Isolation of $rfk-2^{UV}$, a mutation that blocks spore killing by Neurospora Spore killer-3

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Abstract

Neurospora Spore killer-3 (Sk-3) is a selfish genetic element that kills spores to achieve gene drive. Here, we describe the isolation and mapping of $rfk-2^{UV}$, a mutation that disrupts spore killing. The $rfk-2^{UV}$ mutation is located 15.6 cM from $mus-52$ on Chromosome III. The significance of this discovery with respect to Sk-3 evolution is discussed.
Figure 1. The rfk-2\textsuperscript{UV} mutation disrupts spore killing and gene drive.
(A) Spore killing and gene drive are present in SkS (ISU-3037) × Sk-3 (ISU-3291) crosses. Left: Asci possess a spore killing phenotype. Asci develop asynchronously in N. crassa. Asci with dark pigmented ascospores are more mature than those with light pigmented ascospores. Right: Offspring were randomly collected and examined for Sk-3 gene drive with a PCR-based gene drive assay. All 34 offspring possess the Sk-3 genotype, demonstrating that Sk-3 gene drive occurs in this cross. Lanes: K, Sk-3 control, ISU-3291; S, SkS control, FGSC 10340; two lanes contain a DNA ladder, the remaining 34 lanes correspond to 34 offspring. (B) Spore killing and gene drive are absent from SkS (ISU-3036) × Sk-3 rfk-2 UV (ISU-4684) crosses. Left: Spore killing is absent from asci. Right: Of the 34 offspring examined, there are 18 with an Sk-3 genotype and 16 with an SkS genotype, demonstrating that rfk-2 UV disrupts gene drive ($\chi^2 = 0.12$, p-value = 0.73). (C) The Sk-3 interval is on Chromosome III. The relative positions of the centromere, hph$^A$, hph$^B$, and mus-52Δ are shown in the diagram. (D) Left: A three-point cross was performed with strains Sk-3 rfk-2 UV hph$^A$ (ISU-4685/6) and Sk-3 mus-52Δ (ISU-4689). Right: A total of 213 offspring were collected and genotyped for rfk-2 UV, hph$^A$, and mus-52Δ alleles. Recombination analysis indicates that rfk-2 UV is located centromere-proximal of hph$^A$ and mus-52Δ. “Y” means that the indicated allele is present in a genotype. Abbreviations: SCO1, genotypes result from a crossover between rfk-2 UV and hph$^A$; SCO2, genotypes result from a crossover between hph$^A$ and mus-52Δ; DCO, genotypes result from a double crossover. (E) Left: A three-point cross was performed with strains Sk-3 rfk-2 UV hph$^B$ (ISU-4687/8) and Sk-3 mus-52Δ (ISU-4689). Right: A total of 186 offspring were collected and genotyped for rfk-2 UV, hph$^B$, and mus-52Δ alleles. Recombination analysis indicates that rfk-2 UV is located centromere-proximal of hph$^B$ and mus-52Δ.

Description

Spore killer-3 (Sk-3) is a selfish genetic element that was discovered over four decades ago in the filamentous fungus Neurospora intermedia (Turner and Perkins 1979). Sk-3 is interesting because it is transmitted to nearly all offspring of an Sk-3 × SkS cross, where Sk-3 refers to a strain carrying the selfish genetic element, and SkS refers to a strain that is sensitive to Sk-3-based spore killing. The biased transmission of Sk-3 is an example of gene drive that occurs through spore killing (Zanders and Johannesson 2021). Specifically, during an Sk-3 × SkS cross, spore killing eliminates ascospores (offspring) with an SkS genotype while sparing those with an Sk-3 genotype. As a result, Sk-3 × SkS crosses produce asci with four viable and four inviable ascospores, rather than the eight viable ascospores typical of Neurospora crosses.

Sk-3 has been mapped to a 30 cM interval of Chromosome III (Turner and Perkins 1979). This interval contains hundreds of genes and it is transmitted to offspring as a single unit (Campbell and Turner 1987). At least two genes within the interval are thought to mediate gene drive. One gene is rsk, which is required for resistance to spore killing but not for spore killing itself (Hammond et al. 2012). The second gene has yet to be identified, but it is believed to encode Sk-3’s killer (Hammond et al. 2012).

Sk-3 is one of two complex selfish genetic elements known to exist in Neurospora fungi. The second is Sk-2 (Turner and Perkins 1979). Sk-2 shares many similarities with Sk-3. Sk-2 is transmitted to offspring in a biased manner, resides on a similar interval of Chromosome III, and uses rsk for resistance to spore killing but not spore killing itself. Despite these similarities, Sk-2 and Sk-3 are distinct elements. For example, Sk-2’s rsk allele (rsk$^{Sk-2}$) provides resistance to spore killing by Sk-2 but not Sk-3, and Sk-3’s rsk allele (rsk$^{Sk-3}$) provides resistance to spore killing by Sk-3 but not Sk-2 (Hammond et al. 2012).

A recent finding suggests that some of the similarities between Sk-2 and Sk-3, such as their complex genomic rearrangements, may have evolved by convergent evolution (Svedberg et al. 2018). Other similarities, such as the role of rsk in the drive mechanisms of both Sk-2 and Sk-3, appear to be the result of descent from a common ancestral selfish genetic element. However, a complete understanding of the evolutionary relationship between Sk-2 and Sk-3 will likely require additional knowledge, such as the identity of Sk-3’s killer. The Sk-2 killer is encoded by rfk-1 and spore killing is absent in Sk-2 rfk-1Δ × Sk$^k$ crosses (Rhoades et al. 2019). In contrast, deletion of the most likely rfk-1 ortholog from an Sk-3 strain had no effect on spore killing, leaving the identity of Sk-3’s killer unknown (Svedberg et al. 2018).

Here, to help identify Sk-3’s killer, we performed a genetic screen for required for killing (rfk) mutations (see methods). The genetic screen uses Sk-3 rskΔ × SkS crosses, which abort development before the production of viable ascospores (Hammond et al. 2012; Harvey et al. 2014). We isolated a few candidate rfk mutations with our genetic screen and chose the most promising candidate, rfk-2 UV, for additional analysis. As demonstrated in Figure 1 (A and B), rfk-2 UV disrupts spore killing and gene drive.

To determine the approximate genomic location of rfk-2 UV, we performed two sets of three-point crosses (Figure 1C). Recombination analysis of 213 offspring from the first set of crosses (rfk-2 UV hph$^A$ × mus-52Δ) indicates that rfk-2 UV is
located 2.8 cM from \(hph^A\) and 15.0 cM from \(mus-52\Delta\) (Figure 1D). For the second set of crosses \((rfk-2^{UV} hph^B \times mus-52\Delta)\), recombination analysis of 186 offspring indicates that \(rfk-2^{UV}\) is located 7.5 cM from \(hph^B\) and 16.1 cM from \(mus-52\Delta\) (Figure 1E).

In addition to providing genetic distances from physical positions on Sk-3 Chromosome III, our recombination data indicate that \(rfk-2^{UV}\) is located centromere-proximal of \(hph^A\), \(hph^B\), and \(mus-52\Delta\) (Figure 1, D and E). This is somewhat surprising given that Sk-2 \(rfk-1\) is located at the junction of Sk-2 and SkS sequences on the right arm of Chromosome III (Rhoades et al. 2019). \(rfk-1\)'s location within Sk-2 allows it to escape inactivation by a genome defense process called meiotic silencing by unpaired DNA (MSUD) (Aramayo and Selker 2013; Hammond 2017; Rhoades et al. 2019), and thus, given the importance of \(rfk-1\)'s location, we initially predicted that Sk-3's killer gene would be found centromere-distal of \(hph^A\), \(hph^B\), and \(mus-52\Delta\). Our finding that \(rfk-2^{UV}\) is centromere-proximal to all three of these genetic markers indicates that Sk-2 and Sk-3 may have evolved different relative positions for their killer genes. In summary, the future cloning and characterization of \(rfk-2^{UV}\) should help clarify the organizational patterns of critical gene drive genes within Sk-2 and Sk-3, as well as the evolutionary relationship between these two complex selfish genetic elements.

Methods

Strains and alleles used in this study

Sk-3 was introgressed into \(N.\ crassa\) for genetic analysis shortly after its discovery in \(N.\ intermedia\) (Turner and Perkins 1979). Only \(N.\ crassa\) strains were used in the present study. The \(rid\) genotype suppresses a genome defense process called RIP, which mutates duplicated sequences during sexual reproduction (Freitag et al. 2002; Aramayo and Selker 2013). \(mus-51\), \(RIP^{70}\), \(mus-51\), and \(mus-52\Delta\) alleles suppress NHEJ, thereby increasing the efficiency of genetic transformation (Ninomiya et al. 2004). The \(sad-2\Delta\) allele inhibits MSUD, which suppresses the expression of unpaired genes during meiosis (Aramayo and Selker 2013; Hammond 2017). The \(his-3\) and \(leu-1\) genes are required for histidine and leucine biosynthesis, respectively, and \(fl\) controls macroconidiation (Perkins et al. 2000).

Culture conditions and ascus analysis

Vegetative propagation was performed on VMM/VMA and crosses were performed on SCA as previously described (Harvey et al. 2014; Rhoades et al. 2020). For imaging asci, syringe needles were used to dissect asci from perithecia into 50% glycerol at 18 days post fertilization. Asci were imaged by standard light microscopy.

Screen for Sk-3 \(rfk\) mutations

To isolate Sk-3 \(rfk-2^{UV}\), we made one change to a previously developed screen for Sk-2 \(rfk\) mutations (Harvey et al. 2014). Specifically, we irradiated Sk-3 \(rsk\Delta\) conidia (from strain ISU-4677) instead of Sk-2 \(rsk\Delta\) conidia. We then followed the protocol as previously described by fertilizing \(Sk^S\) protoperithecia with the UV irradiated conidia, incubating the mating cultures for four weeks, collecting shot ascospores from the lids of crossing plates, germinating ascospores on VMA, and transferring individual germlings (offspring) to culture tubes containing VMA for vegetative propagation. Each offspring was genotyped for Sk-3 and examined for an ability to kill spores in crosses with an \(Sk^S\) mating partner (strains F2-23, F2-26, ISU-3036, and/or ISU-3037). Offspring with an Sk-3 genotype that displayed defects in spore killing were considered \(rfk\) mutant candidates. The \(rfk-2^{UV}\) mutation was first identified in strain MAV214. The following series of crosses was used to move \(rfk-2^{UV}\) from MAV214 into strain ISU-4684: Cross 1) MAV214 \(\times\) ISU-3036 = ISU-4678; Cross 2) ISU-4678 \(\times\) ISU-4679 = ISU-4681; Cross 3) ISU-4681 \(\times\) F2-23 = ISU-4682; Cross 4) ISU-4682 \(\times\) ISU-3291 = ISU-4683; and, Cross 5) ISU-4683 \(\times\) ISU-3291 = ISU-4684.

Genetic modifications

A standard electroporation-based transformation procedure was used to make genetic modifications to \(N.\ crassa\) (Margolin et al. 1997; Rhoades et al. 2020). All transformation vectors were constructed by double-joint (DJ)-PCR (Yu et al. 2004; Hammond et al. 2011), using oligonucleotide PCR primers. Sk-3 genomic DNA was used for amplification of left and right DJ-PCR fragments. The genome sequence of Sk-3 strain FGSC 3194 (Svedberg et al. 2018) was used for primer design and Chromosome III position information. Plasmid pTH1256.1 was used for amplification of \(hph\) center fragments for DJ-PCR (GenBank: MH550659.1). Plasmid pNR28.12 was used for amplification of \(nat\) center fragments for DJ-PCR (GenBank: MH553564.1). Both plasmids can be obtained from the Fungal Genetics Stock Center (McCluskey et al. 2010). Strain ISU-3291 and transformation vectors v14b, v260, and v337 were used to create \(rsk\Delta::hph, leu-1\Delta::nat, \) and \(mus-52\Delta::nat\) alleles, respectively. Strain ISU-4684 and transformation vectors v322 and v324 were used to create \(hph^A\) and \(hph^B\) alleles,
respectively. Primers for v14b construction: 1001b/1002b (center), 1003b/1004b (left), 1005b/1006b (right), and 1007b/1008b (nested). Primers for v260 construction: 297/298 (center), 1907/1908 (left), 1909/1910 (right), and 1911/1912 (nested). Primers for v322 construction: 585/586 (center), 2204/2158 (left), 2159/2160 (right), and 2161/2162 (nested). Primers for v324 construction: 585/586 (center), 2169/2170 (left), 2171/2172 (right), and 2173/2174 (nested). Primers for v337 construction: 297/298 (center), 2205/2214 (left), 2215/2208 (right), and 2216/2217 (nested).

PCR-based assay for gene drive

Genomic DNA was isolated from offspring and control strains with IBI Scientific’s mini genomic DNA kit for plants and fungi. PCR primer set 49/50 amplifies a 596 bp product from \(\text{Sk-3}\) genotypes and an 896 bp product from \(\text{SkS}\) genotypes. PCR products were examined by standard agarose-gel electrophoresis with ethidium bromide staining.

Three-point crosses

The position of rfk-2\textsuperscript{UV} was mapped relative to three markers: \(hph^A\), \(hph^B\), and \(\text{mus-52}\Delta\). \(hph^A\) was created by inserting the hygromycin resistance cassette \((hph)\) between genes \textit{ncu05694} and \textit{ncu05695} at approximately 1.0 Mb on Chromosome III of ISU-4684. \(hph^B\) was created by inserting \(hph\) between genes \textit{ncu07875} and \textit{ncu07876} at approximately 1.6 Mb on Chromosome III of ISU-4684. \(\text{mus-52}\Delta\) was created by replacing \(\text{mus-52}\) in strain ISU-3291 with \(\text{nat}\), a nourseothricin resistance cassette. Offspring were genotyped for \(hph^A\) or \(hph^B\) with hygromycin resistance assays, for \(\text{mus-52}\Delta\) with nourseothricin resistance assays, and for rfk-2\textsuperscript{UV} with spore killing assays.

Reagents

| Strain    | Genotype                  | Source                      |
|-----------|---------------------------|-----------------------------|
| F2-23     | \( \text{rid}; \text{fl}\ A\) | Hammond et al. 2012         |
| F2-26     | \( \text{rid}; \text{fl}\ a\) | Hammond et al. 2012         |
| F3-14     | \( \text{rid}; \text{fl}; \text{Sk-3}\ A\) | Hammond et al. 2012         |
| FGSC 10340| \( \text{rid}; \text{mus-51}^{\text{RIP70}\ a}\) | Smith et al. 2016           |
| ISU-3036  | \( \text{rid}; \text{fl}; \text{sad-2}\Delta::hph\ A\) | Samarajeewa et al. 2014     |
| ISU-3037  | \( \text{rid}; \text{fl}; \text{sad-2}\Delta::hph\ a\) | Samarajeewa et al. 2014     |
| ISU-3291  | \( \text{rid}; \text{Sk-3}; \text{mus-51}\Delta::\text{bar}\ A\) | P8-42 \(\times\) F3-14     |
| ISU-4677  | \( \text{rid}; \text{Sk-3}\ rsk\Delta::hph; \text{mus-51}\Delta::\text{bar}\ A\) | Trans. ISU-3291 with v14bc |
| ISU-4678  | \( \text{rid}; \text{Sk-3}\ rsk\Delta::hph \text{rfk-2}\text{UV}; \text{mus-51}\Delta::\text{bar}; \text{sad-2}\Delta::hph\ A\) | ISU-4773 \(\times\) ISU-3036 |
| ISU-4679  | \( \text{rid}; \text{Sk-3}\ leu-1\Delta::\text{nat-1}; \text{mus-51}\Delta::\text{bar}\ A\) | Trans. ISU-3291 with v260  |
| ISU-4681  | \( \text{rid}; \text{Sk-3}\ \text{rfk-2}\text{UV}; \text{mus-51}\Delta::\text{bar}; \text{sad-2}\Delta::hph\ a\) | ISU-4678 \(\times\) ISU-4679 |
| ISU-4682  | \( \text{rid}; \text{Sk-3}\ \text{rfk-2}\text{UV}\ a\) | ISU-4681 \(\times\) F2-23   |
| ISU-4683  | \( \text{rid}; \text{Sk-3}\ \text{rfk-2}\text{UV}\ a\) | ISU-4682 \(\times\) ISU-3291 |
| ISU-4684  | \( \text{rid}; \text{Sk-3}\ \text{rfk-2}\text{UV}; \text{mus-51}\Delta::\text{bar}\ a\) | ISU-4683 \(\times\) ISU-3291 |
| PCR Primer Number | Sequence |
|-------------------|----------|
| 49                | CCGCTGGTTTGTGGTTCTTGATG |
| 50                | CAGCCACGGATCGCTTATCGTTT |
| 297               | GAGGGAGTGTGGGAAATGGTGTC |
| 298               | GTTGGTTAGTGGAACGCTTGT |
| 585               | CCGTCCACGCCCCCTTAATACGACT |
| 586               | CTTGATTGACAGCGAAACGAAACC |
| 1001b             | CTCTGCTCTTCTTCCCTCCGGCTCCAAACTGATATTGAAGGAGCAT |
| 1002b             | AACCTCGATCTCAATGAAGCCGGAACGTGGTTCCTCGGCGCAT |
| 1003b             | ATAGGGGTGAAAGTTGGCTTC |
| 1004b             | ATGCTTCATTCAATATCGTTGAGCAGGGAAAGAAGAGCAGAG |
| 1005b             | ATGCCGACCAGCAGTGGGCTTCATTTGAGATCGAGGT |
| 1006b             | CCAGGCACCATCCAAGACAGT |
| 1007b             | CTGGTCGCTTTTTTGCTCTTGTTC |
| 1008b             | GTAATTCCAGGTGCCCAAGCTCA |
| 1907              | TGGGTGAAAGCTTTGGGAACGAG |
| 1908              | TGAATGCTAAAGACACCATTTCCTCCCCACACTCCCTCCTCGTGCAGGAGCTGGAATTATCAA |
|   |   |
|---|---|
| 1909 | GCTGGCTGCAATACAAGCGTTCCCACCTAACCAACGGGGCAGTGCAA ACAATGCTCTTT |
| 1910 | CACCTCATAATACACAGCTCACT |
| 1911 | GGACCTCGGGCAAGGATTGTAAG |
| 1912 | CTTTTCACAAACTGCTCGCTCCT |
| 2158 | AGTCGTATTAAGGGCGTGAGGCGGAGGAG |
| 2159 | GGGTTTCGTTGCCTGTAATCAAGTCCCCGAAGATAATAGCTAGGAG |
| 2160 | AGTCTAGAAAACGGGCGGAG |
| 2161 | GCCAGAGCTGGCTCCAGGG |
| 2162 | AGGGTAAATGTACGGACGAAGCT |
| 2169 | GCCGACTGTTGGAATGGAAGCG |
| 2170 | AGTCGTATTAAGGGCGTGGACGGGAG |
| 2171 | GGGTTTCGTTGCCTGTAATCAAGGAAATGAGGCTAGGTAGTAAGT |
| 2172 | TTGACCCCGACGTCAAGATC |
| 2173 | TGCAATTGAGTCATCATTGGCATGG |
| 2174 | ACATCTTGCGCTCATTTCCCT |
| 2204 | TTCAATTTGGAGCCGGACTT |
| 2205 | GGGATGTCAGGGGAAGACG |
| 2208 | GCGTAATTGAGGCTCCCAACA |
| 2214 | TGAATGTCAAAAGACACCATTCGCTCAGGTGGGGTCTAGG |
| 2215 | GGCTGGCTGCAATACAAGCGTTCCCACCTAACCAACGGGGCAGTGCAA ACAATGCTCTTT |
| 2216 | GAGGAATTCGGGCGGAG |
| 2217 | GCCCCACTGTAAGGTACAAAGGACG |

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