Introduction

In many organisms, genetic factors, called Meiotic Drive Elements (MDs), complex nuclear genetic loci found in various eukaryotic genomes and distorting segregation in their favor. Here, we identify and characterize in the model fungus *Podospora anserina* Spok1 and Spok2, two MDs known as Spore Killers. We show that they are related genes with both spore-killing distorter and spore-protecting responder activities carried out by the same allele. These alleles act as autonomous elements, exert their effects independently of their location in the genome and can act as MDs in other fungi. Additionally, Spok1 acts as a resistance factor to Spok2 killing. Genetical data and cytological analysis of Spok1 and Spok2 localization during the killing process suggest a complex mode of action for Spok proteins. Spok1 and Spok2 belong to a multigene family prevalent in the genomes of many ascomycetes. As they have no obvious cellular role, Spok1 and Spok2 Spore Killer genes represent a novel kind of selfish genetic elements prevalent in fungal genome that proliferate through meiotic distortion.

Abstract

Mendel laws of inheritance can be cheated by Meiotic Drive Elements (MDs), complex nuclear genetic loci found in various eukaryotic genomes and distorting segregation in their favor. Here, we identify and characterize in the model fungus *Podospora anserina* Spok1 and Spok2, two MDs known as Spore Killers. We show that they are related genes with both spore-killing distorter and spore-protecting responder activities carried out by the same allele. These alleles act as autonomous elements, exert their effects independently of their location in the genome and can act as MDs in other fungi. Additionally, Spok1 acts as a resistance factor to Spok2 killing. Genetical data and cytological analysis of Spok1 and Spok2 localization during the killing process suggest a complex mode of action for Spok proteins. Spok1 and Spok2 belong to a multigene family prevalent in the genomes of many ascomycetes. As they have no obvious cellular role, Spok1 and Spok2 Spore Killer genes represent a novel kind of selfish genetic elements prevalent in fungal genome that proliferate through meiotic distortion.

Results

Identification of Spok1

In crosses between the S and T strains of *P. anserina*, only half the progeny reaches maturity in 90% of the asci (spore sacs; n > 200), while all ascospores reach maturity in control S×S and T×T crosses (Fig. 1A). Back cross of the progeny retrieved from S×T crosses to the parental S and T strains showed 90% 2-spored and 100% 4-spored asci, respectively, indicating the presence of at least one Sk in strain T to which strain S is sensitive. It likely corresponds to the first described Sk in fungi [15,17]. In our hands, this Sk triggers death in nine out of ten asci, and hence harbors a first division segregation (FDS) of 90%, suggesting a close linkage to a centromere (Fig. 1B). During *P. anserina* genome assembly verification by microsatellite genotyping of the progeny from a cross between the S and T strains [18], we observed a strong bias towards the transmission of the T centromere region of chromosome 5 in 50 progeny (Fig. S1) and this was not the case for the other chromosomes, pinpointing the Sk locus close to the centromere of chromosome 5.

P. anserina, at least eight Sks have been observed [15]. One of them has been associated with deleterious effects during ascospore formation of the Het-s prion [16]. However, several additional Sks remain uncharacterized [15], including the first one discovered in fungi [17]. Here, we identify the distorters and responders of two *P. anserina* Sks. Unlike previously known MDs, both activities for these Sks are carried out by single genes acting autonomously irrespective of their position in the genome or of the fungal species and whose homologues are prevalent in many fungi.
To narrow the region containing the Sk, we backcrossed a progeny (ST1) of the ST × T cross twenty times to strain S, selecting each time for FDS asc. At each generation, we observed the Sk effect (i.e., 90% of 2-spored asc) and thus the final backcrossed strain (SKT20, Table S1) had a genome coming mostly from strain S, except for a small region containing the Sk locus from the T strain. Molecular analysis of polymorphic markers showed that SKT20 had its entire chromosome 5 coming from strain S except for a small region of 70 kb bordered by markers 5PGK and 5PGM (Table S2, Fig. 2). 5PGK differs between S and T for several SNPs that can be detected by sequence analysis. 5PGM differs by the presence of a 15 kb-region present in strain T and absent in strain S. The final backcrossed strain, SKT20, had the 5PGK and 5PGM markers of strain S. This strain displayed 90% 2-spored asc when crossed with S and 100% 4-spored asc when crossed with T, as expected if it contains the distorter and responder of the Sk from strain T (Table 1, Figure 3A). Final identification of the Sk was made by nested deletions in SKT20. A 6 kb-region located between coding sequences (CDS) Pa_5_4070 and Pa_5_4075 was found to be responsible for meiotic drive. This region encompassed a retroposon LTR and a single predicted gene, which we called Spok1 (Spore killer 1, Fig. 2, Fig. S2). Spok1 is absent in strain S, which has transposable elements at the same chromosomal location (i.e., between Pa_5_4070 and Pa_5_4075, Fig. 2, Fig. S2).

To validate that Spok1, and not an additional non-annotated gene present in the 6-kb region, was necessary and sufficient for spore killing, we first replaced solely its coding sequence with a hygromycin B-resistance marker (Fig. S2, see Materials and Methods for gene deletions and Fig. S3 for Southern Blot validations). The SKT20 strain had thus a genotype identical to SKT20, except that the Spok1 coding sequence was replaced. We observed the production of 100% 4-spored asc in crosses of SKT20S with S and 90% 2-spored asc in crosses of SKT20S with SKT20 (Fig. 3A), showing that Spok1 was responsible for both killing and resistance. Secondly, we inserted Spok1 in the PaPKS1 gene of strain S. PaPKS1 is located at the centromere of chromosome 2 and segregates with 99% FDS [19]. It encodes a polyketide synthase that controls the first step of melanin biosynthesis and PaPKS1 mutants are devoid of pigment at all stages of their life cycle [19]. This allows for easy screening of colorless recombinant transgenic strains carrying an insertion in the PaPKS1 gene. Additionally, ascospores carrying Spok1 should be devoid of pigment enabling their easy identification in crosses. Strains carrying Spok1 at PaPKS1 (PaPKS1::Spok1) yielded 99% of unpigmented 2-spored asc in crosses with the S strain (Fig. 3C). The 1% 4-spored asc recovered resulted from the expected second division segregation (SDS) of the PaPKS1::Spok1 locus. Thus, insertion of solely Spok1 into the PaPKS1 gene of strain S was sufficient to trigger both spore killing and resistance.

Phenotypic analysis of the whole life cycle of SKT20 and SKT20A (i.e., ascospore maturation and germination, mycelium growth, heterokaryon incompatibility and sexual reproduction including differentiation of fruiting body) showed no defects other than a lack of Sk activity in SKT20A, with SKT20 × SKT20 and SKT20A × SKT20A homozygous crosses produced 100% 4-spored asc (Table 1, Fig. 3B). Sequence analysis of the 734 amino acid-long Spok1 did not reveal any functional domain (Fig. 4). However, PSORT [20] predicted a nuclear localization.

Strain S contains Spok2, a parologue of Spok1, which also causes spore killing

Surprisingly, when we inactivated Spok1 in parental strain T (Spok1- strain), this did not result in the expected absence of 2-spored asc in Spok1- × S crosses, as 40% of the asc were 2-spored (Table 1, Fig. 3A). However, the strain yielded 100% 4-spored asc when crossed with itself and 90% 2-spored asc when crossed with strain T, as expected (Table 1, Fig. 3A and 3B). Strain S, but not strain T, carries Pa_5_10, a CDS with 87% amino-acid identity to Spok1 (Fig. 4) and bordered by two large regions composed of Repeat Induced Point mutation (RIP)-inactivated transposons [21] (Fig. 2 and Fig. S2). Pa_5_10 is 600 kb away from Spok1 on the same chromosome arm, in a region with an expected FDS of 40% (Fig. 2). In strain T, this position is occupied by a segmental duplication of chromosome 6 (Fig. S2). The Pa_5_10 gene (hereafter named Spok2 for Spore killer 2) was thus a good candidate for the killing of ascospores in 40% of the asc of the Spok1A × S crosses. Spok2 was deleted by replacing its coding sequence with a hygromycin B-resistance marker to yield strain Spok2A (Fig. S3). When Spok2A was crossed with the parental strain S, 40% 2-spored asc were observed (Fig. 3A). Analysis of the homokaryotic ascospores recovered from a Spok2A × S cross showed that they were all sensitive to hygromycin B, indicative of a specific killing of the ascospores carrying Spok2A (hygromycin B-resistant) by those carrying wild-type Spok2. As expected from its chromosomal location, Spok2 causes ascospore death in only 40% of asc (n =200; Fig. 3A). Moreover, when the Spok2 coding sequence was inserted in the PaPKS1 gene of the Spok2 strain, using the same strategy as for Spok1 (PaPKS1::Spok2), it caused ascospore death in 99% of the asc when crossed to the Spok2A strain (Fig. 3C). This showed that Spok2 can also be responsible for spore killing. In crosses between Spok1A (strain T) and Spok2A (strain S), 100% 4-spored asc were observed (Table 1, Fig. 3A), showing that Spok2 was responsible for the 40% 2-spored asc present in the Spok1A × S crosses.

Like Spok1, Spok2 does not appear to be involved in any aspect of the physiology and development of P. anserina, as we could not detect any defect in the mycelium, fruiting body and ascospores of the Spok2A strain, with the homozygous cross of this strain yielding 100% 4-spored asc (Table 1, Fig. 3B). Sequence analysis of the Spok2 protein predicted with low probability an ATP binding site of a kinase domain acting on low molecular weight molecules. However, this domain is not predicted for Spok1 despite the great sequence identity (87%) between the two proteins (Fig. 4). Like Spok1, Spok2 was predicted by PSORT to be in nuclei.

Spok1 is a resistance factor to Spok2

In S × T crosses, we did not detect any obvious meiotic drive created by Spok2, i.e., excess transmission of the S genotype in the
region surrounding Spok2 (Fig. S1). This was surprising since in this cross both Spok1 and Spok2 are in heterozygous configuration, which should enable killing by both Spok1 and Spok2 (Table 1). Possibly, Spok1 could act as a resistance factor to Spok2. To directly test this hypothesis, the 2-spored-asci progeny of Spok1<sup>Δ</sup> x S crosses was analyzed. Data showed that all the recovered ascospores had two nuclei containing both the Spok2 gene (11 asci analyzed), suggesting that Spok2 exerted spore killing only when the cross was

Figure 1. Structure of <i>P. anserina</i> asci. (A) S x S or T x T crosses yield 100% four binucleated ascospores per ascus. In S x T cross, 2-spored asci are indicative of spore killing. The Sk locus is linked to the centromere since only 10% of the asci have four ascospores (arrow in S x T). (B) Schematic representation of <i>P. anserina</i> FDS and SDS asci with Sk. FDS: no crossover between the Sk locus and the centromere results in First Division Segregation of Sk, triggering death of the two ascospores lacking the Sk locus. SDS: a crossover between the Sk locus and the centromere results in Second Division Segregation of Sk, generating four surviving heterokaryotic ascospores. Proportion of FDS and SDS asci depends upon the frequency of crossover and thus upon the genetic distance between Sk and the centromere. The 90% of 2-spored asci in S x T cross (A) is indicative of a close linkage of the Sk with the centromere.

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Figure 2. Spok1 and Spok2 DNA regions. Double arrows define the sequences deleted to identify Spok1. 4-spored asci identify the deletions that abolish spore killing. LTR are Long Terminal Repeats of the crapaud retroposon and discoglosse is a DNA transposon [18]. SD: Segmental Duplication. 5 kb and 11 kb regions bordering Spok2 contain inactivated transposons. Pa_x_xxxx are <i>P. anserina</i> predicted CDS.

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devoid of Spok1 (Table 1). Homokaryotic ‘‘SKT20 Spok2'A' strains, carrying a functional Spok1 recombined with a deleted Spok2, were successfully isolated in the progeny of a cross between SKT20 (which contain functional Spok1 and Spok2; Table 1) and Spok2A. Upon crossing these SKT20 Spok2'A' strains with SKT20, approximately 40% of homokaryotic descendants (9 of 23) carried the Spok2 deletion. Altogether, this showed that Spok2 triggered spore killing only in the absence of Spok1. On the contrary, Spok2 did not confer any resistance to Spok1, as we never obtained homokaryotic progeny that did not carry Spok1, in S×T crosses.

Spok2 but not Spok1 is prevalent in P. anserina strains

Because Spok1 and Spok2 behaved as selfish genetic elements propagating through meiotic drive, we evaluated their presence by PCR amplification of a 630 bp product using primers hybridizing in regions conserved in both Spok1 and Spok2 (Table S2) in various strains of P. anserina and its sibling species, P. comata (Table 2). PCR amplification products were obtained for 19 out of the 22 tested strains. Among the three remaining ones, two (X and CBS411.78) behaved as expected if they lacked both Spok1 and Spok2 in crosses with Spok1- or Spok2- containing strains. The third one (A406) exhibited a surprising behavior, as it was non-killing but resistant to both Spok1 and Spok2. Similar strains with non-killing activities but resistant to all Sks have been found in N. crassa [22].

The 630 pb amplification products were sequenced to assess whether they originated from Spok1 or Spok2. Spok1 was not found in any of the other strains tested here and is thus so far only present in strain T, while analysis of the remaining strains showed that all but two (Y and CBS235.71) contained Spok2 or a variant of it. Strains B, D, E, F, I, M, U and PSN14 carried a Spok2 variant with a silent nucleotide polymorphism (A to G at nucleotide N1194) and these strains behaved like strain S. Strain A carried another Spok2 variant with the silent A194 to G substitution, an A to G substitution at position 1029, resulting in a Tyr343 to Cys polymorphism, and a GCCGGT insertion at position 1041 resulting in the insertion of two amino acids (Arg-Cys) after amino acid n’346. This Spok2 allele was active for resistance to Spok2 as shown by the recovery of 100% 4-spored ascii from A×S crosses. The A×SKT20 crosses yielded 90% of 2-spored ascii, showing that Spok1 was still able to act as a Sk in presence of the Spok2 allele from strain A. Interestingly, A×Spok1A and A×Spok2A crosses produced 100% 4-spored ascii, showing that this allele was inactive for killing in such crosses (Table 2). Inoperativeness for killing was confirmed by analysis of A×Spok2A progeny, in which 50% homokaryotic hygromycin B-resistant ascospores carrying the Spok2 deletion were present.

P. comata CBS237.11 and P. anserina strain Y contained the same Spok-related gene having 14 and 9 differences with S and T, respectively, in the sequenced region. This gene may be another distinct functional Sk. Indeed, strain Y was previously reported as containing a Sk in crosses with strain S [15]. We confirm this (Table 2), as Y×S crosses presented 90% 2-spored ascii. Unfortunately, fertility of strain Y was very low when crossed with strain T and SKT20. However, the few recovered ascii suggested a complex interaction between the Sk of strain T and Y, a phenomenon previously seen in crosses with strain O and Us5 [23]. Therefore, it is highly probable that a third Spok gene (Spok3) endowed with spore killing activity and segregating with 90% SDS is present in Y. Spok3 could also be present in P. comata CBS237.11, which also displays 90% 2-spored ascii when crossed with strain S (data not shown). Unfortunately, this strain cannot be crossed with strain T.

### Table 1. Progeny analysis of Spok crosses.

| cross | Spok genotype | progeny | segregation of Spok2 in progeny |
|-------|---------------|--------|-------------------------------|
| S×S   | Spok2 x Spok2 | 100% 4-spored asci | / |
| T×T   | Spok1 x Spok1 | 100% 4-spored asci | / |
| S×T   | Spok2 x Spok1 | 90% 2-spored all with Spok1, 10% 4-spored asci | 50% Spok2: 50% Spok20 |
| SKT20×S | Spok1 Spok2 x Spok1 | 100% 4-spored asci | / |
| SKT20×T | Spok1 Spok2 x Spok1 | 100% 4-spored asci | / |
| SKT20×SKT20 | Spok1 Spok2 x Spok1 Spok2 | 100% 4-spored asci | / |
| SKT20A×SKT20 | Spok1+ Spok2 x Spok1+ Spok2 | 100% 4-spored asci | / |
| Spok1A×T | Spok1+ x Spok1 | 90% 2-spored all with Spok1, 10% 4-spored asci | / |
| Spok1A×Spok1A | Spok1+ x Spok1+ | 100% 4-spored asci | / |
| Spok2A×S | Spok2+ x Spok2 | 40% 2-spored all with Spok2, 60% 4-spored asci | 100% Spok2 |
| Spok2A×Spok2A | Spok2+ x Spok2+ | 100% 4-spored asci | / |
| Spok1A×Spok2A | Spok1+ x Spok2+ | 100% 4-spored asci | / |
| SKT20×Spok2A | Spok1 Spok2 x Spok2+ | 90% 2-spored all with Spok1, 10% 4-spored asci | 50% Spok20: 50% Spok2A |
| SKT20 Spok2A×SKT20 | Spok1 Spok2+ x Spok1 Spok2 | 100% 4-spored asci | 50% Spok20: 50% Spok2A |
| SKT20 Spok2A×Spok2A | Spok1 Spok2+ x Spok2+ | 100% 4-spored asci | / |

Spok2A corresponds to the locus in strain T devoid of Spok2 but located at the same chromosomal location.
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GFP and mCherry tagging alter distorter or responder activities of Spok proteins

To gain some insight into the molecular mechanisms of Spok1 and Spok2 action, we tagged the two proteins at the carboxy- and amino-termini with GFP (Spok1) and mCherry (Spok2). Spok1-GFP and Spok2-mCherry proteins tagged at their C-termini were obtained by introducing the GFP or mCherry CDS upstream of the stop codon of Spok1 and Spok2. GFP-Spok1 and mCherry-Spok2 proteins tagged at their N-termini were obtained by inserting in vitro the GFP or mCherry CDS downstream of the Spok genes start codons. The chimaeric constructs were then inserted at the PaPKS1 locus. When crossed with the S, T, SKT20, SKT20D, Spok1D and Spok2D strains, the strains carrying the GFP and mCherry constructs exhibited unexpected patterns. Crosses of Spok1-GFP with all strains yielded 100% asci with four spores, indicating that, while unable to promote killing, the transgene enabled resistance to Spok1 and Spok2 killing. Similarly, crosses with Spok2-mCherry showed that this allele was unable to kill Spok2D, yet was resistant to Spok2. N-terminally tagged mCherry-Spok2 produced empty asci when crossed with Spok2D (Fig. 3D), as if the transgene conserved the killing activity but lost the responder activity. However, in crosses with the strain having Spok2 at the PaPKS1 locus, only 4-spored asci were obtained, as if the responder activity was restored in the presence of a wild-type copy of Spok2. Both Spok1 and Spok2 enabled resistance to mCherry-Spok2 killing, as crosses with S, SKT20 and PaPKS1::Spok1 yielded ascosporas with the expected segregation if full resistance occurred (Fig. 3E). On the contrary, GFP-Spok1 exhibited a pattern of asci expected for a protein endowed with both distorter and responder activities (data not shown). These data indicated that it was possible to independently inactivate either the responder or the distorter activities of Spok proteins, as previously gathered from the variant present in strain A.

Spok1 distorter and responder domains cannot be easily dissociated

In view of the above results, we tried to determine whether two separate domains carrying either the distorter or the responder activities could be identified in the Spok1 protein. In frame deletions in Spok1 were made in vitro and the truncated genes were reintroduced in the PaPKS1 gene, as done with full length Spok1 (Fig. S4). None of the construct carried any functional killing and resistance activity (n=30), showing that the two functions could not easily be separated on two independent DNA fragments.

Figure 3. Rosettes of asci in indicated crosses. (A) Spok1 and Spok2 are Sks with 90% and 40% FDS, respectively; (B) Spok1 and Spok2 have no role in ascospore determination; (C) Spok1 and Spok2 are functional at the PaPKS1 locus; the ascii with four dark spores results from SDS of the PaPKS1 locus; (D) complex phenotypes of mCherry-Spok2; (E) Spok1 and Spok2 confer resistance to mCherry-Spok2 spore killing; (F) complex phenotypes of Spok2AA. See text and table S1 for full genotype of strains.

Figure 4. Comparison of Spok1 and Spok2 protein sequences. The amino acids corresponding to the codons changed in the Spok2AA allele are underlined. Differences between Spok1 and Spok2 are shaded in grey.
Aspartate707/Glutamate708 are important for Spok2 activity

As mentioned above, a putative kinase domain was predicted at the C-terminus of Spok2, but not in Spok1. Pfam analysis [24] identified aspartate707 as a potential catalytic residue in Spok2. Because the next residue (n708) was a glutamate, which may substitute to aspartate707 in the catalytic center, we mutated both the aspartate707 and glutamate708 to alanines. The recovered mutator, Spok2AA, was inserted in the PaPKS1 gene in the Spok2 strain. When crossed with Spok2AA, the strain carrying Spok2AA yielded empty ascii, as if the distorter activity was active and the responder one was inactive (Fig. 3F). However, cross of Spok2AA with the strain carrying a wild-type Spok2 allele at the PaPKS1 locus produced ascii with four colorless ascospores (Fig. 3F). In such cross, both the Spok2 and Spok2AA ascospores survive, as if the Spok2AA responder activity was active. The behavior of the Spok2AA allele was thus identical to the one of N-terminally tagged mCherry-Spok2. Finally, when crossed with a strain carrying Spok1 at the PaPKS1 locus, ascii containing two colorless ascospores were obtained in 99% of the ascii, as if Spok1 acted alone.

Table 2. Spok Sk in P. anserina geographic races.

| strain | Spok1 | S/T | x S | x T | x SKT20 | x Spok1 | x Spok2 |
|--------|-------|-----|-----|-----|---------|---------|---------|
| A      | +     | 8/25| 0   | /   | 90      | 0       | 0       |
| B      | +     | 1/18| 0   | /   | 90      | /       | 40      |
| D      | +     | 1/18| 0   | /   | 90      | /       | 40      |
| E      | +     | 1/18| /   | /   | /       | /       | /       |
| F      | +     | 1/18| 0   | 90  | 90      | 40      | 40      |
| H      | +     | 0/19| 0   | 90  | 90      | 40      | 40      |
| I      | +     | 1/18| 0   | 90  | 90      | 0       | 40      |
| M      | +     | 1/18| 0   | /   | 90      | /       | 40      |
| N      | +     | 0/19| 0   | /   | 90      | 40      | 40      |
| s      | +     | 0/19| 0   | 90  | 90      | 40      | 40      |
| R      | +     | 0/19| 0   | 90  | 90      | 40      | 40      |
| U      | +     | 1/18| 0   | 90  | 90      | 0       | 40      |
| V      | +     | 1/18| 0   | /   | 90      | /       | 40      |
| W      | +     | 0/19| 0   | 90  | 90      | 40      | 40      |
| X      | –     | /   | 50  | 90  | 90      | 0       | 0       |
| Y      | +     | 14/9| 90  | 100  | 90      | /       | 90      |
| Z      | +     | 0/19| 0   | 90  | 90      | 40      | 40      |
| PSCJ14 | +     | 0/19| 0   | /   | 90      | /       | 40      |
| PSN14  | +     | 1/18| 0   | /   | 90      | /       | 40      |
| A406   | –     | /   | 0   | 0   | /       | 0       | 0       |
| CBS411.78 | –   | /   | 50  | /   | 90      | /       | 40      |
| CBS237.71 | +  | 14/9| 90  | 50  | 90      | /       | 90      |

The table gives the percentage of 2-spored ascii when crossed with the indicated strain. / means no progeny could be recovered due to sterility of the cross. CBS411.78 and CBS237.11 are labeled as P. comata in the Baarn collection, but are fully fertile when crossed with our P. anserina strains. PSCJ14 and PSN14 were recently isolated from nature (in 2003 and 2007, respectively). A406 was kindly obtained from D. P. Mahoney and A. E. Bell.

The genomes of P. anserina and other fungi contain genes related to Spok1 and Spok2

Mining available databases of complete genome sequences showed that homologues of Spok genes are present and prevalent in the genomes of many filamentous ascomycetes (spore sac fungi; Fig. 6). They are present in all major classes of Pezizomycotina except in the basal classes Orbiliomycetes and Pezizomycetes, but in a patchy distribution with closely related species having or lacking up to the post-meiotic mitosis: a diffuse cytoplasmic presence and an accumulation inside nuclei, as predicted by PSORT (Fig. 5). After this mitosis, at the stage at which two nuclei are present in each ascospore [23] (Fig. 1B), fluorescence was clearly discernible in nuclei of all ascospores in the ascii were spore killing occurred (mCherry-Spok2 x S and GFP-Spok1 x S crosses). At the beginning of ascospore development, fluorescence was detected in the nuclei of all spores (Fig. 5C and 5D); including those undergoing death. At later stages, fluorescence persisted only in the surviving two ascospores, while the two others degenerated (Fig. 5E and 5F). In ascii of crosses where no death occurs (Spok2-mCherry x S and Spok1-GFP x S), we could not detect fluorescence in two out of the four ascospores as early as after post-meiotic mitosis; the remaining two survived. This suggests that lack of death in these crosses was due to reduced levels of Spok1 or Spok2 in sensitive ascospores.
Spok-related genes. Numbers can go up to 9 and 11 Spok-like genes in the genomes of Fusarium oxysporum and Microsporum canis, respectively. Interestingly, tree construction with selected species showed that the Spok-like gene phylogeny did not follow the known evolution of fungi, indicative of possible horizontal transfers (Fig. 6). Moreover, they were often present as pseudogenes, identified by the presence of mutations interrupting the coding sequence or by truncation. P. anserina contains three more Spok-like genes (Pa_7_3950, Pa_4_4000 and Pa_1_5015), all with transposable elements in their vicinity. They are all present in S and T, each occupying the same locus in both strains.

**Spok triggers spore killing in other fungi**

To determine whether Spok1 was able to trigger ascospore death in another species, it was introduced under the expression of its own promoter in Sordaria macrospora along with a hygromycin B-
resistance marker. This fungus is related to *P. anserina*, even if the genetic distance (i.e., average percentage identity between orthologous proteins) between *Laesiophaeriaceae* to which *P. anserina* belongs and *Sordariaceae* to which *S. macrospora* belongs is equivalent to that between mammals and fishes [18]. It is homothallic and ascospore morphogenesis is different, as eight ascospores are differentiated around single nuclei [26]. Genome sequence analysis indicates that *S. macrospora* is devoid of *Spok* genes (Fig. 6). Eight transformants carrying *Spok1* were recovered and crossed to a strain devoid of *Spok1*. Resulting asci contained four wild-type-looking darkly-pigmented spores and four smaller often-abnormal unpigmented spores (Fig. 7). Wild-type-looking and abnormal ascospores were germinated and tested for resistance to hygromycin B and spore killing activity. 45 out of 66 wild-type-looking ascospores germinated, all were resistant to hygromycin B. 17 were successfully crossed to the strain devoid of *Spok1*. All showed a segregation of four normal and four abnormal ascospores. Two white ascospores out of 66 germinated. Both were resistant to hygromycin B. One was successfully crossed to the strain devoid of *Spok1*. Progeny was composed of asci with four wild-type-looking spores and four abnormal spores. The two germinated spores contained *Spok1* and likely corresponded to unripe ascospores devoid of pigments, as sometime seen in crosses. Therefore, *Spok1* is able to create meiotic drive in *S. macrospora*.

Finally, we introduced the *Necha2_82228* gene from *Nectria haematococca* (Fig. 6) into the *PaPKS1* gene of strain *Spok2*³, with its own promoter and terminator sequences. Despite being the closest relative of *Spok1/Spok2* on the tree of Fig. 6, the *Spok*N protein is only 34% and 33% identical to *Spok1* and *Spok2*, respectively. Note that the evolutionary gap between *P. anserina* and *N. haematococca*, is much larger than that between *S. macrospora* and *P. anserina*, arguing for strong differences in modality of ascospore differentiation [27]. *PaPKS1::Spok N* x *Spok2*³ crosses were barren, as all asci were empty, indicating that the *SpokN* protein had a non-autonomous killing action, reminiscent of the *Spok2AA* and mCherry-*Spok2* proteins that kills but does not allow for resistance. As for these two proteins, *PaPKS1::SpokN* x *PaPKS1::Spok1* and *PaPKS1::SpokN* x *S* crosses showed that *Spok1* and *Spok2* promote resistance to *SpokN*, respectively. Therefore, despite the great divergence between *Spok2* and *Spok1/Spok2*, killing activity is retained and meiotic drive could be promoted by linking genetically *SpokN* with a resistance factor such as *Spok1* or *Spok2*.

Figure 6. Phylogenetic tree of *Spok* and *Spok*-like genes in representative fungal species. ψ: putative pseudogenes. Right: species in grey: no detected *Spok* or *Spok*-like gene and pseudogene. The other species were color coded according to the known phylogeny.
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Discussion

The Spok MDs that we report here are constituted of single genes that carry both distorter and responder activities, unlike those of animals, plants and Neurospora, which are large and complex loci with two major genes, the distorter and the responder, and several additional ones that quantitatively modify the effects of either the distorter or the responder [3,5,6,8,14]. Unlike other MDs, Spok MDs may not have originated from cellular genes that have acquired additional functions disrupting normal gamete/spore formation, as they do not appear to be endowed with any physiological function. In P. anserina, another MD has been linked to the Het-s prion [16], in which spore killing results from an incompatibility reaction triggered by the co-presence of both the het-s protein in a prion-aggregated form and the soluble Het-S protein. A similar reaction is observed in vegetative hyphae when the two proteins are mixed together. From a genetic point of view, both the Het-s and Het-S alleles need to be present in crosses to see the MD effects. Spok genes act differently from the Het-s/Het-S MD, since their mere presence at a locus is sufficient to trigger the preferential transmission of this locus during meiosis. Spok genes thus define a new class of selfish elements that propagate vertically via meiotic drive and possibly horizontally in association with mobile elements. Indeed, at least in P. anserina, Spok-like genes are always in the vicinity of transposons and they do not seem to play any role in normal development.

Bioinformatic analysis did not provide many clues regarding the potential mode of action of Spok genes. An ATP-binding site of a kinase domain was predicted at the C-terminus of Spok2 with low probability, but not in Spok1, questioning its validity. A first model
based on the presence of such a domain to explain the dual activity of the Spok genes can be put forward as follows. Spok proteins could be bifunctional enzymes that catalyze both the formation of a toxin from cellular metabolites and its inactivation. The toxin could diffuse in the ascus, while the enzyme and hence the detoxifying activity could not. This should result in the death of the ascospores not expressing the Spok proteins. If the kinase domain is involved in the formation of the toxin, its mutation should result in an allele inactive for killing but active for resistance. On the contrary, if the kinase domain is involved in detoxification, the mutation should produce an allele active for killing and inactive for resistance. However, our data show that this model is unlikely since the mutation of the putative catalytic residues, Aspartate707/Glutamate708, has a more complex effect as it creates a Spok2Δ4 allele unable to resist the toxin it produces, which is fully resistant in the presence of a wild-type Spok2 allele in the cross. Therefore, it is as if wild-type Spok2 could activate the responder activity in Spok2Δ4, while Spok2Δ4 could not. A second model for the dual mode of action of Spok proteins could be inspired from the yeast killer toxins for which the preprotoxin is a precursor of the toxin but can confer resistance by complex formation with the toxin and subsequent degradation [28]. In yeasts, the preprotoxin genes are carried by double-stranded RNA viruses and are not known to trigger meiotic drive. Although, Spok1 and Spok2 do not present any obvious sequence similarity with the yeast killer toxin genes, we could propose that they would operate in a similar manner in which the preprotoxin (responder) is involved in resistance to the toxin (distorfer). In such model, killing and resistance will depend on a sublte balance between toxin production from preprotoxin processing and preprotoxin/toxin complexes degradation. This may account for all the features presented by Spok Sks, including cross-resistance triggered by Spok1 to the Spok2 Sk (Spok1 could inactivate the Spok2 toxin, while Spok2 could remove the Spok1 toxin), the inability to separate two domains by deletion analysis as well as the phenotype of the mCherry-Spok2, Spok2ΔΔ, SpokΔΔ alleles. Indeed, interactions between the Spok2 preprotoxin or toxin and the mCherry-Spok2, Spok2ΔΔ, SpokΔΔ proteins could result in their rapid degradation leading to inability to produce sufficient amounts of toxins. Cytological observations are also compatible with this model as GFP- and mCherry-tagged proteins that do not promote killing disappear very early during ascus maturation. A last model would posit that the Spok proteins may be the toxins and the Spok genes the responders that would inactivate the toxins by sequestering them at a defined place inside the nucleus. This would require protein/DNA as well as protein/protein interaction to allow the binding of many toxin molecules on few DNA sequences. In this model, the putative ATP binding site could thus be involved in DNA (nucleotide) binding rather than in a kinase activity. In this model, the Spok2 protein could bind the Spok1 gene, while the converse would not be possible explaining the resistance of Spok1 over Spok2 effects. The mCherry-Spok2, Spok2ΔΔ, SpokΔΔ proteins would be unable either to enter the nucleus or bind directly the Spok2ΔΔ gene, but could do so in the presence of the wild-type Spok2 protein through protein/protein interactions. It is also compatible with the nuclear localization of the Spok proteins after delimitation of the ascospores.

Spok1 and Spok2 have many similar homologues in a wide array of filamentous ascomycetes, including in P. anserina itself. These are present in a patchy phylogenetic distribution, even in the P. anserina populations, do not follow the known fungal evolution and are often present as pseudogenes. The hypothesis that meiotic drive elements are invasive, can result in fewer progeny and can transport bad hitchhikers may explain both the unusual phylogeny and pseudogenes. The fact that a Spok-like gene can be a resistance factor to other Spok-like Sks also complicates the evolution of this family of genes. As Spok1 alone is able to trigger meiotic drive in S. macrospora and because SpokΔΔ may do so in P. anserina when associated with a Spok resistance factor(s), we surmise that Spok-like genes may account, in part, for the additional Sks detected in many other fungi, including P. anserina itself.

Materials and Methods

All protocols for cultivation, genetic and molecular analysis with P. anserina are available at http://podospora.igmors.u-psud.fr. Similar culture techniques were used for S. macrospora. Crosses were performed on M2 minimal medium using the S-derived strains as females and T-derived strains as males (supplementary Table S1). Sk was detected by the presence of 2-spored asci in the F1 progeny and assignments to Spok1 or Spok2 were made by measuring the ratio of 2-spored versus 4-spored asci and by backcrossing the F1 progeny to the S, T, Spok1ΔΔ and Spok2ΔΔ strains and observing the F2 progeny. The sequence of Spok1 has been deposited in GenBank with accession n° JX560967.

Polyomarker maker analysis

Genomic DNA was extracted from 50 progenies of an S×T cross (ST1 to ST50). Markers were amplified by PCR with 5 min denaturation at 94°C followed by 30 cycles [30 sec 94°C, 30 sec 55°C, 1 min 72°C] and finished by 10 min elongation at 72°C. The primer pairs used are given in Supplementary Table S2. DNA was separated on 2% agarose gel. DNA extracted from the parental S and T strains was used as a control.

Deletion

Spok1 and Spok2 deletions were made on strains TΔmus51 and SΔmus51, respectively, as described for PaTLK2 in [29] using Hygromycin B resistance as a selection marker. Table S2 gives the primers used for deletions. The deletions were verified by Southern blotting as in [30].

Insertion of Spok alleles at the centromere of chromosome 2

Insertion of Spok alleles was made with a strategy involving integration of a plasmid with a single crossover into the PaPKS1 gene resulting in its inactivation. A 1395 bp DNA fragment from PaPKS1 was amplified by PCR using S genomic DNA and primers 193SsFSI and 193SsRSI. A 4344 bp DNA fragment surrounding Spok1 was amplified by PCR with the Pfu DNA polymerase from Promega (Madison, WI, USA) using T genomic DNA and primers 510FSI and 510RNI. The PaPKS1 segment was digested with SacI and NotI enzymes, the Spok1 fragment with SacI and NotI and both were ligated into pBG-phleo vector [31] cut with SacI and NotI to yield pEnterprise1. pEnterprise1 was introduced by transformation into the TΔmus51:sub-1 strain and one transformant devoid of pigment, was selected for further analysis. Spok2 and SpokΔΔ, including their own promoters and terminators, were fused by PCR with the 1395 bp PaPKS1 DNA fragment. The fused PCR fragments were digested with SacI and NotI and cloned into pB-Gphleo and pBC-Genet vectors to yield pEnterpise2 and pEnterpise-Nectria, respectively. Both plasmids were then introduced into P. anserina as for pEnterprise1. The same strategy was used to introduce GFP-Spok1 and mCherry-Spok2 and the truncated alleles (used primers in Table S2).
Creation of the Spok2<sup>2A</sup> allele
To create the Spok2<sup>2A</sup> allele, the plasmid pEnterprise2 was amplified by PCR using primers Spok2MutF and Spok2MutR (Table S2, in bold red are the nucleotides used to change the aspartate and glutamate codons to alanine ones). The PCR product was transformed into Escherichia coli and candidates were selected by sequencing the mutated region. One candidate was completely sequenced, found devoid of mutations and introduced in a Spok<sup>2A</sup> Anau51 strain. Two transformants were selected based on their lack of pigments and used for further analysis.

Insertion of Spok1 in S. macrospora
The Spok1 fragment was excised from pEnterprise1 and cloned into the pBC-Hygro plasmid cut with SalI and NotI. The plasmid Hygro containing Spok1 plasmid was transformed into the spo11 mutant of S. macrospora. The wild-type strain and 27 hygromycin B-resistant transformants were crossed with the spo55 mutant to force outcrossing.

Microscopy analysis
Perithecium contents for fluorescence analysis were prepared as in [26]. Pictures were taken with a Leica DMIRE 2 microscope coupled with a 10-MHz Cool SNAP HQ charge-coupled device camera (Roper Instruments). They were analyzed with ImageJ.

Phylogenetic analysis
The phylogenetic analysis of Supplementary Fig. S2 was carried out by aligning the sequences with MAFFT [32] and trimming them with Jalview to retain informative positions [33]. The tree was constructed using PhyML [34] with the default parameters [35] and 100 bootstrapped data sets. The tree was visualized with the iTOL server [36]. Pseudogenes were defined by the presence of several mutations (deletions, framemshifts or read-through) inactivating the coding sequences.

Supporting Information
Figure S1 Preferential transmission of T markers near the centromere (red dot) of chromosome 5 in 50 descendants of S x T cross. Letters indicate the parental origin of the markers in the progeny strains. Positions of markers on chromosome 5 are indicated at the top. Bracket defines the region with strongly biased transmission of T markers around 5PH1. The red arrow marks the position of *P. 5_10* (Spok2) in the genome of strain S. (TIF)

Figure S2 Comparison of Spok1 and Spok2 loci in strains S and T. The comparisons were drawn with the ACT genome comparison tool [37]. Identical regions are linked by red connections. Spok genes are in green. Neighboring genes are in light blue and mobile elements in other colors. The red lines depict the different deleted regions. (TIF)

Figure S3 Southern blot analysis of SKT20<sup>A</sup>, Spok1<sup>A</sup> and Spok2<sup>2A</sup> strains. Genomic DNA was extracted from the indicated strains and cut with appropriate restriction enzymes. (A) and (C) predicted structures of Spok1 and Spok2 loci before and after marker replacement. (B) and (D) results of Southern blots showing the expected bands. The DNA fragment labeled with * were used as probe. (TIF)

Table S1 Strains used in this study. (DOCX)
Table S2 Primers used for polymorphic marker analysis, gene deletions and cloning. (DOCX)

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Author Contributions
Conceived and designed the experiments: PG HL PS. Performed the experiments: PG HL FM. Analyzed the data: PG FM HL PS. Contributed reagents/materials/analysis tools: PS. Wrote the paper: PG FS.

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