary to harness gene drivers for practices that mitigate agricultural problems.

In this report, we have shown that the *F. verticillioides SKC1* gene driver achieves gene drive through spore killing and that the success of spore killing is dependent on *SKC1*′s competing allele. Specifically, *SKC1* kills spores when its competing allele is a 754 bp interval of intergenic DNA (as in Fv149-*SkS*) but not when its competing allele consists of the same intergenic DNA plus a 2551 bp geneticin resistance cassette (as in $GENR^{3165\text{-}64}$). A similar phenomenon has been reported in *Neurospora*, where placing a hygromycin resistance cassette (*hph*) within an intergenic region immediately flanking the *Neurospora rfk-1* spore killer gene eliminates spore killing in an MSUD-dependent manner. This finding indicates that the native location of *rfk-1* in the *Neurospora* genome allows it to avoid effective detection and silencing by MSUD, and that this ability to avoid detection and silencing is lost when *hph* is inserted next to *rfk-1*. The findings of the current study indicate that the native location of *F. verticillioides SKC1* allows it to avoid effective detection and silencing by MSUD when *SKC1*′s competing allele is the 754 bp interval of intergenic DNA found in strain Fv149-*SkS*, but not when *SKC1*′s competing allele is $GENR^{3165\text{-}64}$. We propose a model in which *SKC1* is inefficiently detected, or completely missed, by the unpaired DNA detection (UDD) proteins in Fv999-*SKC1* × Fv149-*SkS* crosses, thereby allowing *SKC1* to be expressed in meiotic cells (Fig. 4A). In Fv999-*SKC1* × $GENR^{3165\text{-}64}$ crosses, the presence of the 2.5 kb *GENR* cassette increases the level of unpaired DNA shared between *SKC1* and its completing allele. This increases the likelihood of *SKC1* detection by UDD proteins and subsequent silencing through MSUD or an MSUD-like genome defense process (Fig. 4B).

Our proposal that MSUD exists in *F. verticillioides* is based on two lines of evidence. First, MSUD produces masiRNA with sequences matching unpaired DNA during meiosis and we found that small RNAs with masiRNA characteristics in perithecial tissue (containing meiotic cells) were substantially higher in perithecial tissue than vegetative tissue for 10 randomly selected unpaired genes (Set U genes). In contrast, we found no such relationship between small RNA levels and tissue type for 10 randomly selected paired genes (Set P genes). Second, *F. verticillioides* contains orthologs of all but three *Neurospora* MSUD proteins. Our inability to detect orthologs for three *Neurospora* MSUD proteins (SAD-1, SAD-4 and SAD-5) does not preclude *F. verticillioides* from possessing a functioning MSUD system because *F. graminearum* also lacks orthologs of the same genes despite the formal demonstration of MSUD in this species (Son et al., 2011).

5. Conclusions

In summary, our findings demonstrate that *SKC1*-based spore killing and gene drive can be disrupted by increasing the length of *SKC1*′s competing allele. We also provide evidence that MSUD exists in *F. verticillioides* and that MSUD is the mechanism by which *SKC1*′s competing allele can disrupt spore killing and gene drive. It remains to be determined, however, whether modifications to *SKC1*, which would be necessary for *SKC1*-based control practices, will also disrupt *SCK1*-induced gene drive. Overall, our findings indicate that efforts to use *SKC1* or other fungal gene drive systems to control mycotoxin contamination and fungal-based crop diseases will likely need to overcome fungal genome defense processes such as MSUD. It may be possible to incorporate UDD protein or MSUD suppressors into gene drive systems. This will require identification of UDD/MSUD proteins in *Fusarium* fungi. Additionally, it will be interesting to have a more complete understanding of the parameters of unpaired DNA detection in *F. verticillioides* to better understand how to hide gene drivers from genome defense processes.

Data availability

Datasets reported in this work are available from NCBI (BioProject ID: PRJNA388208). Small RNA datasets can be downloaded from NCBI SRA with the following accession numbers: SRR20576709 (Fv999-*SKC1* vegetative), SRR20576710 (Fv149-*SkS* vegetative), SRR20576708 (Fv149-*SkS* × Fv999-*SKC1* perithecia), and SRR20576707 ($GENR^{316564}$ × Fv999-*SKC1* perithecia). Genome sequencing reads can be downloaded from NCBI SRA with accession number SRR20576711 (strain TJML20.45-$GENR^{3165\text{-}64}$).

CRediT authorship contribution statement

Jessica M. Lohmar: Investigation, Formal analysis, Methodology, Writing – original draft. **Nicholas A. Rhoades:** Investigation, Writing – review & editing. **Thomas M. Hammond:** Conceptualization, Formal analysis, Funding acquisition, Software, Writing – review & editing. **Daren W. Brown:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

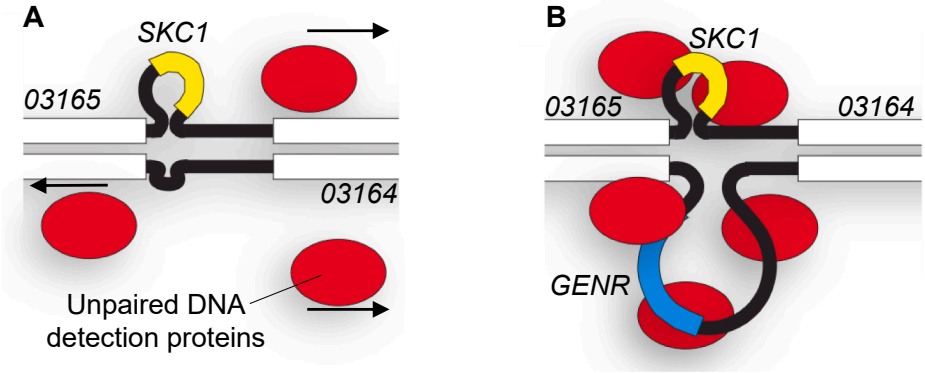

Fig. 4. Model: Unpaired DNA detection (UDD) proteins and the detection of *SKC1* during meiosis. (A) *SKC1*-based spore killing and gene drive occur in *Fv999-SKC1* × *Fv149-SkS* crosses. These findings suggest that hypothetical UDD proteins (red circles) are unable to detect *SKC1* (yellow) within the meiotic cells of these crosses because the level of unpaired DNA shared between *SKC1* and its competing allele (black loops) is too low. (B) In contrast, *SKC1* spore killing and gene drive fail in *Fv999-SKC1* × $GENR^{3165\text{-}64}$ crosses, presumably because the addition of a 2.5 kb *GENR* marker (blue) to *SKC1*′s competing allele increases unpaired DNA locally and attracts UDD proteins, thereby leading to *SKC1* silencing through MSUD or an MSUD-like phenomenon. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2022.103749.

References

Alberts, J.F., van Zyl, W.H., Gelderblom, W.C.A., 2016. Biologically based methods for control of fumonisin-producing *Fusarium* species and reduction of the fumonisins. Front. Microbiol. 7, 548. https://doi.org/10.3389/fmicb.2016.00548.

Alexander, W.G., Raju, N.B., Xiao, H., Hammond, T.M., Perdue, T.D., Metzenberg, R.L., Pukkila, P.J., Shiu, P.K.T., 2008. DCL-1 colocalizes with other components of the MSUD machinery and is required for silencing. Fungal Genet. Biol. 45, 719–727. https://doi.org/10.1016/j.fgb.2007.10.006.

Aramayo, R., Selker, E.U., 2013. *Neurospora crassa*, a model system for epigenetics research. Cold Spring Harb. Perspect. Biol. 5 (10) https://doi.org/10.1101/cshperspect.a017921.

Basenko, E., Pulman, J., Shanmugasundram, A., Harb, O., Crouch, K., Starns, D., Warrenfeltz, S., Aurrecoechea, C., Stoeckert, C., Kissinger, J., Roos, D., Hertz-Fowler, C., 2018. FungiDB: An integrated bioinformatic resource for fungi and oomycetes. J. Fungi Basel Switz. 4 (1), 39.

Boone, E.C., Xiao, H., Vierling, M.M., Decker, L.M., Sy, V.T., Kennedy, R.F., Bonham, M. A., Schmitz, S.F., John, A.M., Hammond, T.M., Shiu, P.K.T., 2020. An NCBP3-domain protein mediates meiotic silencing by unpaired DNA. G3 10, 1919–1927. https://doi.org/10.1534/g3.120.401236.

Brown, D.W., Butchko, R.A.E., Busman, M., Proctor, R.H., 2007. The *Fusarium verticillioides FUM* gene cluster encodes a Zn(II)2Cys6 protein that affects *FUM* gene expression and fumonisin production. Eukaryot. Cell 6, 1210–1218. https://doi.org/10.1128/EC.00400-06.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., Madden, T.L., 2009. BLAST+: architecture and applications. BMC Bioinf. 10, 421. https://doi.org/10.1186/1471-2105-10-421.

Decker, L.M., Xiao, H., Boone, E.C., Vierling, M.M., Shanker, B.S., Kingston, S.L., Boone, S.F., Haynes, J.B., Shiu, P.K.T., 2017. The nuclear cap-binding complex mediates meiotic silencing by unpaired DNA. G3 7 (4), 1149–1155. https://doi.org/10.1534/g3.116.038679.

Gardiner, D.M., Rusu, A., Barrett, L., Hunter, G.C., Kazan, K., 2020. Can natural gene drives be part of future fungal pathogen control strategies in plants? New Phytol. 228, 1431–1439. https://doi.org/10.1111/nph.16779.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symp. Ser. 41, 95–98.

Hammond, T.M., 2017. Sixteen years of meiotic silencing by unpaired DNA. Adv. Genet. 97, 1–42. https://doi.org/10.1016/bs.adgen.2016.11.001.

Hammond, T.M., Xiao, H., Boone, E.C., Perdue, T.D., Pukkila, P.J., Shiu, P.K.T., 2011. SAD-3, a putative helicase required for meiotic silencing by unpaired DNA, interacts with other components of the silencing machinery. G3 1, 369–376. https://doi.org/10.1534/g3.111.000570.

Hammond, T.M., Spollen, W.G., Decker, L.M., Blake, S.M., Springer, G.K., Shiu, P.K.T., 2013a. Identification of small RNAs associated with meiotic silencing by unpaired DNA. Genetics 194, 279–284. https://doi.org/10.1534/genetics.112.149138.

Hammond, T.M., Xiao, H., Boone, E.C., Decker, L.M., Lee, S.A., Perdue, T.D., Pukkila, P. J., Shiu, P.K.T., 2013b. Novel proteins required for meiotic silencing by unpaired DNA and siRNA generation in *Neurospora crassa*. Genetics 194, 91–100. https://doi.org/10.1534/genetics.112.148999.

Kathariou, S., Spieth, P.T., 1982. Spore killer polymorphism in *Fusarium moniliforme*. Genetics 102, 19–24. https://doi.org/10.1093/genetics/102.1.19.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359. https://doi.org/10.1038/nmeth.1923.

Lee, D.W., Pratt, R.J., McLaughlin, M., Aramayo, R., 2003. An argonaute-like protein is required for meiotic silencing. Genetics 164, 821–828. https://doi.org/10.1093/genetics/164.2.821.

Lee, D.W., Millimaki, R., Aramayo, R., 2010. QIP, a component of the vegetative RNA silencing pathway, is essential for meiosis and suppresses meiotic silencing in *Neurospora crassa*. Genetics 186, 127–133. https://doi.org/10.1534/genetics.110.118422.

Leinonen, R., Sugawara, H., Shumway, M., 2011. The sequence read archive. Nucleic Acids Res. 39, D19–D21. https://doi.org/10.1093/nar/gkq1019.

Leslie, J.F., Summerell, B.A., 2006. In: The *Fusarium* Laboratory Manual. Wiley-Blackwell. https://doi.org/10.1002/9780470278376.

Li, D., Liu, C.-M., Luo, R., Sadakane, K., Lam, T.-W., 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinforma. Oxf. Engl. 31, 1674–1676. https://doi.org/10.1093/bioinformatics/btv033.

Lohmar, J.M., Rhoades, N.A., Patel, T.N., Proctor, R.H., Hammond, T.M., Brown, D.W., 2022. A-to-I mRNA editing controls spore death induced by a fungal meiotic drive gene in homologous and heterologous expression systems. Genetics 221 (1). https://doi.org/10.1093/genetics/iyac029.

Madeira, F., Pearce, M., Tivey, A.R.N., Basutkar, P., Lee, J., Edbali, O., Madhusoodanan, N., Kolesnikov, A., Lopez, R., 2022. Search and sequence analysis tools services from EMBL-EBI in 2022. Nucleic Acids Res. 50 https://doi.org/10.1093/nar/gkac240.

McCluskey, K., Wiest, A., Plamann, M., 2010. The Fungal Genetics Stock Center: a repository for 50 years of fungal genetics research. J. Biosci. 35, 119–126. https://doi.org/10.1007/s12038-010-0014-6.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, 621–628. https://doi.org/10.1038/nmeth.1226.

Pyle, J., Patel, T., Merrill, B., Nsokoshi, C., McCall, M., Proctor, R.H., Brown, D.W., Hammond, T.M., 2016. A meiotic drive element in the maize pathogen *Fusarium verticillioides* is located within a 102 kb region of chromosome V. G3 6, 2543–2552. https://doi.org/10.1534/g3.116.029728.

Raju, N.B., 1994. Ascomycete Spore killers: chromosomal elements that distort genetic ratios among the products of meiosis. Mycologia 86, 461–473. https://doi.org/10.2307/3760737.

Raju, N.B., Metzenberg, R.L., Shiu, P.K.T., 2007. *Neurospora* Spore killers *Sk-2* and *Sk-3* suppress meiotic silencing by unpaired DNA. Genetics 176, 43–52. https://doi.org/10.1534/genetics.106.069161.

Rhoades, N.A., Harvey, A.M., Samarajeewa, D.A., Svedberg, J., Yusifov, A., Abusharekh, A., Manitchotpisit, P., Brown, D.W., Sharp, K.J., Rehard, D.G., Peters, J., Ostolaza-Maldonado, X., Stephenson, J., Shiu, P.K.T., Johannesson, H., Hammond, T.M., 2019. Identification of *rfk-1*, a meiotic driver undergoing RNA editing in *Neurospora*. Genetics 212, 93–110. https://doi.org/10.1534/genetics.119.302122.

Rhoades, N.A., Webber, E.K., Hammond, T.M., 2020. A nonhomologous end-joining mutant for *Neurospora sitophila* research. Fungal Genet. Rep. 64 https://doi.org/10.4148/1941-4765.2172.

Samarajeewa, D.A., Sauls, P.A., Sharp, K.J., Smith, Z.J., Xiao, H., Groskreutz, K.M., Malone, T.L., Boone, E.C., Edwards, K.A., Shiu, P.K.T., Larson, E.D., Hammond, T.M., 2014. Efficient detection of unpaired DNA requires a member of the rad54-like family of homologous recombination proteins. Genetics 198, 895–904. https://doi.org/10.1534/genetics.114.168187.

Samarajeewa, D.A., Manitchotpisit, P., Henderson, M., Xiao, H., Rehard, D.G., Edwards, K.A., Shiu, P.K.T., Hammond, T.M., 2017. An RNA recognition motif-containing protein functions in meiotic silencing by unpaired DNA. G3 7, 2871–2882. https://doi.org/10.1534/g3.117.041848.

Shiu, P.K., Raju, N.B., Zickler, D., Metzenberg, R.L., 2001. Meiotic silencing by unpaired DNA. Cell 107, 905–916. https://doi.org/10.1016/S0092-8674(01)00609-2.

Shiu, P.K.T., Zickler, D., Raju, N.B., Ruprich-Robert, G., Metzenberg, R.L., 2006. SAD-2 is required for meiotic silencing by unpaired DNA and perinuclear localization of SAD-1 RNA-directed RNA polymerase. Proc. Natl. Acad. Sci. 103, 2243–2248. https://doi.org/10.1073/pnas.0508896103.

Sidhu, G.S., 1984. Genetics of *Gibberella fujikuroi* V. Spore killer alleles in *G. fujikuroi*. J. Hered. 75, 237–238. https://doi.org/10.1093/oxfordjournals.jhered.a109923.

Son, H., Min, K., Lee, J., Raju, N.B., Lee, Y.-W., 2011. Meiotic silencing in the homothallic fungus *Gibberella zeae*. Fungal Biol. 115, 1290–1302. https://doi.org/10.1016/j.funbio.2011.09.006.

Svedberg, J., Vogan, A.A., Rhoades, N.A., Sarmarajeewa, D., Jacobson, D.J., Lascoux, M., Hammond, T.M., Johannesson, H., 2021. An introgressed gene causes meiotic drive in *Neurospora sitophila*. Proc. Natl. Acad. Sci. 118 https://doi.org/10.1073/pnas.2026605118.

Wang, Y., Smith, K.M., Taylor, J.W., Freitag, M., Stajich, J.E., 2015. Endogenous small RNA mediates meiotic silencing of a novel DNA transposon. G3 5, 1949–1960. https://doi.org/10.1534/g3.115.017921.

Xiao, H., Alexander, W.G., Hammond, T.M., Boone, E.C., Perdue, T.D., Pukkila, P.J., Shiu, P.K.T., 2010. QIP, a protein that converts duplex siRNA into single strands, is

required for meiotic silencing by unpaired DNA. Genetics 186, 119–126. https://doi.org/10.1534/genetics.110.118273.

Xu, J.R., Leslie, J.F., 1996. A genetic map of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*). Genetics 143, 175–189. https://doi.org/10.1093/genetics/143.1.175.

Zanders, S., Johannesson, H., 2021. Molecular mechanisms and evolutionary consequences of spore killers in ascomycetes. Microbiol. Mol. Biol. Rev. 85, e00016–21. https://doi.org/10.1128/MMBR.00016-21.