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G3 Genes | Genomes | Genetics

G3, 2021, 11(8), jkab159
DOI: 10.1093/g3journal/jkab159
Advance Access Publication Date: 11 May 2021
Fungal Genetics and Genomics

Important role of melanin for fertility in the fungus *Podospora anserina*

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Abstract

Melanins are pigments used by fungi to withstand various stresses and to strengthen vegetative and reproductive structures. In *Sordariales* fungi, their biosynthesis starts with a condensation step catalyzed by an evolutionary-conserved polyketide synthase. Here we show that complete inactivation of this enzyme in the model ascomycete *Podospora anserina* through targeted deletion of the *PaPks1* gene results in reduced female fertility, in contrast to a previously analyzed nonsense mutation in the same gene that retains full fertility. We also show the utility of PaPks1 mutants for detecting rare genetic events in *P. anserina*, such as parasexuality and possible fertilization and/or apomixis of nuclei devoid of mating-type gene.

Keywords: melanin; sexual reproduction; *Podospora anserina* strain S; parasexuality; mating type

Introduction

Melanins are brown-green pigments widely used by living organisms for protection, especially against damaging UV radiations, as seen by tanning in human. Many fungi produce melanins that can block to some extent harmful radiations from a wide range of wavelength, scavenge reactive oxygen species, sequester metal ions, and strengthen cell wall [see Cordero and Casadevall (2017) for a review]. Melanins thus enable fungi to cope with extreme conditions, such as cold, heat, dryness, and pollution. Melanins also facilitate host invasion in parasitic species and participate in the proper development and toughening of vegetative and reproductive structures (*e.g.*, mycelia, sclerotia, sporophores, and spores).

In many ascomycetes, melanins are produced through the DHN (dihydroxynaphthalene) pathway that starts with the production of 1,3,6,8 tetrahydroxynaphtalene from acetyl-CoA by a polyketide synthase (PKS). This pathway appears to be the one used for melanin production at all stages of the lifecycle by all investigated fungi belonging to the *Sordariales* order, which includes model species such as *Neurospora crassa*, *Sordaria macrospora*, and *Podospora anserina*, but also species of industrial importance such as *Chaetomium* spp. used for the biodegradation of agricultural wastes, and for the production of enzymes and secondary metabolites (Coppin and Silar 2007; Engh *et al.* 2007; Hu *et al.* 2012; Ao *et al.* 2019). Intriguingly, inactivation of this PKS has different effects on fruiting body development depending upon the fungal species. In *N. crassa* and *S. macrospora*, inactivation of PKS results in apparently fertile unpigmented perithecia (Engh *et al.* 2007; Ao *et al.* 2019), while its inactivation by RNAi results in sterility in *Chaetomium globosum* (Hu *et al.* 2012). In this latter species, the PKS seems to be also involved in the production of chaetoglobosin, a secondary metabolite with antitumoral, antibacterial, and antifungal properties. Nevertheless, in *Ophiostoma piliferum*, belonging to the closely related *Ophiotsomatales* order, color mutants produce tiny and hyaline fruiting bodies containing few ascospores (Zimmerman *et al.* 1995). Because addition of scytalone, an intermediate of DHN melanin biosynthesis, partly restores pigmentation and normal development, it is likely that the PKS gene is affected in some of these *O. piliferum* color mutants.

In *P. anserina*, we previously reported (Coppin and Silar 2007) that the *PaPks1-193* mutants of the *PaPKS1* gene that contains a UGA stop codon after the acyl transferase domain (and hence most likely producing a protein lacking the dehydratase, acyl carriers, and thioesterase domains) differentiate normal-looking (albeit slightly less chubby) fertile perithecia, as observed for *N. crassa* and *S. macrospora*. Here, we show that the complete deletion of the PKS gene in *P. anserina* results in drastically reduced female fertility. This indicates that, while the tiny amount of 1,3,6,8 tetrahydroxynaphtalene potentially produced by the *PaPks1-193* mutant through translation readthrough may be sufficient to enable normal sexual development, the complete lack of PKS severely impairs fruiting body maturation. We also show that *PaPKS1* deletion mutants, as well as *PaPks1-193* mutants, can be used to detect rare genetic events such as parasexuality and, more surprisingly, to recover ascospores with nuclei deleted for their mating-type locus.

Materials and methods

Strains, media, and chemicals

The strains used in this study derived from the "S" (uppercase S) wild-type strain (Rizet and Delannoy 1950; Boucher *et al.* 2017)

Received: March 8, 2021. **Accepted:** May 5, 2021

used for sequencing (Espagne *et al.* 2008; Grognet *et al.* 2014). Recovery of the *PaPks1-193* mutant was described in Picard (1971) as the weakly pigmented *NG193* mutant. The sequence of the *PaPks1-193* allele and the recovery of transgenes complementing *PaPKS1* mutants have been previously described (Coppin and Silar 2007), as was the construction of the Δ*mat* strain (Coppin *et al.* 1993). Standard culture conditions, media, and genetic methods for *P. anserina* have been described previously (Rizet and Engelmann 1949; Silar 2013, 2020).

Deletion and complementation of *PaPKS1*

The *PaPKS1* coding sequence was replaced by a phleomycin-resistance gene with the split marker method (Silar 2013, 2020), using the primers depicted in Supplementary Table S1 (see also Supplementary Figure S1 for inactivation scheme). Transformation was made in a Δ*mus51::NourR* strain which favors integration of DNA at the homologous region (El-Khoury *et al.* 2008; Silar 2020). A primary phleomycin-resistant transformant with the expected lack of pigmentation was analyzed by PCR to validate that the correct gene replacement occurred (Supplementary Figure S1) and was then crossed to the wild type. In the progeny, we recovered strains having the *PaPKS1* gene correct replacement (hence being phleomycin-resistant) but lacking the Δ*mus51::NourR* mutation (hence being nourseothricin-sensitive). Their mating types were determined by crossing with *mat+* and *mat−* tester strains. One *mat+* and one *mat−* offspring were selected for further investigations. These strains were named *PaPks1-Δ mat+* and *PaPks1-Δ mat−*.

Complementation was assayed by crossing the *PaPks1-Δ mat+* strain with a *PaPks1-193 mat−* strain carrying a *PaPKS1*$^+$ wild-type allele associated with a hygromycin B resistance marker (Coppin and Silar 2007). This transgene carries only the *PaPKS1* gene and not the neighboring ones (Supplementary Figure S1). In the progeny, we recovered strains carrying both the *PaPks1-Δ* mutation and the ectopic *PaPKS1*$^+$ wild-type allele (these were resistant to both phleomycin and hygromycin B).

Data availability

Strains are available upon request. All Supplementary data have been uploaded to figshare. All data necessary for confirming the conclusions of the article are present within the article and its figures. Supplementary material is available at figshare: https://doi.org/10.25387/g3.14533716.

Results and discussion

The deletion of PaPKS1 results in female sterility

The replacement of *PaPKS1* with a phleomycin-resistance marker impaired sexual reproduction. Indeed, when grown in optimal conditions, *i.e.*, on M2 medium at 27°C under permanent light, the *PaPks1-Δ* mutant produced mostly tiny perithecia lacking the neck through which ascospore is normally ejected (Figure 1), with only a few perithecia with a neck, while *PaPks1-193* produced perithecia with well-formed necks under the same conditions. Moreover, perithecia contained much fewer asci in *PaPks1-Δ* than in the wild-type strain or than in *PaPks1-193* (Figure 1). Overall, *PaPks1-Δ* was able to eject few asci per Petri plates (<100), instead of the hundreds of thousand produced by the wild-type strain and *PaPks1-193*. This defect had a maternal origin, as shown by the normal maturation of perithecia in ♂ *PaPks1-Δ* × ♀ wild-type crosses and abnormal maturation in ♀ *PaPks1-Δ* × ♂ wild-type crosses (Figure 1). In addition, *PaPks1-Δ mat+* × *PaPks1-Δ mat−* × Δ*mat* mosaics differentiated a mixture of wild-type looking, *PaPks1-Δ*-looking and intermediate-looking perithecia (Figure 1), indicating that *PaPKS1*$^+$ hyphae provided by the Δ*mat* partner of the mosaics could rescue the fertility defect of *PaPks1-Δ*. Complementation with a transgene carrying the *PaPKS1*$^+$ allele resulted in complete restoration of fertility, indicating that the female fertility defect was actually due to the lack of *PaPKS1* and not to an additional mutation (Figure 1).

To assess whether the maternal defect of *PaPks1-Δ* was linked to problems in the mycelium or in the developing fruiting bodies, we grafted wild-type, *PaPks1-193* and *PaPks1-Δ* developing perithecia onto wild-type, *PaPks1-193* and *PaPks1-Δ* mycelia (Figure 2). While wild-type and *PaPks1-193* perithecia completed their maturation on all tested mycelia, no mature *PaPks1-Δ* perithecia could be obtained on any mycelium type, indicating that the *PaPks1-Δ* female fertility defect was due to problems within maturing *PaPks1-Δ* perithecia and not to defects in *PaPks1-Δ* mycelia (Silar 2014). The lack of melanin likely impaired neck formation as this structure is heavily melanized, altering further development.

In addition to the drastically reduced female fertility of the *PaPks1-Δ* mutant, we observed that many *PaPks1-Δ* ascospores burst upon landing on water agar (Figure 3). On the contrary, fewer *PaPKS1-193* ascospores burst upon landing, although they did so when touched, confirming that they likely still contained minute amounts of melanin. For both mutants, ascospores viable after landing germinated spontaneously (Figure 3), providing an easy way to recover mutant progeny.

Overall, these data showed that *PaPKS1* was essential in the fruiting bodies for proper maturation in *P. anserina*, as observed in *C. globosum* (Hu *et al.* 2012) and *O. piliferum* (Zimmerman *et al.* 1995). Intriguingly, the *PaPks1-Δ* phenotype was different from those observed in *S. macrospora* and *N. crassa PKS* mutants. However, neither in *S. macrospora* nor *N. crassa*, the *PKS* mutants analyzed so far had full gene deletions. In *S. macrospora*, the mutation was an insertion of a transposon at position 4710 of the *pks* gene (Engh *et al.* 2007) and in *N. crassa* the *per-1* mutant analyzed for fertility (Ao *et al.* 2019) was a 1-bp deletion causing frameshift at codon 38 (McCluskey et al. 2011). It is possible that the *PaPKS1-193* mutant, despite containing a UGA stop codon that truncates the PaPKS1 protein from essential catalytic domains, still produced tiny amounts of 1,3,6,8 tetrahydroxynaphtalene, in sufficient quantity to allow full development. Similar hypotheses can be put forward for the *S. macrospora* and *N. crassa* mutants. Interestingly, *PaPKS1-193* was originally described as being weakly pigmented, especially at low temperature (Picard 1971); incubation of *PaPKS1-193* at 18°C during ascospore production indeed resulted in ascospores pigmented in green, while those of *PaPks1-Δ* remained completely devoid of pigments at 18°C (Supplementary Figure S2). The best explanation to account for this production of melanin at low level is the occurrence of translation readthrough at the *PaPKS1-193* UGA stop codon. Note that *PaPKS1-193* has extensively been used to screen for mutants affected in the accuracy of translation that alter readthrough level (Picard 1973; Picard-Bennoun 1976). It is, however, not clear if fertility of *PaPKS1-193* is linked to the production of melanin or to another metabolite produced from 1,3,6,8 tetrahydroxynaphtalene that would signal development. In *C. globosum*, *pks-1*, the ortholog of *PaPKS1*, has indeed been shown to participate in both the biosynthesis of melanin and chaetoglobosin (Hu *et al.* 2012).

The *PaPKS1* mutants as tools to provide evidence for parasexuality and ascospores carrying Δ*mat* nuclei in *P. anserina*

PaPKS1 mutants have extensively been used to address genetic questions, starting with the fine structure of eukaryotic genes (Picard 1971; Touré and Picard 1972) and the genetic control of

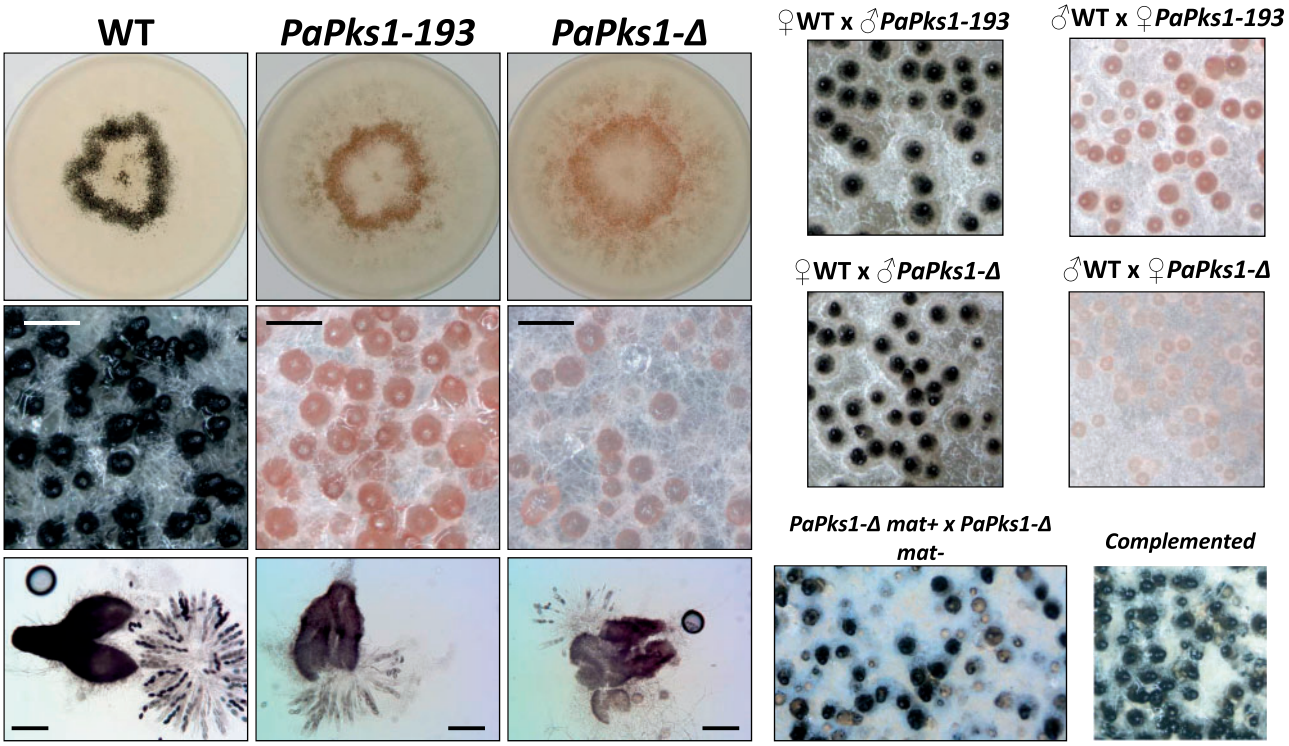


Figure 1 Sterility of *PaPks1-Δ*. Left: (Top) Eight-cm Petri plates were inoculated with *mat+/mat−* heterokaryotic strains of the indicated genotypes and incubated for 8 days, at which point the pictures were taken. While *PaPks1-193* differentiated a ring of perithecia with a morphology similar to the wild type except for its color, *PaPks1-Δ* differentiated a more diffuse ring. (Middle) Enlargement of the perithecia in the ring region; bar = 0.5 mm. (Bottom) typical perithecia were squashed to analyze their content; bar = 0.25 mm. Right: fruiting bodies from the indicated crosses obtained after incubation of 8 days. In all cases, further incubation of up to 3 weeks did not promote further maturation of fruiting bodies when *PaPks1-Δ* is used as female.

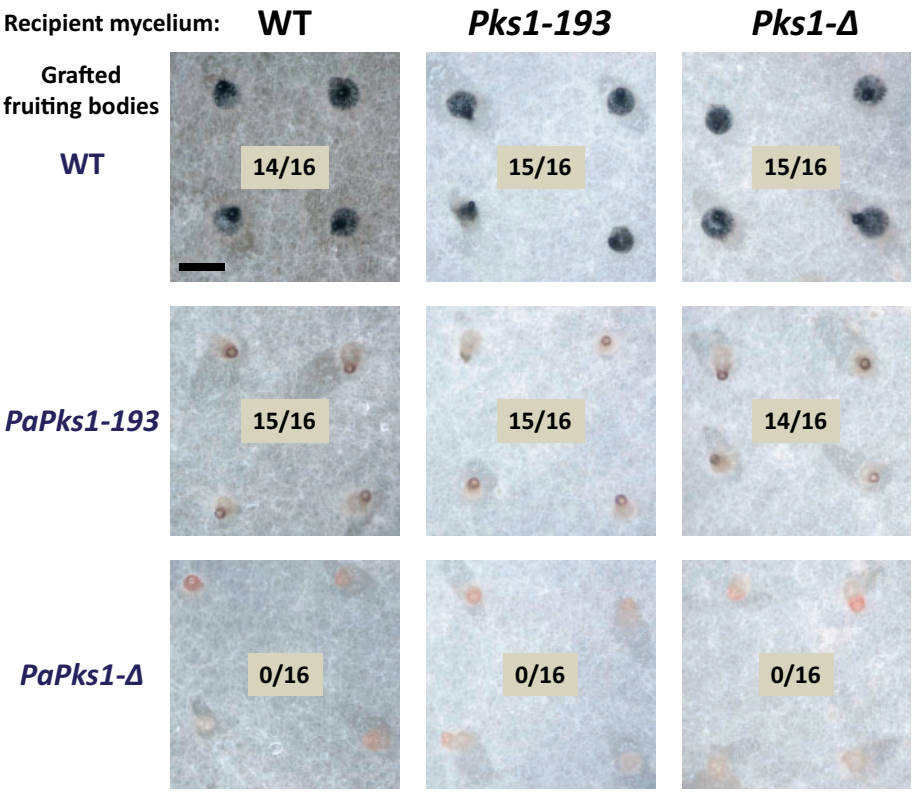


Figure 2 Graft analysis. Two-day-old fruiting bodies were grafted onto recipient mycelia grown onto M2 for 5 days and having the indicated genotype. The grafts were then incubated in optimal conditions for three additional days, at which time pictures were taken. Bar = 0.5 mm. Successful grafts among 16 attempts (number in the middle of the pictures) were determined by the observation of the differentiation of necks and expulsion of mature ascospores. Note that the lighter area in the center of the grafted *PaPks1-Δ* perithecia is due to the lighting required to take the pictures and not the presence of necks.

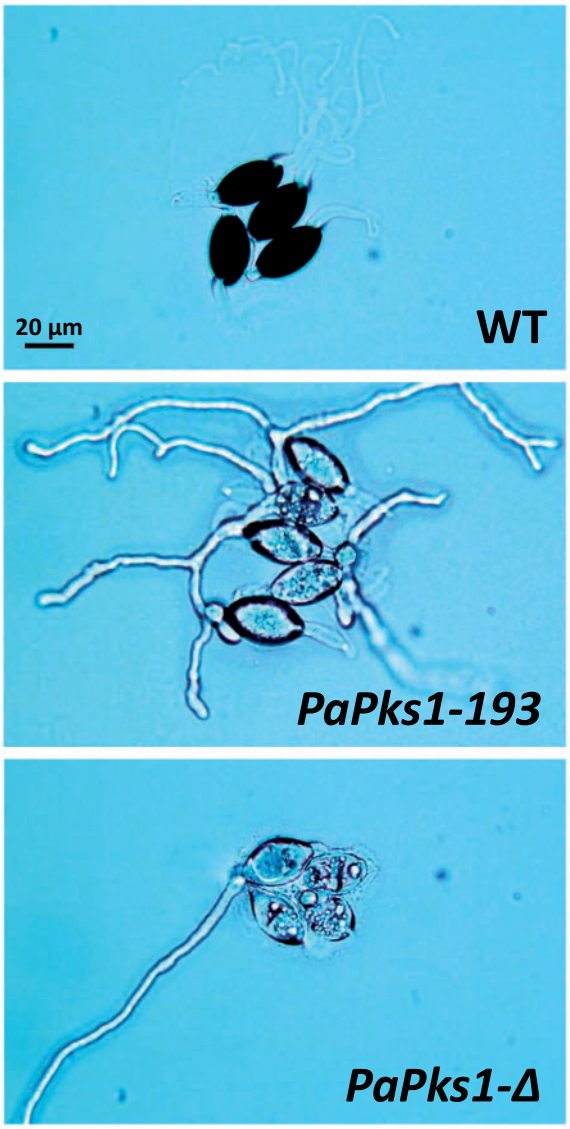
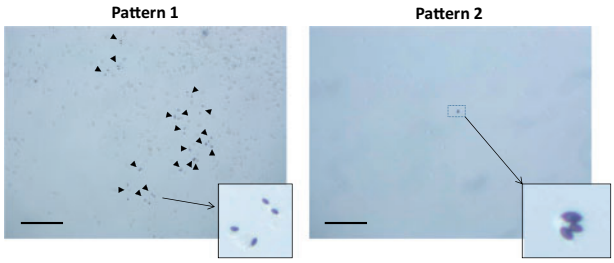


Figure 3 Ascospores of *PaPks1*-Δ. *PaPks1*-193 ascospores (middle) usually landed on water agar after ejection without collapsing, unlike *PaPks1*-Δ ones (bottom); in the depicted ascus, three ascospores collapsed and one did not. This viable *PaPks1*-Δ ascospore germinated spontaneously, as did the four viable *PaPks1*-193 ascospores. As seen on the top panel, wild-type ascospores did not germinate under these conditions and required a special G medium to do so.



	Pattern 1	Pattern 2
PaPks1-Δ mat+ x PaPks1-Δ mat- x Δmat	2	6
PaPks1-193 mat+ x PaPks1-193 mat- x Δmat	6	2

Figure 4 Patterns of presence of black ascospores in mosaics with Δ*mat*. The bottom table gives the observed number of cases among 47 examined plates for each genotype. Scale bar = 0.5 mm.

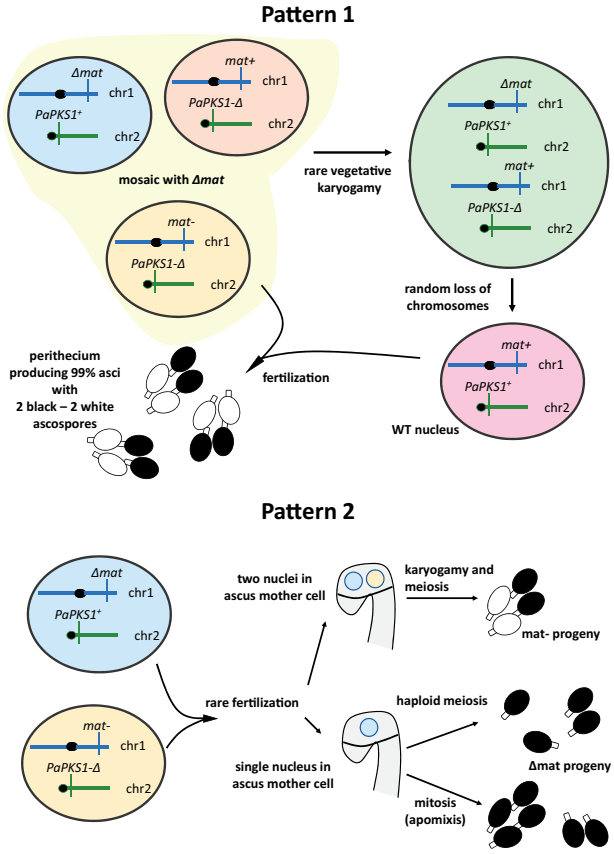


Figure 5 Possible models to explain pattern 1 and pattern 2 asci. In the case of a rare fertilization events involving Δ*mat* nuclei (pattern 2), most asci may proceed through haploid meioses as seen for other mating-type mutants (Zickler *et al.* 1995) and would abort due to abnormal migrations of chromosomes; hence few ascospores would end up with the correct set of seven nuclear chromosomes required for correct maturation of the ascospores.

translation accuracy through suppressor and antisuppressor screening (Picard 1973; Picard-Bennoun 1976). More recently, *PaPKS1* mutants were also used to assess the efficiency of RIP (repeat-induced point mutation) in *P. anserina* (Coppin and Silar 2007) and the ontogeny of fruiting bodies (Silar 2014). During our analyses of the *PaPks1*-Δ *mat*+ × *PaPks1*-Δ *mat*− × Δ*mat* mosaics, we serendipitously discovered that *PaPKS1* mutants also enabled to detect rare genetic events in *P. anserina*. Indeed, in the progeny of *PaPks1*-Δ *mat*+ × *PaPks1*-Δ *mat*− × Δ*mat* mosaics, we spotted unexpected black ascospores (Figure 4). Note that these black ascospores were very rare (<0.01%), most of the progeny being devoid of pigment as expected. We also identified rare black ascospores in *PaPks1*-193 *mat*+ × *PaPks1*-193 *mat*− × Δ*mat* mosaics. In both cases, black ascospores were detected in two distinct patterns on Petri dishes.

In the first pattern, numerous (>15) asci with two black spores clustered together in the middle of abundant colorless progeny (pattern 1 of Figure 4). This suggested that black spores were most likely produced by a single fruiting body or closely located ones on the Petri dishes. Phenotypic analyses of the progeny showed that these were self-fertile phleomycin-sensitive offspring and produced upon sexual reproduction wild-type asci. Hence, the black ascospores had the following genotype: *PaPKS1*$^{+}$

mat+/*PaPKS1*$^{+}$ *mat*−. Because *PaPks1*-Δ had no sequence homologous to *PaPKS1*, this indicated that, in the thalli of the mosaics prior to fertilization, some rare events led to recombination in the *PaPKS1*$^{+}$ gene, which is located near the centromere of chromosome 2 and is present in the Δ*mat* strain, with a functional mating-type locus carried by chromosome 1 and present in the *PaPks1*-Δ partners of the mosaics (Figure 5). The most likely explanation for this is the occurrence of parasexuality, *i.e.*, the vegetative formation of diploid nuclei by fusing Δ*mat* nuclei with *PaPks1*-Δ ones, followed by spontaneous and random loss of chromosomes to restore a haploid state. Parasexuality can thus result in the production of *PaPKS1*$^{+}$ nuclei carrying a functional mating-type locus (*i.e.*, wild-type nuclei) that can differentiate gametes that would engage fertilization with *PaPks1*-Δ ones of opposite mating type. This should result in the production of fruiting bodies producing asci with two black ascospores and two colorless ones, as seen in pattern 1. The occurrence of parasexuality was previously shown in *P. anserina* using strains carrying spindly growth-promoting translocations (Berteaux-Lecellier *et al.* 1995).

The second pattern (pattern 2) was more diverse and encompassed isolated ascospores, single two-spored asci and more rarely few (<5) clustered asci. Most offspring of such progeny were sterile and could not engage in mating with either *mat*+ or *mat*− wild-type strains. Hence, these ascospores carried Δ*mat* nuclei. This was surprising since the Δ*mat* strain has never been observed to undergo sexual reproduction (Coppin *et al.* 1993; Zickler *et al.* 1995). Intriguingly, in two instances, the two recovered black ascospores carried only the *mat*− allele, although the ascospore size indicated that they were typical binucleated ascospores. Overall, such pattern was reminiscent of the one obtained with *mat* mutants carrying point mutations in the genes present at the mating-type locus (Zickler *et al.* 1995) (Figure 5). In this instance, it was proposed that asci, obtained in low numbers, may result either from haploid meioses involving a *mat*-mutant single nucleus or from typical meioses triggered by the karyogamy of *mat* mutant nuclei with nuclei carrying a functional *mat* locus (in the two events detected here, it would have been *mat*− ones). Because fertilization involving a Δ*mat* nucleus has never been observed, it is possible that the genetic mosaics analyzed here increase the probability of obtaining or detecting such fertilization event involving a Δ*mat* nucleus. However, an appealing alternative explanation may be that apomixis occurs at low level in *P. anserina*. Apomixis is the production of "ascospores" in typical looking perithecia, but following only mitoses, without any meiosis. In this model, few Δ*mat* nuclei could end up in ascus mother cells, divide by mitosis, and produce few ascospores (Figure 5). Note that *Arnium arizonese* (= *Podospora arizonensis*), a close relative of *P. anserina*, reproduces exclusively by apomixis (Mainwaring 1971).

Conclusion

Complete lack of the PaPks1 enzyme results in severely diminished female fertility in *P. anserina*, while minute activity of this enzyme allows a normal sexual process. Mutants of PaPks1 proved again useful to analyze genetical features of *P. anserina*. They can reveal parasexuality, which had previously been shown to occur in *P. anserina*, and more surprisingly the possible involvement of nuclei lacking their mating type in the sexual process, either through some very rare fertilization events or through as-yet undescribed and rare apomictic events.

Acknowledgments

The authors thank Sylvie Cangemi for her expert technical assistance.

Funding

This work was supported by a Louis D. Foundation Award (Institut de France) and the EvolSexChrom ERC advanced grant no. 832352.

Conflicts of interest

None declared.

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Communicating editor: J. Berman