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Identifying the location of the rfk-2 spore killer gene on Chromosome III of Neurospora crassa

Princy A. Patel
Illinois State University, ppate26@ilstu.edu

IDENTIFYING THE LOCATION OF THE *RFK-2* SPORE KILLER GENE ON CHROMOSOME III OF *NEUROSPORA CRASSA*

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Meiotic drivers are selfish genetic elements that skew transmission in their favor. In the filamentous fungus *N. crassa,* one such meiotic driver is *Spore killer-3* (*Sk-3*). In a cross between *Sk-3* and a spore killer-sensitive mating partner (*Sk-S*), only half of the ascospores (sexual spores) survive. Nearly all of the survivors inherit the genes for spore killing. Previous studies have established that a gene called *rfk-2* (*required for spore killing*) is essential for the spore killing activity of *Sk-3*. The *rfk-2* gene has been mapped to Chromosome III, but its exact location is unknown. The goal of this study is to help identify the exact location of *rfk-2*. Towards this goal, I investigated a DNA interval called *v378*. Preliminary findings suggested that this interval may be important for spore killing. To determine if *v378* is required for spore killing, I constructed and used a transformation vector to replace *v378* with a hygromycin resistance gene (*hph+*) in an *N. crassa Sk-3* strain. Strains deleted of *v378* were then crossed with two spore killing-sensitive tester strains. The spore sacs containing ascospores from these crosses were imaged to analyze the effects of replacement of *v378* on *Sk-3*-based spore killing. My findings demonstrate that *v378* is required for spore killing. The potential implications of my findings with respect to our understanding of meiotic drive elements, and their potential applications, is discussed.

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PRINCY PATEL

School of Biological Sciences

ILLINOIS STATE UNIVERSITY

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COMMITTEE MEMBERS

Dr. Thomas Hammond (Chair)

Dr. Kyle Floyd

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CHAPTER I
INTRODUCTION

1.1 Mendelian genetics

Mendelian Genetics dictates that gene alleles are inherited as separate units, one from each parent. Mendel's proposed Law of Segregation, which states that during meiosis when gametes are formed, gene alleles are separated from their pairs so that each gamete contains only one allele of that gene. Mendel also proposed the Law of Independent Assortment which states that segregation of one pair of alleles is independent of the segregation of another allelic pair. Although these laws appear to be followed by most genes in eukaryotic organisms during sexual reproduction, there are some exceptions to them as well.

1.2 Meiotic drivers

Scientists have identified meiotic drivers in the genomes of multiple organisms. These meiotic drivers skew the transmission of genetic elements in their favor, and therefore, they are also called selfish genetic elements. Hence, they change the probability of inheriting each pair of alleles and overtime they distort the equal segregation ratio where the one allele is at a higher ratio compared to the other allele. These meiotic drivers benefit the offspring that contain it while the rest of the offspring that either do not possess the meiotic driver, or it is not functional, are often inviable. Eventually the population only contains the selfish gene, and the other allele becomes extinct from the population.

1.3 Spore killing in *Neurospora crassa*

In filamentous fungi, examples of meiotic drivers are spore killer genetic elements (consisting of one or more genes) discovered in the genus Neurospora (Turner and Perkins, 1979). Researchers have found a total of three spore killer genetic elements in Neurospora fungi: *Sk-1*^{k} in *N. sitophila,* and *Sk-2*^{k} and *Sk-3*^{k} in *N. intermedia* (Turner and Perkins, 1979). Although none of the spore killer strains were originally discovered in *N. crassa,* researchers have introduced *Sk-2*^{k} and *Sk-3*^{k} into *N. crassa* through introgression because *N. crassa* is better suited to genetics studies. *N. crassa* produces spore sacs called asci during sexual reproduction. A homozygous cross between either two same-type Spore killer strains (*Sk-K*), or two Spore killer sensitive strains (*Sk-S*), produces asci with 8 viable ascospores (Turner and Perkins, 1979). Whereas a heterozygous cross between an *Sk* strain and an *Sk-S* strain produces asci with 4 viable ascospores and four inviable ascospores. The ascospores that possess the *Sk* genetic element are the survivors. Hence, through spore killing, the *Sk* genetic elements transmit themselves to all the surviving offspring of a heterozygous cross (Zanders and Johannesson, 2021).

1.4 *Sk-2*-based spore killing

Previous studies suggest two specific genes control the meiotic drive activity of *Sk-2*. One of these genes is *rsk* (*resistant to spore killer*), which protects the fungus from the effects of spore killing but does not cause spore killing itself (Hammond et. al. 2012). Another gene is *rfk-1* (*required for spore killing-1*), which directly influences the spore killing activity of an *Sk-2* strain (Harvey et. al. 2014). Previous research showed that *rfk-1* is at the far-right edge of the *Sk-2* element on the right arm of Chromosome III (Rhoades et. al. 2019). The location of *rfk-1* is vital

to prevent its discovery and silencing by a genome defense process called meiotic silencing by unpaired DNA (MSUD) (Rhoades et. al. 2019).

The mechanism that *Sk-2* uses to kill spores is thought to involve a poison and an antidote, where both the poison and antidote genes are encoded within the complex *Sk-2* genetic element (Zanders and Johannesson, 2021). *Sk-2 rfk-1* encodes two protein variants – the 102 amino acid *RFK-1*^{A} and the 130 amino acid *RFK-1*^{B} (Rhoades and Hammond, 2021). Of the two variants, only *RFK-1*^{B} acts as a poison. *RFK-1*^{B} expression requires A-to-I editing of an early stop codon in *rfk-1* mRNA (Rhoades and Hammond, 2021). *Sk-2* ascospores are thought to neutralize *RFK-1*^{B} with a specific antidote: RSK^{Sk-2}.

1.5 *Sk-3*-based spore killing

The importance of *rfk-1* in *Sk-2*-based spore killing suggests that a related gene may be involved in *Sk-3*-based spore killing. However, when researchers deleted the closest matching sequence to *rfk-1* from an *Sk-3* strain, the deletion had no impact upon spore killing (Svedberg et. al. 2018). Researchers then isolated a mutation, named *rfk-2*^{UV}, that disrupts spore killing by *Sk-3*. It was discovered that this mutation is centromere-proximal to three genetic markers, called *hph*^{A}, *hph*^{B}, and *mus-52Δ*, on the right arm of Chromosome III (Velazquez et. al. 2022). This finding suggests that a gene called *rfk-2* exists, and that this gene controls spore killing by *Sk-3.* Unlike *rfk-1* in *Sk-2*, this gene is not found at the far-right edge end of the *Sk-3* genetic element (Velazquez et. al. 2022). It is important to know the exact location of *rfk-2*^{UV} on Chromosome III to better understand the mechanism used by *Sk-3* to kill ascospores, and to determine how *Sk-3's* poison and antidote genes escape inactivation by MSUD. To aid the discovery of *rfk-2*^{UV}, I deleted a

DNA interval called *v378.* This interval was chosen for deletion based on preliminary results indicating that a related DNA interval called *v350* is required for *Sk-3*-based spore killing (Rhoades and Hammond, unpublished results). My initial hypothesis, when beginning this project, was that deletion of the *v378* interval from an *Sk-3* strain will have no effect on spore killing.

CHAPTER II

METHODS

2.1 Overview

In this study, *N. crassa Sk-3* strain RDGR170.3 (**Table 1**) was transformed with a DNA replacement vector that was created using Double-Joint PCR (Yu et. al. 2004). This vector, called v378 (DNA vectors are named identically to the interval they are designed to delete) replaces interval *v378* on Chromosome III with hygromycin resistance gene (*hph+*). The resulting transformants are isolated and crossed to obtain homokaryotic offspring with a *v378Δ::hph+* genotype. The homokaryotic offspring were used in test crosses to determine the effect of *v378* deletion on spore killing. An overview of my methods is provided in **Figure 1**.

2.2 DNA Vector Construction

For this thesis project, transformation vector *v378* was created with Q5® High-Fidelity DNA polymerase (New England Biolabs) and DJ-PCR. In the first PCR reactions, left and right flanks surrounding interval *v378* in RDGR170.3 genomic DNA were amplified with primers V0378-A/V0378-B and V0378-C/V0378-D, respectively (**Table 2**). The flanks are predicted to be 825 bp (left flank) and 846 bp (right flank), and the predicted sequences of the flanks are shown in **Figures 2 and 3**. To amplify the flanks, two primer mixes were created by mixing 6.25 µl of the forward primer (100 pmol / µl) and reverse primer (100 pmol / µl) with 487.5 µl of sterile water and stored on ice after mixing them by vortexing and centrifuging to collect liquid at the bottom of the tubes. Next, 5 µl of left and right flank primer were transferred to two PCR tubes each followed by 1 µl of template DNA (RDGR170.3; approximately 10 ng / µl) was added to each

tube and the tubes were stored on ice. An enzyme master mix was made for five reactions (1 extra to account for pipetting errors). The enzyme master mix contained 67 µl of water, 25 µl of Q5 reaction buffer, 2.5 µl of 10 mM dNTP mix, and 0.5 µl of Q5 enzyme, and the contains were gently mixed by pipetting up and down five times. To all PCR tubes, 19 µl of enzyme master mix was added. The PCR reactions were cycled under the following parameters: denaturation at 98 °C for 30 seconds, annealing at 62.5 °C for 10 seconds, and an extension at 72 °C for 90 seconds. This cycle was repeated 25 times, ending with a single 10-minute extension at 72 °C and indefinite hold at 4 °C. A similar PCR protocol was used for all the PCR reactions to create the replacement vector. The center fragment which was hygromycin resistant marker (*hph*) was amplified from plasmid pTH1256.1 (GenBank MH550659.1) using HPH-CEN-F as the forward primer and HPH-CEN-R as the reverse primer (**Table 2**). The predicted length of the center fragment is 1412 bp, and the predicted sequence is shown in **Figure 4**.

Before fusion by DJ-PCR, PCR products of the center fragment and the flanks were gel-purified using IBI Scientific's Gel Extraction and PCR Cleanup Kit. The gel was made by mixing 1.8 g of agarose and 200 ml of 1x TAE buffer in a 1-liter flask, heating it in microwave for 1 minute, and repeating until all agarose had dissolved. Then 10 µl of 10 mg / ml ethidium bromide solution was added to the molten agarose and mixed by swirling. When the flask containing the molten agarose was still hot, but no longer painful to the touch, the solution was poured into a 13 × 15 cm gel tray with gel comb and allowed to solidify. The gel was transferred within the gel tray to a gel electrophoresis chamber, which was filled with 1× TAE buffer. The PCR samples were prepared for gel electrophoresis by adding 5 µl of 6× Gel Loading Buffer (30% glycerol, 60 mM Tris-HCl pH 7.5, 60 mM EDTA, 0.6% Orange G) to each reaction. The

PCR samples were mixed and loaded into the wells of the gel. A 10 µl aliquot (50 ng / µl) of Gene Ruler 1 kb Plus DNA ladder (Thermo Scientific) was also loaded into a well to serve as a size marker. The gel was run at 120V for 90 minutes. The PCR products were visualized with a UV transilluminator, excised from the gel with a razor blade, and transferred to microcentrifuge tubes (MCTs). DNA was then extracted from the gel slices with IBI Scientific's Gel Purification Kit. Gel electrophoresis was performed on 5 µl aliquots of the gel purified left flank and right flank. The lengths of purified molecules were consistent with the predicted lengths of the left flank (825 bp, **Figure 2** and **Figure 5**), right flank (846 bp, **Figure 3** and **Figure 5**), and center fragment (1412 bp, **Figure 4** and data not shown).

The purified center fragment and flanks were fused by DJ-PCR and the fusion product was used as template for amplification with primers V0378-E and V0378-F (**Table 2**). The amplified product (i.e., Vector v378) was column purified using IBI Scientific's PCR Cleanup Kit. Gel electrophoresis was performed on a 5 µl aliquot of purified Vector v378 to check the length and integrity of the vector. The predicted length of vector v378 is 2857 bp, which is consistent with the length of the purified product detected by gel electrophoresis (**Figure 6**). The predicted sequence of Vector v378 is shown in **Figure 7.** The predicted sequence of interval *v378* is shown in **Figure 8.** The relationship between interval *v378* and interval *v350* is diagrammed in **Figure 9**.

2.3 *Neurospora crassa* Transformation

Neurospora crassa strain RDGR170.3 was grown for about 4 weeks on 50 ml Vogel's minimal agar (VMA: VMM + 2% agar; Vogel 1956), and the conidia from this culture were used in the

transformation process. Before transformation, bottom agar plates were created by mixing 172 ml milliQ water, 8 ml of 25x Vogel salts, and 3 g of agar in a 500 ml glass bottle which was autoclaved on liquid cycle. Once the media cooled, 150 µl of hygromycin B (400 mg / ml; Gold Biotechnology) and 20 ml of 10× FIGS (20% sorbose, 0.5% D-Fructose, 0.2% Inositol, 0.5% D-Glucose) was added to the media. Next, using sterile technique, 20 ml of the media was transferred to 100 mm petri dishes. These petri dishes were stored at room temperature for 24 hours, and later for up to one week at 4 °C. Top agar media was created by mixing and autoclaving 105 mL of milliQ water, 27.3 grams of sorbitol, 6 mL of 25× Vogel salts, and 2 grams of agar. After cooling the medium to a safe handling temperature, 15 mL of 10× FIGS was added to the medium, and then 40 mL aliquots of the medium was transferred to 50 ml conical tubes, which were then stored in an oven set at 50 °C.

N. crassa strain RDGR170.3 was transformed with Vector v378 by electroporation (Margolin et al., 1997; Rhoades et al., 2020). Conidia from the 4-week-old culture of RDGR170.3 were collected into 30 ml of ice-cold 1 M sorbitol and filtered with 100 µm cell strainer. The conidial suspension was centrifuged in a swinging bucket rotor at 2000 × g for 10 minutes to form a conidial pellet. After removing the supernatant, the conidial pellet was resuspended with 20 ml of ice-cold 1 M sorbitol. The suspension was centrifuged again at the same settings, and after removing supernatant, the pellet was resuspended to 1 billion conidia per ml in ice-cold 1 M sorbitol. To a 100 µl aliquot of the conidial suspension, approximately 300 ng (10 µl) of transformation vector was added, and the mixture was transferred to 0.1 cm gap-width electroporation cuvette (BTX model 613). The mixture was then electroporated at 1500 volts with an Eppendorf Eporator. Immediately after electroporation, 750 µl of ice-cold 1 M sorbitol

was added to the electroporated conidia. The resulting suspension of electroporated conidia was then transferred to a 50 ml conical tube containing 5 ml of rescue medium (VMM + 1 M sorbitol) and stored on ice. This is the “DNA” rescue culture. A negative control transformation was performed by following the same process, except that 10 µl of Elution Buffer (IBI Scientific) was added instead of the transformation vector. This created the “CON” rescue culture. The “DNA” conical tubes with electroporated conidia in rescue medium were placed in a tray in a shaker set at 32 °C and 40 RPM for a 3.5-hour incubation.

Next, five bottom agar plates were labelled “DNA”, while another five were labelled “CON”. Both sets of plates were placed in a biosafety hood that had been cleaned with 70% ethanol. One of the three conical vials containing 40 ml of molten top agar was designated as the temperature check vial and its cap was replaced with a thermometer cap to estimate the temperature of top agar. When the temperature of the molten top agar was just below 50 °C, 100 µl of the “DNA” rescue culture was added to a sterile 40 ml volume of top agar in the biosafety hood and mixed by inversion 5x. Then 10 ml of this top agar was added to a petri dish with bottom agar labelled “DNA”. To the same top agar vial, a 500 µl aliquot of the “DNA” rescue medium was added and then poured on a different bottom agar petri dish labeled "DNA". The same process was repeated with 1000 µl of the “DNA” rescue culture, and then the remaining volume of the rescue culture. A similar process was used to plate the "CON" rescue culture.

The bottom agar plates with "DNA" and "CON" electroporated conidia were incubated right side up at room temperature overnight, and after 24 hours, they were inverted and placed at 32 °C. The transformants were allowed to grow for 3–5 days at 32 °C after which sterile syringe

needles were used to transfer single hygromycin resistant colonies to 16 × 125 mm glass culture tubes containing VMA and 200 µg / ml of hygromycin. These “slant cultures” were allowed to grow for 1–2 days at 32 °C before transferring to room temperature.

2.4 Isolation of hygromycin resistant offspring from transformants

Conidia from 12 transformants, named *TPP1.1, TPP1.2, TPP1.3,* etc., were used to make conidial suspensions in sterile water. The conidial suspensions were used to initiate unidirectional crosses with strain RTH1005.2 on synthetic crossing agar (SCA, 1.5% sucrose, pH6.5; Westergaard and Mitchell, 1947) at room temperature, essentially as described by Samarajeewa et al. (2014). These crosses were incubated for 24 days at room temperature. Afterwards, ascospores were collected from the lids of two crossing plates by gently pipetting 50 µl of sterile water up and down onto the dry ascospores. These were mixed with 500 µl of sterile water to create ascospore suspensions, which were then stored in MCTs at 4°C in dark. The ascospore suspensions were named *RPAP1* and *RPAP3*.

The ascospore suspension *RPAP3* was used to create a 500 µl working suspension of ascospores with 1-5 ascospores per 1 µl, and the remaining stock suspension was stored at 4 °C. The ascospore concentration of the stock suspension was estimated by examining 10 µl of the stock suspension with a dissecting microscope. The working suspension was placed in a heat block at 60 °C for 30 minutes before cooling to room temperature on a bench top. Using sterile technique under a biosafety hood, 50 µl, 100 µl, and 200 µl aliquots of the heat- shocked ascospore suspension were spread across VMA plus + hygromycin (200 µg / ml) plates. These plates were placed “right side up” at room temperature for 24 hours. After this, sterile syringes

and a dissecting microscope were used to transfer hygromycin-resistant germlings to VMA plus + hygromycin (200 µg / ml) in 16 × 125 mm culture tubes. A total of 12 germlings, named RPAP3.1, RPAP3.2, RPAP3.3, etc., were transferred to slants. The procedure was repeated a few months later, resulting in isolation of germlings named RPAP3.101, RPAP3.102, RPAP3.103, etc. In both cases, the germlings in the slants were incubated at 32 °C for 2 days before transferring to room temperature.

2.5 Test crosses for the presence or absence of spore killing

Test crosses were performed by first growing strains RTH1623.1 and RTH1623.2 on SCA in 60 mm plates for 10 days at room temperature. For the first set of test crosses, conidial suspensions in 500 µl sterile water were made for RPAP3.1, RPAP3.2, RPAP3.5, RPAP3.6, RPAP3.9, and RPAP3.12. For the second set of crosses, conidial suspensions were made for RPAP3.101, RPAP3.107, RPAP3.108, RPAP3.109, RPAP3.111, and RPAP3.112. Conidial suspensions were also made for control strains RDGR170.3 (*Sk-3*) and RZS27.10 (*Sk-S*). The conidial suspensions were vortexed and stored on ice. Each conidial suspension was then used to fertilize both RTH1623.1 and RTH1623.2 by adding 100 µl of the suspension in 10–20 drops to the surface of the plates. **Figure 12** shows the second set of crossing plates after fruiting bodies had developed. The asci (ascospore sacs) from the first and second sets of crosses were dissected from fruiting bodies in 25 % glycerol at 14-days post fertilization. The asci were then imaged with a Leica DMBRE microscope and Zeiss imaging system.

2.6 DNA Isolation

This protocol was used twice to isolate DNA for genotyping. To conserve resources, only a few RPAP3 offspring were genotyped. The first group of RPAP3 offspring to be genotyped included RPAP3.1, RPAP3.2, and RPAP3.5. The second group of RPAP3 offspring to be genotyped included RPAP3.107, RPAP3.108, RPAP3.109, and RPAP3.111.

Mycelia for each strain was generated by transferring a small amount of conidia to 2–4 ml of VMM in 15 ml "snap cap" tubes using sterile technique. These tubes were then placed in shaking incubator for 24–48 hours at 150 rpm, 32 °C. Mycelia was harvested from the cultures with sterile wooden applicators and transferred to a sheet of filter paper on a stack of 5–10 clean paper towels. The mycelia were dried with paper towels and clean forceps were used to transfer the mycelia to MCTs. The mycelial tissue was then lyophilized before storage in a dry cabinet. DNA was then isolated from the lyophilized mycelia with IBI Scientific's Mini Genomic DNA Kit for Plants/Fungi.

2.7 Genotype screening by PCR

This protocol was used to confirm the genotype of select hygromycin-resistant offspring (RPAP3.1, RPAP3.2, RPAP3.5, RPAP3.107, RPAP3.108, RPAP3.109, and RPAP3.111), all of which were predicted to have the *v378Δ::hph+* genotype. First, forward primer V0378-E, reverse primer V0378F, DNA templates (@ 10 ng / µl), and Q5 DNA polymerase buffer were thawed at 65 °C in a heat block, while a 10 mM dNTP solution was thawed between gloved hands. These reagents were vortexed and quickly spun in centrifuge and stored on ice along with Q5 DNA polymerase. Next, a primer mix was created by mixing 6.25 µl of each primer (100 pmol / µl)

with 487.5 µl of sterile water in a MCT tube, vortexing the tube, collecting the contents of the tube at its base by centrifugation, and storing the tube on ice. A PCR tube rack was placed on ice with labelled PCR tubes. A 5 µl aliquot of primer mix plus 1 µl of template DNA was added to each tube. Next, an enzyme mix was created for 5 reactions by adding 67 µl of sterile water, 25 µl Q5 reaction buffer, 2.5 µl 10 mM dNTP solution, and 0.5 µl Q5 enzyme to an MCT tube. The enzyme master mix was mixed by pipetting. To the PCR tubes with primer mix and DNA template, 19 µl of the enzyme master mix was added to each tube and mixed by gentle pipetting up and down. The PCR reactions were transferred to the PCR machine and cycled according to manufacturer's recommendations for Q5 DNA polymerase. The PCR products were then examined by gel electrophoresis with ethidium bromide and imaged with a gel documentation system. **Figures 7** and **10** show the predicted sequence of PCR products from strains with a *v378Δ::hph+* genotype and *v378+* genotype, respectively. **Figures 11** and **12** show the images of the gels generated during genotyping of RPAP3.1, RPAP3.2, RPAP3.5, RPAP3.107, RPAP3.108, RPAP3.109, and RPAP3.111. These data indicate that all RPAP3 strains are homokaryotic with the genotype *v378Δ::hph+*.

CHAPTER III

RESULTS

Preliminary results have shown that deletion of interval *v350* eliminates *Sk-3*-based spore killing. In **Figure 9**, interval *v378* is mapped to show its location on the Chromosome III with respect to interval *v350*. I initially hypothesized that deletion of *v378* by replacement with a hygromycin resistance gene would not disrupt *Sk-3* based spore killing.

The images of the asci dissected from the perithecia of both sets of test crosses and control strains RDGR170.3 (*Sk-3*) and RZS27.10 (*Sk-S*) with female strains RTH1623.1 and RTH1623.2 are shown in **Figures 14-21.** Spore killing was not detected in any of the test crosses between an RPAP3 parent and an RTH1623 parent. For example, in all the rosette images, asci with eight black ascospores are visible, indicating that *Sk-3* based spore killing did not occur. Hence, the hypothesis that replacement of vector *v378* with *hph* marker would not affect the *Sk-3*-based spore killing was not supported.

CHAPTER IV

DISCUSSION

Based on **Figures 14-21,** the replacement of intreval *v378* with hygromycin resistance marker in *N. crassa* strain RDGR170.3 disrupts *Sk-3* based spore killing. The test cross rosette images are similar to those from the cross between male control RZS27.10 (*Sk-S*) with female strain RTH1623.1, where spore killing does not occur. In all the test cross images, eight ascospores are clearly visible. This shows that interval *v378* is critical for successful *Sk-3*-based spore killing to occur. Therefore, my hypothesis was not supported.

These findings increase our knowledge about the location of the *rfk-2* gene on Chromosome III as there is a possibility that interval *v378* is a part of the *rfk-2* gene. It could also be an important promotor or enhancer sequence that influences the transcription of *rfk-2* or a different gene required for spore killing. These results further our understanding about the location of the *rfk-2* gene and bring us a step closer to understanding the molecular mechanism by which a meiotic drive element functions.

One of the practical applications of knowing the mechanism of action of a spore killer genetic element is to develop them into synthetic driver systems. In the future, it may be possible to adapt the meiotic driver properties of the Neurospora Spore killers to help introduce certain traits into various populations of fungi. Gene drivers could also be used to eliminate certain populations of fungi that are harmful to human health or agriculture.

A recent study by Gardiner *et. al.* (2020) used spore killer elements (Spok1) to eliminate virulence traits from fungal colony of *Fusarium graminearum* by distorting inheritance pattern at two loci that controls virulence in *F. graminearum*. Gene drivers like the Neurospora spore killers and Spok1 could be used in agriculture to help eliminate fungal infections in crops. Instead of using harmful chemical treatment to combat such infections, spore killer genes could be introduced into a population of harmful fungi in a way that would reduce the ability of the population to be infectious. Thus, better understanding of the mechanism of spore killer elements could be beneficial in controlling pathogenic population of fungus depending on the reproductive properties of the species.

Furthermore, meiotic drivers are important to understand evolutionary biology as spore killer elements allow us to study natural selection at a multiple level in the biological hierarchy (Zanders and Johannesson, 2021). As spore killing is a costly process for the organism's fitness, it gives evidence for intragenomic conflict that helps drive the evolution of species (Burt and Trivers, 2006).

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Table 1 Strains used in this study

Strain name	Genotype
RTH1005.2	*rid; fl; Sk-S^{ChrIII} a+*
RZS27.10	*rid; Sk-S^{ChrIII}; mus-51^{RIP70} a+*
RTH1623.1	*rid; fl; Sk-S^{ChrIII}; sad-2Δ::hph+ A+*
RTH1623.2	*rid; fl; Sk-S^{ChrIII}; sad-2Δ::hph+ a+*
RDGR170.3	*rid; Sk-3^{ChrIII}; mus-51Δ::bar+ A+*
TPP1.1	*v378*-based hygromycin resistant transformant of RDGR170.3
TPP1.2	*v378*-based hygromycin resistant transformant of RDGR170.3
TPP1.3	*v378*-based hygromycin resistant transformant of RDGR170.3
RPAP3.1	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.2	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.5	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.6	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.9	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.12	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.101	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.107	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.108	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.109	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.111	hygromycin resistant offspring of TPP1.3 × RTH1005.2
RPAP3.112	hygromycin resistant offspring of TPP1.3 × RTH1005.2

Predicted genotype based of lineage of strain and hygromycin resistance (not genotyped by PCR). *Sk-3*^{ChrIII} indicates the strain carries the *Sk-3* genetic element, while *Sk-S*^{ChrIII} indicates the strain does not carry the *Sk-3* genetic element. The *rid* allele disrupts Repeat Induced Point Mutation (Freitag et al. 2002). The *fl* allele disrupts macroconidiation (Perking et al. 2001). The *mus-51*^{RIP70} and *mus-51Δ::bar+* allele disrupts Non-Homologous End Joining (Ninomiya et al. 2004; Smith et al. 2016). The *sad-2Δ::hph+* allele suppresses Meiotic Silencing by Unpaired DNA (Shiu et al. 2006).

Table 2 Primers used in this study

Name (Alias)	Sequence (5′ > 3′)
HPH-CEN-F	AACTGATATTGAAGGAGCATTTTTTGG
HPH-CEN-R	AACTGGTTCCCGGTCGGCAT
V0378-A	AACAGGCAACTGCGTTAGGGCCA
V0378-B	AAAAAATGCTCCTTCAATATCAGTTCTTACAGCACCCACTAGGCCCGC
V0378-C	GAGTAGATGCCGACCGGGAACCAGTTAGCAACAGCCCCGAGCCCATCC
V0378-D	GGCTTGGTAAGAACGCGCCGAGG
V0378-E	GCAATGGGATGTCCAGACGAGGGC
V0378-F	TGCATATTCGGTAGGTGGCGGGGA

Underlined nucleotides were used for the fusion of the left and right flanks to the center fragment (*hph* marker).

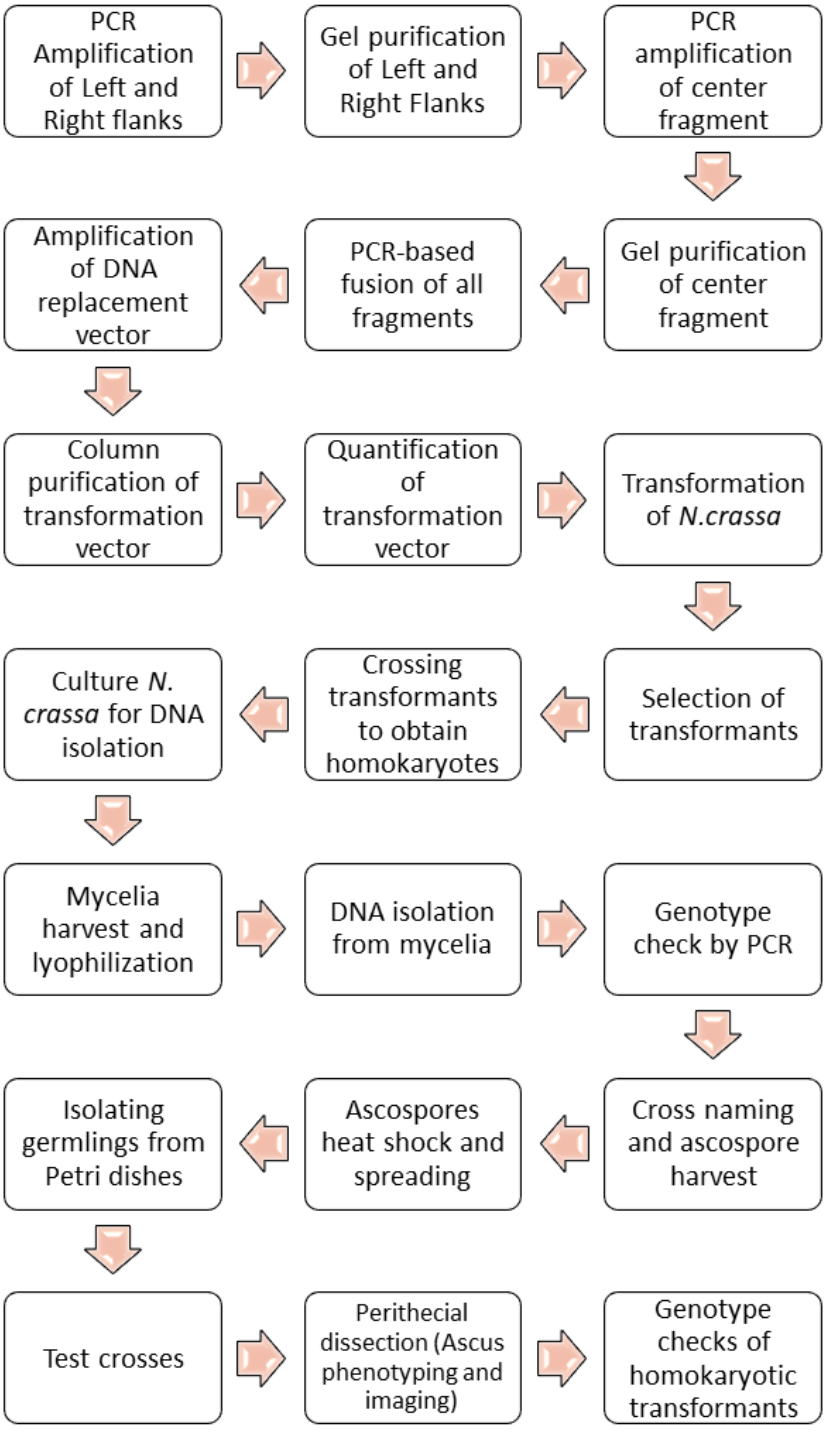

Figure 1 Methods Overview

>v378 left flank

AACAGGCAACTGCGTTAGGGCCATGAAACATGTGCAGCTTCGTTCCAGTCCCCAAGCCCGAAT
GATAGACGGATGAGTAAGGAGTGTCCGGCCTGCACTGGAAAAGAGAATTTGGATGGCTCCCAT
GCGCACTTGCACATCATGATCATGACACCATATTAACAACAGTAGGCAATGGGATGTCCAGAC
GAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGCCCGTCTCGCTCTTCCCGG
GCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGCCC
CGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAG
GCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAG
ATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGGAAA
GAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAA
AGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAACATCG
TTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGCAT
TCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTG
TATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGC
TGTAAG

Figure 2 The v378 left flank. The 825 bp sequence of the v378 left flank is shown in the 5′ to 3′ direction. This sequence was PCR-amplified with primers V0378-A and V0378-B from RDGR170.3 genomic DNA.

>v378 right flank

AGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCATGGAGCAGCA
ATGGCAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGG
CCAAATGTCCATGTCCCCTCGAGACTACGCCAGCAACAGCCAGGTCCAGGCGCAGCAGCAGCC
GCCGGCCGGATACAAGTATGATTCATACCAGGCCGGTCTGAACCCGAGCGCGCAACCACAGTC
CTCCTCCATTTCCCCAATGACGTCGTCCCAGTCGCGCGACGCCAACGGCGACGTCGCTATGCA
GGATGCCCATGATCCACACTCATCCCAGAACGCTACCATCAAGTACCCCCTGAGGCCGCATCA
CTCGCTCTCCGGTGGTCGCCCGGCCAGCCGTCAAGCATCCCAACAGCTTCCCCCCATCAACCC
ATACGCACCCGCTCAACACGGCCACAGCTATCCAAACTCGGCAATCAGCTCTACCATGGACGG
CTCTTATATGGACCCCAAGTCGCCGCCAAGGCGCATGAACTCTCAGTCGCAACAGATGCCCAT
GCCCGAGAGGACGCCAGTTCCCGAATTTAGGAAAATGCGAGGACCCCAGGACCTTCGACCAAA
GATTAACAAGCAGCCGGCTCATCGACGAGCTAACCCGGAAGGCGGCTTTATCAGTGTATGTAC
ATGTTCGCGAGCCATAGCTTTGGGCAAGCCTGCTGACGAACCACGATAGCCCCTCCAAGCGCT
AACAGTTCACCTCCCCGCCACCTACCGAATATGCAACCCCGGCTTCAAGTACGAGTCGTCTAG
GAATCCTCGGCGCGTTCTTACCAAGCC

Figure 3 The v378 right flank. The 846 bp sequence of the v378 right flank is shown. This sequence was PCR-amplified with primers V0378-C and V0378-D from RDGR170.3 genomic DNA.

>v378 center fragment

AACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGC
CTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCA
TTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTC
TTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTG
CGTTTGTCAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCT
TTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCATCCAAATGAAAAAGCCTGAACT
CACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCA
GCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCT
GCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATC
GGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCCTGACCTATTG
CATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGT
TCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGG
GTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGC
GATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGT
CGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGT
GCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGA
CTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCC
GTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGG
ATCGCCGCGCCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGT
TGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGG
AGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTG
TGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATA
GAGTAGATGCCGACCGGGAACCAGTT

Figure 4 The v378 center fragment. The 1412 bp sequence of the v378 center fragment is shown. This sequence was PCR-amplified with primers HPH-CEN-F and HPH-CEN-R from plasmid pTH1119.2. The sequence contains *Aspergillus nidulans trpC* promoter sequences upstream of the 1026 bp *hph* coding region. The yellow shaded regions indicate positions of the *hph* start codon and stop codon.

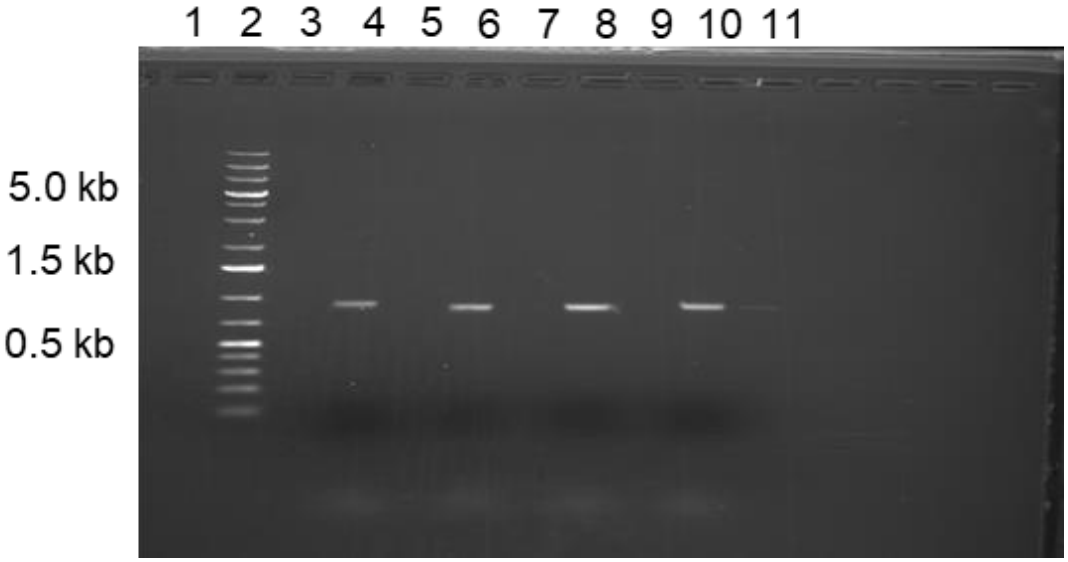

Figure 5 v378 construction: left and right flanks. PCR products for v378 left and right flanks. The left flank for v378 was amplified from RDGR170.3 genomic DNA with primers V378-A and V378-B. The right flank for v378 was amplified from RDGR170.3 genomic DNA with primers V378-C and V378-D. Products were gel purified as described in the methods section. Purified DNA was examined by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 2) 0.5 µg GeneRule1 kb Plus (ThemoFisher); Lanes 3-6) not applicable, Lane 8) 30 µl purified product (v378 left flank trial 1); Lane 10) 30 µl purified product (v378 right flank trial 1). The expected product lengths for the v378 left and right flanks are 825 bp and 846 bp, respectively.

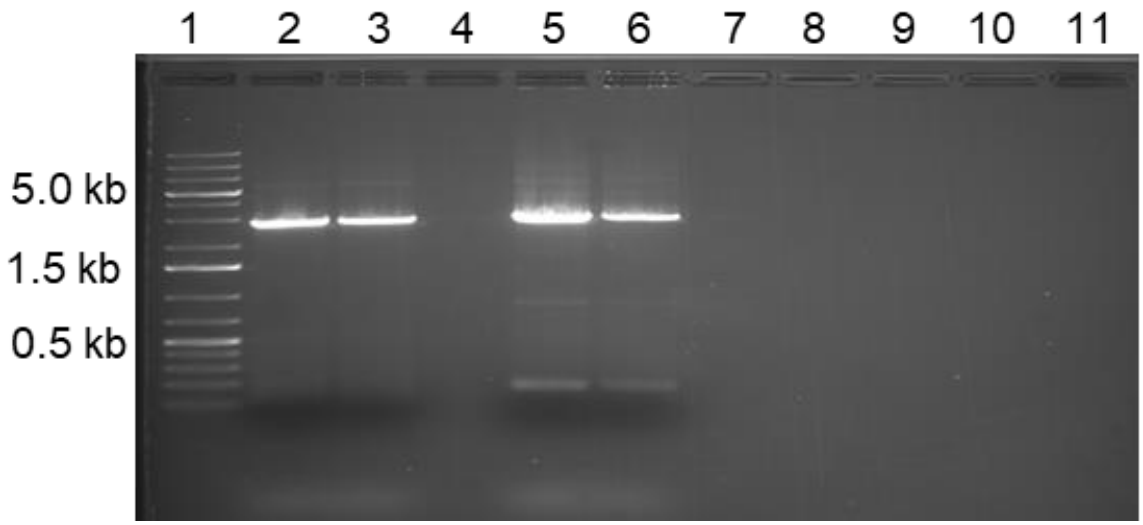

Figure 6 v378 construction: fusion and nested amplification. The v378 left flank, v378 center fragment, and v378 right flank were fused by DJ-PCR. The fusion product was amplified with primers V378-E and V378-F. The amplified product was column purified as described in the methods section. The amplified and purified product was examined by gel electrophoresis with ethidium bromide staining. Purified DNA was examined by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1) 0.5 µg GeneRule1 kb Plus (ThemoFisher); Lanes 2-4) not applicable; Lane 5) 30 µl of amplified and purified v378; Lane 6) 30 µl of amplified and unpurified v378. The expected length of v378 is 2857 bp.

>*v378Δ::hph*+ PCR product, predicted sequence, primers V378-E and V378-F

GCAATGGGATGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGCCCGTCTCG
CTCTTCCCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTGGCCATCTTCTGC
CCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTCGGGTCCGAGAAGGCCGCA
GCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCGCACTACCAAGATACATTAGAGGTA
CTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGGAAGGGAAAGAGGGGACGGAGAAAACGACTG
AGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCACCCAGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAA
ACTCTCGTTAGATTTTCTTTCTCTCCAACATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCC
TTCTTTCATCCCAACATGTTAGCATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTG
CGGCGTTTGGCTTGTTGTATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCT
AGTGGGTGCTGTAAGAACTGATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAA
TGAATGCCTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCAT
TTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTCTTATTAGCA
GACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTGCGTTTGTCAAGCAAGGT
AAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCTTTATTTCAGATTCAATCTGACTTAC
CTATTCTACCCAAGCATCCAAATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGA
AAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATG
TAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTAT
CGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCCTGAC
CTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTC
TCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCA
TTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGT
GTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGC
TTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCATGCGGATTTCGGCTCCAACAATGTCCTGACG
GACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGC
CAACATCCTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATC
CGGAGCTTGCAGGATCGCCGCGCCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGC
TTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGC
CGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTAC
TCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATAGAGTAGATGCCGACCGGGA
ACCAGTACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCATGGAGCAG
CAATGGCAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAACGGTTCCGGCCAAAT
GTCCATGTCCCCTCGAGACTACGCCAGCAACAGCCAGGTCCAGGCGCAGCAGCAGCCGCCGGCCGGATACA
AGTATGATTCATACCAGGCCGGTCTGAACCCGAGCGCGCAACCACAGTCCTCCTCCATTTCCCCAATGACG
TCGTCCCAGTCGCGCGACGCCAACGGCGACGTCGCTATGCAGGATGCCCATGATCCACACTCATCCCAGAA
CGCTACCATCAAGTACCCCCTGAGGCCGCATCACTCGCTCTCCGGTGGTCGCCCGGCCAGCCGTCAAGCAT
CCCAACAGCTTCCCCCCATCAACCCATACGCACCCGCTCAACACGGCCACAGCTATCCAAACTCGGCAATC
AGCTCTACCATGGACGGCTCTTATATGGACCCCAAGTCGCCGCCAAGGCGCATGAACTCTCAGTCGCAACA
GATGCCCATGCCCGAGAGGACGCCAGTTCCCGAATTTAGGAAAATGCGAGGACCCCAGGACCTTCGACCAA
AGATTAACAAGCAGCCGGCTCATCGACGAGCTAACCCGGAAGGCGGCTTTATCAGTGTATGTACATGTTCG
CGAGCCATAGCTTTGGGCAAGCCTGCTGACGAACCACGATAGCCCCTCCAAGCGCTAACAGTTCACCTCCC
CGCCACCTACCGAATATGCA

Figure 7 PCR product sequence: *Sk-3 v378Δ::hph*+ genotype. The predicted sequence of DNA amplified with primers V378-E and V378-F from a template consisting of *Sk-3 v378Δ::hph*+ genomic DNA is shown in FASTA format. The binding sites of V378-E and V378-F are

indicated with bold font. The start and stop codons of the *hph* coding region are shown with white font on black background. The length of the sequence is 2857 bp.

>v378 DNA interval

TCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCT
CGACGGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCG
GTGGCCCCACCTCCCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCA
GGGGGCTGCTGGGGCCCAGCCCCCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCA
GTGAGTCAATCCATGCTAGGTATTCTCAGGTTATGAAATCTACGATCGCTGACAGTT
GCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGTCCAGGCGGCCACAGAGGTCGT
TCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAGCGATTATCAGCGA
AACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCTTCAAACCCTC
CCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAATTCAT
TGCCAAACCAGACCC

Figure 8 The v378 DNA interval. The DNA sequence of the 528 bp v378 DNA interval is shown. DNA replacement vector v378 was designed to delete this interval by replacing it with *hph*+.

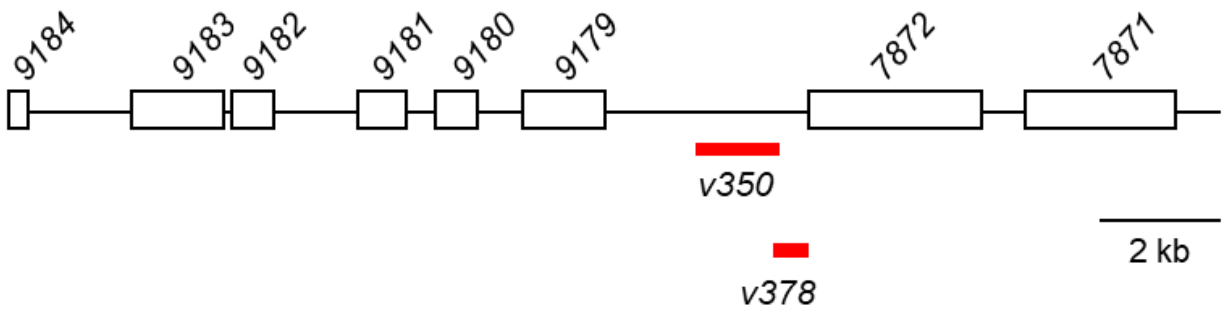

Figure 9 *Sk-3* Chromosome III positions 320,000–340,000. A diagram of Chromosome III, positions 320,000 to 340,000, in *Sk-3* strain FGSC 3194 is shown. White rectangles mark the locations of protein coding genes. Gene numbers were identified with blastx searches of an *N. crassa* protein database. The location of intervals v350 and v378 are indicated with red bars.

>v378+ PCR product, predicted sequence, primers V378-E and V378-F

GCAATGGGATGTCCAGACGAGGGCAACTTGGAACATCGATACGACGACTGCTCCGGCTCACGCCC
GTCTCGCTCTTCCCGGGCCTTTTGTCAGGCAGATGCCCCAGTTCTTCCTGCCTGCTGTCAAGGTG
GCCATCTTCTGCCCCGCCATCGCCACGGCAGTAAAAAAAGTCCAGGACAACTGAATTGACCCGTC
GGGTCCGAGAAGGCCGCAGCGTGAGCGCTCACGTTTGAATTGAAGAAGGCGCAGGCTAGTTTCCG
CACTACCAAGATACATTAGAGGTACTACGTACCACTCCGTTGAAGGAGGTTATGACGGGGAAGGG
AAGGGAAAGAGGGGACGGAGAAAACGACTGAGCTACAGCACGCTTTTCCAGCTCAGTTGGGGCAC
CCAGAAAGCTATAAGATTCCTCTTCCCCCGGCCCAAACTCTCGTTAGATTTTCTTTCTCTCCAAC
ATCGTTAAGGACTTTGTTTCTTTTTTTTTGGAATATCATCCCTTCTTTCATCCCAACATGTTAGC
ATTCATCCTAATGCTCTGGGCCGCAGAGCCCTACAAGGTGGCCATGTGCGGCGTTTGGCTTGTTG
TATTCGGAATACACATGCTGGCGCTGTTCGCGTGCCGCCAGCCATGGCGGGCCTAGTGGGTGCTG
TAAGTCTACTTTATATTCTCATTCGTTTTGGTTTCTTTCTTTCTTTCTCTTCTCATTTCCTCGAC
GGCTTACCTTGTCCTCTCGGTCCTCTCTGTTTTTCGCTAACCAGAAACAGGCGGTGGCCCCACCT
CCCCCCCGACGGGGATGACCTTGCCCCTGTCCCGTACCGGCGGCAGGGGGCTGCTGGGGCCCAGC
CCCCACCGCCCTGGTGGCGGTTGTGGGTCCGCGACCAGTGAGTCAATCCATGCTAGGTATTCTCA
GGTTATGAAATCTACGATCGCTGACAGTTGCACACCAGTGCGTTTCCGGCAGTGGCGGCCTCCGT
CCAGGCGGCCACAGAGGTCGTTCAATTAATCACCACTCTCTAAACGAATTCCCACCATTCTCCAG
CGATTATCAGCGAAACACCACCCCACCCAGGTTAGTGCGCGTCCATCGTCTTCGAAAGCTTCAAA
CCCTCCCTCTCCTTCCCCCCTCTCGCGCTGACGACACCACCGGCCACCGCAACAGAATTCATTGC
CAAACCAGACCCAGCAACAGCCCCGAGCCCATCCCGCGAGCTGGCCTTCGCCCTGCCAGCAGCCA
TGGAGCAGCAATGGCAACCGTACTCTGACTCTGCCGCCAGCGGCTCGTCCAGGAGATACAACAAC
GGTTCCGGCCAAATGTCCATGTCCCCTCGAGACTACGCCAGCAACAGCCAGGTCCAGGCGCAGCA
GCAGCCGCCGGCCGGATACAAGTATGATTCATACCAGGCCGGTCTGAACCCGAGCGCGCAACCAC
AGTCCTCCTCCATTTCCCCAATGACGTCGTCCCAGTCGCGCGACGCCAACGGCGACGTCGCTATG
CAGGATGCCCATGATCCACACTCATCCCAGAACGCTACCATCAAGTACCCCCTGAGGCCGCATCA
CTCGCTCTCCGGTGGTCGCCCGGCCAGCCGTCAAGCATCCCAACAGCTTCCCCCCATCAACCCAT
ACGCACCCGCTCAACACGGCCACAGCTATCCAAACTCGGCAATCAGCTCTACCATGGACGGCTCT
TATATGGACCCCAAGTCGCCGCCAAGGCGCATGAACTCTCAGTCGCAACAGATGCCCATGCCCGA
GAGGACGCCAGTTCCCGAATTTAGGAAAATGCGAGGACCCCAGGACCTTCGACCAAAGATTAACA
AGCAGCCGGCTCATCGACGAGCTAACCCGGAAGGCGGCTTTATCAGTGTATGTACATGTTCGCGA
GCCATAGCTTTGGGCAAGCCTGCTGACGAACCACGATAGCCCCTCCAAGCGCTAACAGTTCACCT
CCCCGCCACCTACCGAATATGCA

Figure 10 PCR product sequence: *Sk-3 v378+* genotype. The predicted sequence of DNA amplified with primers V378-E and V378-F from a template consisting of *Sk-3 v378+* genomic DNA is shown in FASTA format. The binding sites of V378-E and V378-F are indicated with bold font. The V378 interval is indicated with red font. The length of the sequence is 1973 bp.

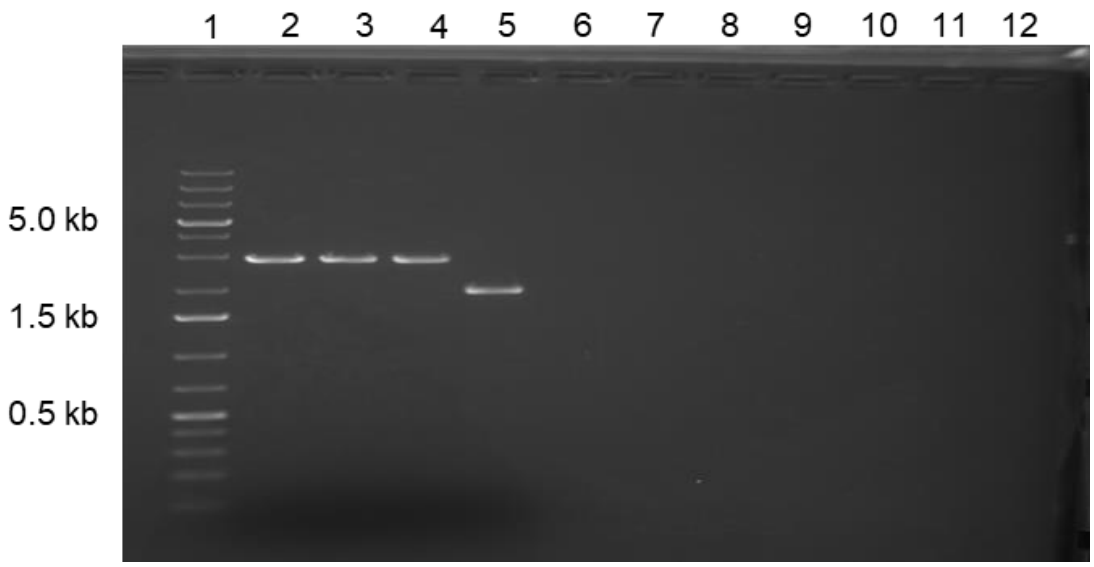

Figure 11 Transformant screening. Genomic DNA samples from candidate transformants RPAP3.5, RPAP3.2, and RPAP3.1, and transformation host RDGR170.3 were used as templates in PCR reactions with primers V0378-E and V0378-F. PCR products were examine by gel electrophoresis with ethidium bromide staining. An image of the gel over transilluminated UV light is shown. Lane 1) 0.5 µg GeneRule1 kb Plus (ThemoFisher); Lanes 2-5) PCR products from templates RPAP3.5, RPAP3.2, RPAP3.1, and RDGR170.3, respectively. The expected PCR product lengths are 1973 bp for *v378+* and 2857 bp for *v378Δ::hph+*.

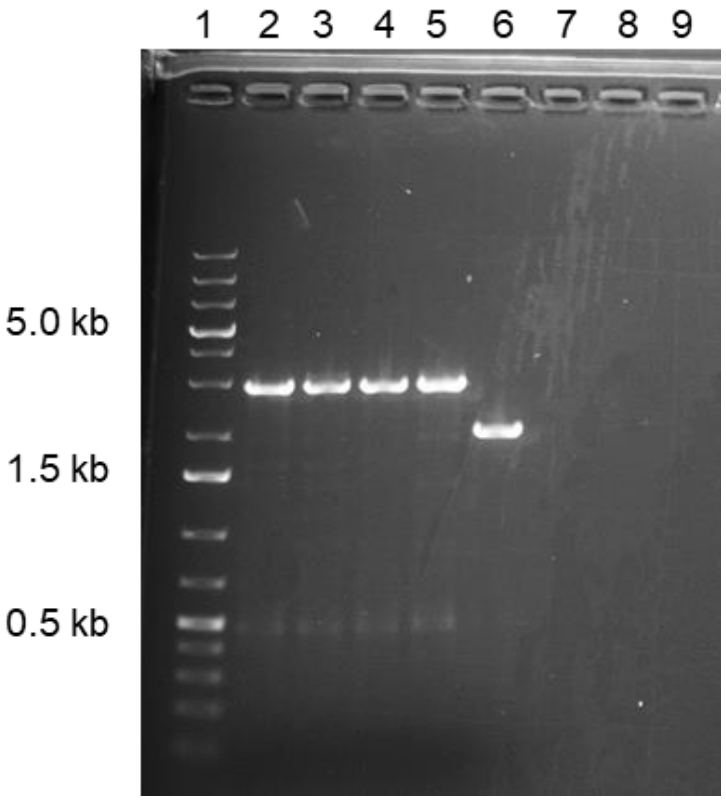

Figure 12 Test strain genotype confirmation. DNA was isolated from test strains and test strains were genotyped by PCR with primers V0378-E and V0378-F. PCR products were examined by gel electrophoresis with ethidium bromide staining. An image of the gel with UV transillumination is shown. The predicted PCR product length for the *v378+* and *v378Δ* genotypes are 1973 bp and 2860 bp, respectively. Lane 1 contains 0.5 µg of Gene Ruler 1 Kb Plus DNA ladder. DNA templates for each PCR reaction are as follows: Lane 2, RPAP3.107; Lane 3, RPAP3.108; Lane 4, RPAP3.109; Lane 5, RPAP3.111; and Lane 6, RDGR170.3; Lanes 7–9) Empty. These results show that test strains RPAP3.107, RPAP3.108, RPAP3.109, and RPAP3.111 have the *v378Δ* genotype.

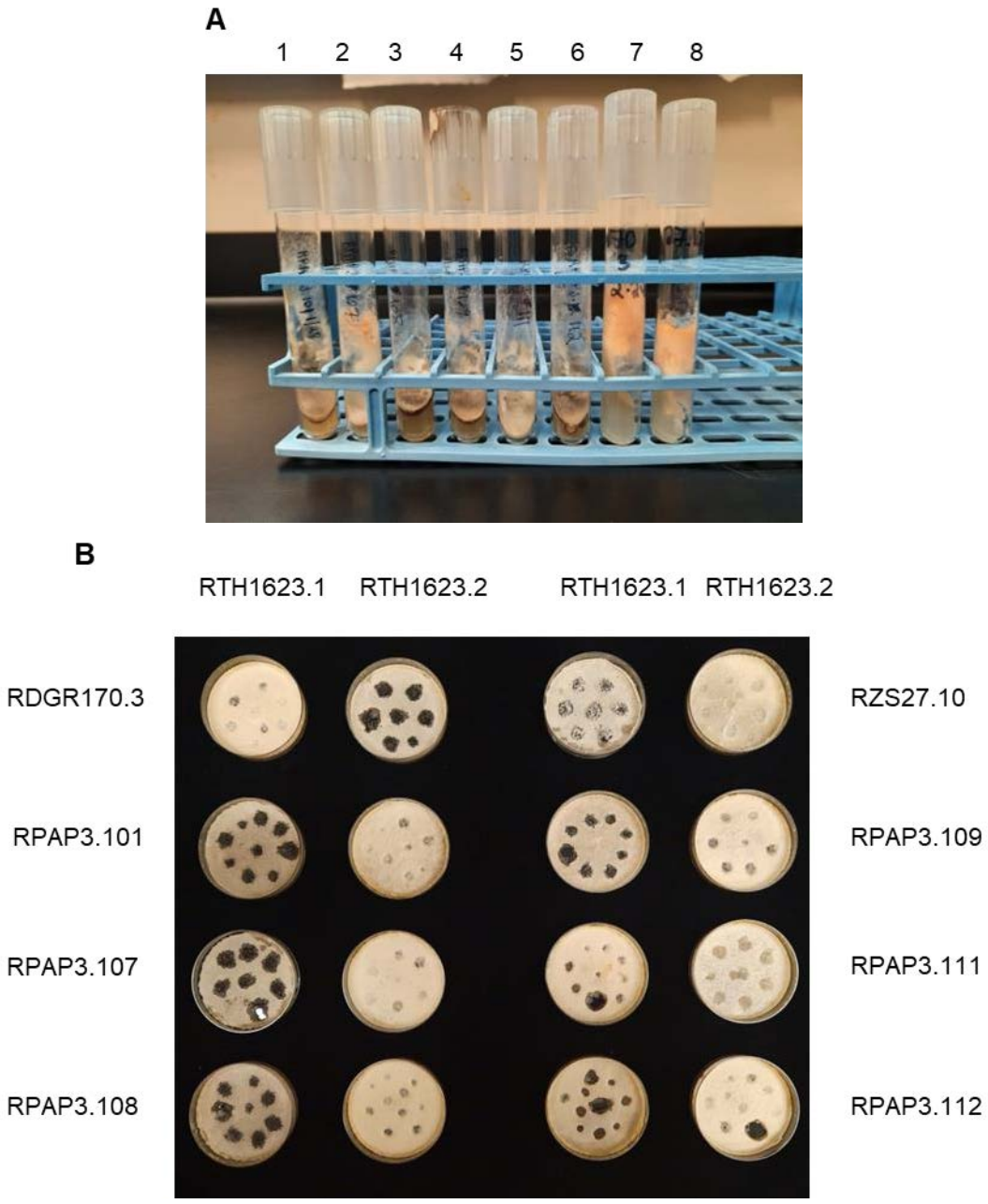

Figure 13 Test strains and crosses. **(A)** Six hygromycin-resistant offspring from cross RPAP3 (TPAP1.3 × RTH1005.2) were selected for test crosses. 1) RPAP3.101, 2) RPAP3.107, 3) RPAP3.108, 4) RPAP3.109, 5) RPAP3.111, and 6) RPAP3.112; 7) RDGR170.3, 8) RZS27.10. (B) Test crosses were performed with RTH1623.1 and RTH1623.2. Images are of crossing dishes approximately three weeks post fertilization.

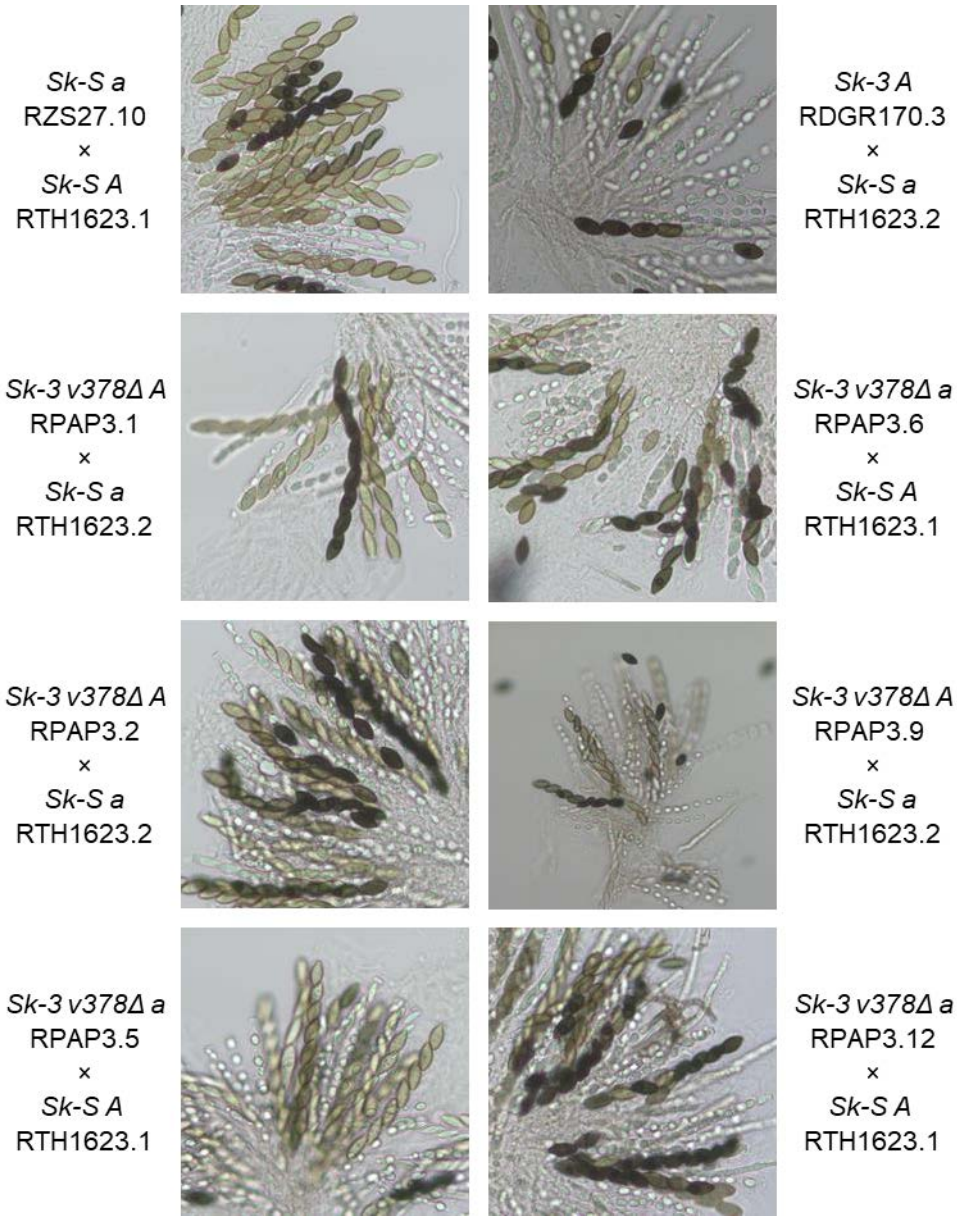

Figure 14 Ascus phenotype summary from first set of test crosses. Asci were dissected from perithecia of eight crosses on day 12 post fertilization and imaged under magnification. Strain names and genotypes are indicated. These results demonstrate that deletion of interval *v378* disrupts spore killing.

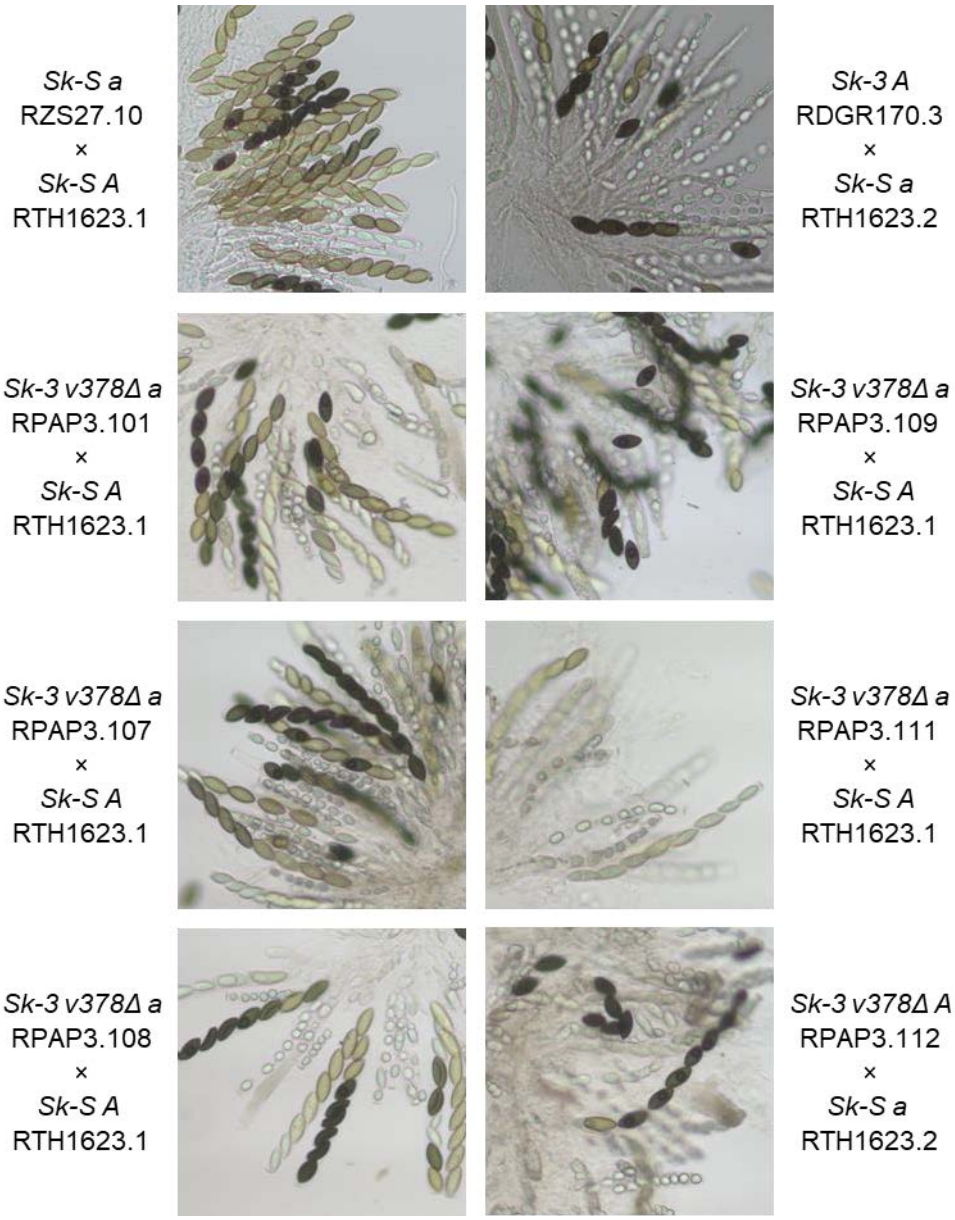

Figure 15 Ascus phenotype summary of second set of test crosses. Asci were dissected from perithecia of eight crosses on day 14 post fertilization and imaged under magnification. Strain names and genotypes are indicated. These results demonstrate that deletion of interval *v378* disrupts spore killing.

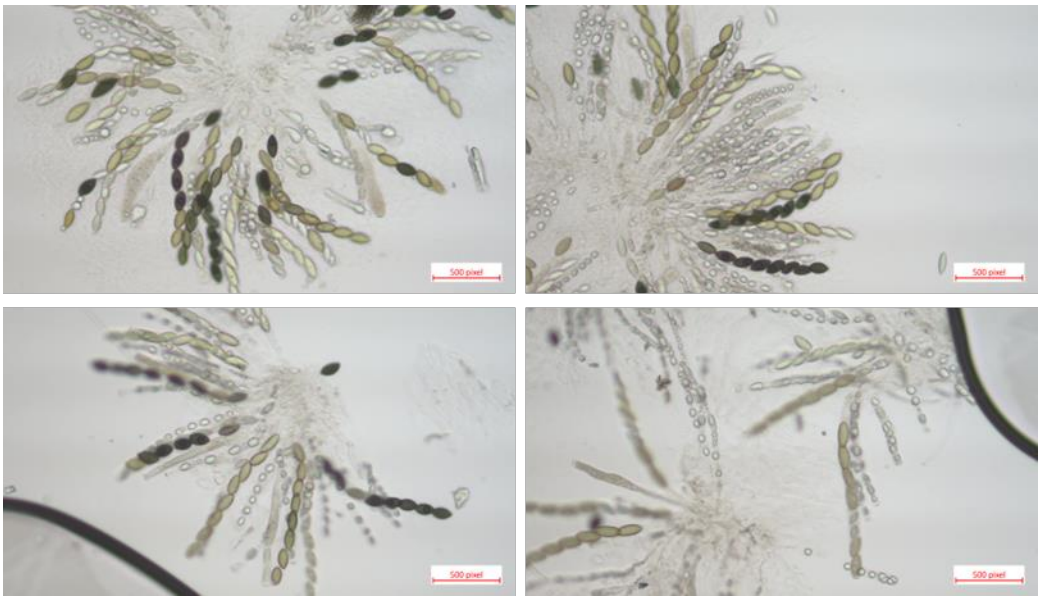

Figure 16 Ascus phenotypes. Asci were dissected from perithecia of RPAP3.101 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

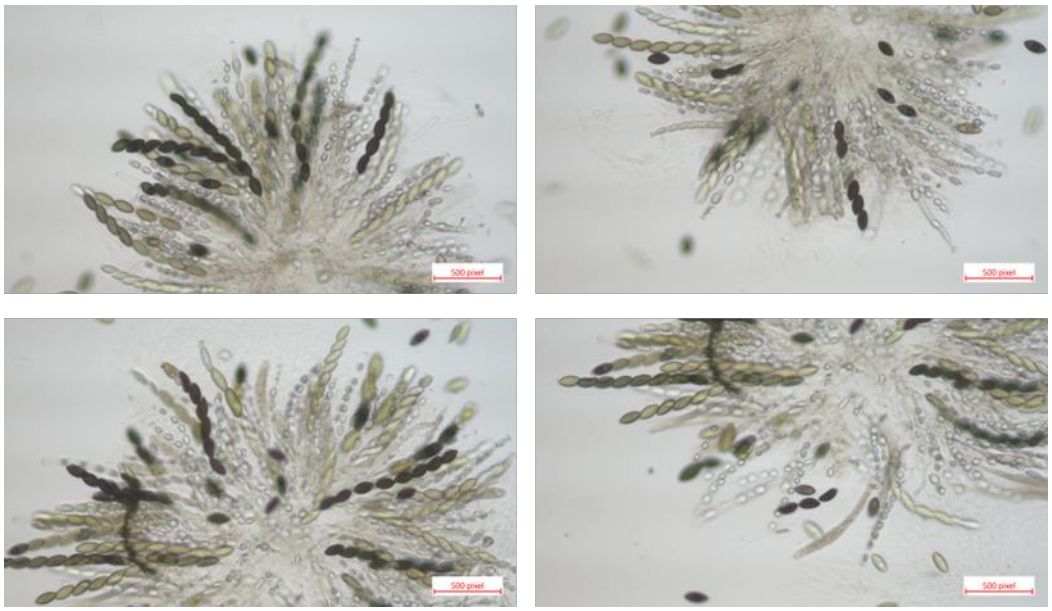

Figure 17 Ascus phenotypes. Asci were dissected from perithecia of RPAP3.107 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

Figure 18 Ascus phenotypes. Asci were dissected from perithecia of RPAP3.108 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

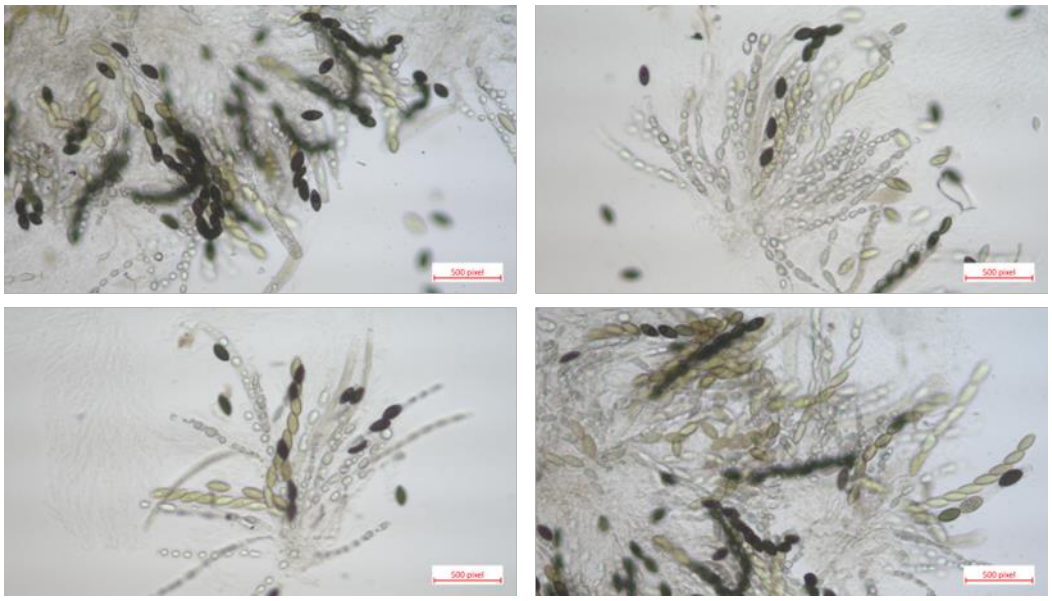

Figure 19 Ascus phenotypes. Asci were dissected from perithecia of RPAP3.109 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

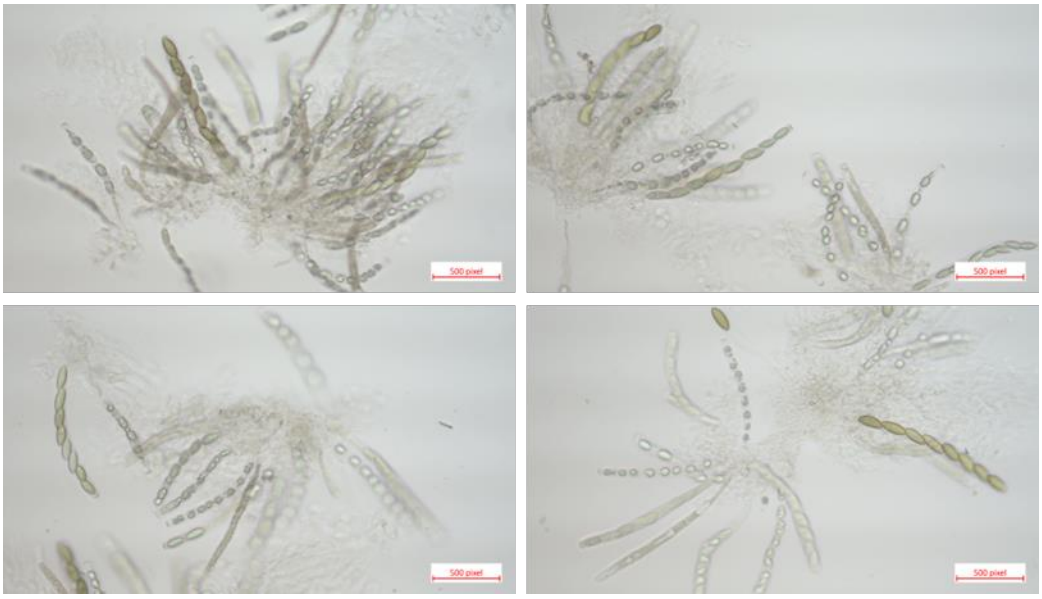

Figure 20 Ascus phenotypes. Asci were dissected from perithecia of RPAP3.111 × RTH1623.1 on day 14 post fertilization and imaged under magnification.

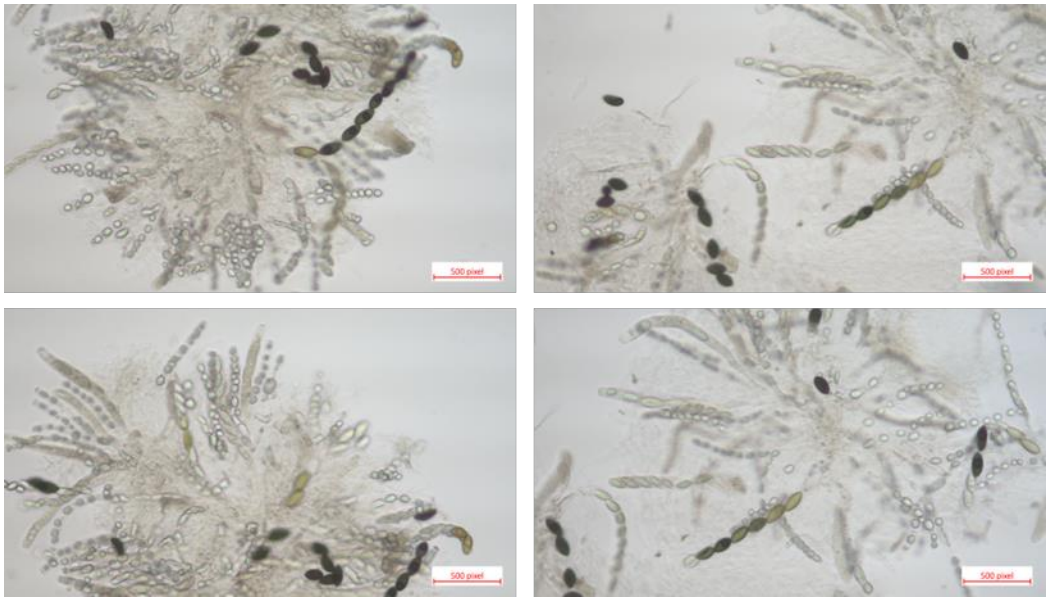

Figure 21 Ascus phenotypes. Asci were dissected from perithecia of RPAP3.112 × RTH1623.2 on day 14 post fertilization and imaged under magnification.