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Functional characterization of the sterigmatocystin secondary metabolite gene cluster in the filamentous fungus *Podospora anserina*: involvement in oxidative stress response, sexual development, pigmentation and interspecific competitions

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Summary

Filamentous fungi are known as prolific untapped reservoirs of diverse secondary metabolites, where genes required for their synthesis are organized in clusters. The bioactive properties of these compounds are closely related to their functions in fungal biology, which are not well understood. In this study, we focused on the *Podospora anserina* gene cluster responsible for the biosynthesis of sterigmatocystin (ST). Deletion of the *PaStcA* gene encoding the polyketide synthase and overexpression (OE) of the *PaAflR* gene encoding the ST-specific transcription factor in *P. anserina* were performed. We showed that growth of *PaStcA*$^{\Delta}$ was inhibited in the presence of methylglyoxal, while *OE-PaAflR* showed a little inhibition, indicating that ST production may enhance oxidative stress tolerance in *P. anserina*. We also showed that the *OE-PaAflR* strain displayed an overpigmented thallus mediated by the melanin pathway. Overexpression of *PaAflR* also led to sterility. Interspecific confrontation assays showed that ST-overexpressed strains produced a high level of peroxides and possessed a higher competitiveness against other fungi. Comparative metabolite profiling demonstrated that *PaStcA*$^{\Delta}$ strain was unable to produce ST, while *OE-PaAflR* displayed a ST overproduction. This study contributes to a better understanding of ST in *P. anserina*, especially with regard to its involvement in fungal physiology.

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Introduction

In filamentous fungi, secondary metabolites (SMs) are low-molecular weight compounds, typically encoded by biosynthetic gene clusters (BGCs) (Smith *et al.*, 1990). They are not strictly required for their normal growth and development but are critical to their lifestyle (Lind *et al.*, 2017). In particular, fungal SMs are known to be involved in protection from abiotic and biotic stressors and establishment of a secure niche, as well as displaying antimicrobial activity (Keller, 2015).

Among the fungal SMs, polyketides are a large class of SMs produced through the sequential condensation of small carboxylic acids mediated by polyketide synthases (PKSs) (Katz and Donadio, 1993). The polyketide sterigmatocystin (ST) was first isolated more than 60 years ago as pale-yellow crystals from *Aspergillus versicolor* (Birkinshaw and Hammady, 1957). This metabolite is produced by several fungal species, belonging to widely different genera, mainly *Aspergillus* spp., but also *Emericella*, *Chaetomium* and *Botryotrichum* (Rank *et al.*, 2011). ST is one of the ultimate precursors in the biosynthesis of the aflatoxins (AF) B1 and G1 produced by *Aspergillus parasiticus*, *Aspergillus flavus* and *Aspergillus nomius*, while it represents an end-product in *Aspergillus nidulans.* AF and ST cause mammalian hepatocarcinomas and animal toxicities (Terao, 1983; Anderson *et al.*, 1990). Ingestion of AF, linked to common contaminations by *A. parasiticus* and *A. flavus* in grain-based

products during storage, is considered a major human health risk (Ismail *et al.*, 2018). Since the discovery of these mycotoxins, many studies have focused on the mechanisms leading to their biosynthesis. In *A. nidulans*, the gene cluster responsible for the ST biosynthesis was identified by Brown *et al.* (1996). The ST cluster covers a 54 kb genomic region on chromosome IV and contains 24 co-regulated genes, most of which encode enzymes coordinately involved in ST biosynthesis, including *stcA* that encodes the core PKS. The *A. nidulans* ST cluster contains two regulatory genes, *aflR* and *aflJ* (Georgianna and Payne, 2009). The transcription factor AflR is a C6-zinc cluster DNA-binding protein required for the transcriptional activation of ST/AF biosynthetic genes. AflJ (also named AflS in *A. flavus* and *A. parasiticus*) may act as an enhancer through its interaction with AflR (Yu *et al.*, 2004; Georgianna and Payne, 2009).

Coprophilous fungi are considered as a potential reservoir for the discovery of novel SMs due to highly interspecific competition by microorganisms inhabiting the complex ecosystem (Bills *et al.*, 2013; Sarrocco, 2016). It is therefore likely that coprophilous fungi are integral to the chemical war occurring in interspecies competition, by producing SMs that kill or inhibit competing microbes. Among them, *Podospora anserina* is a typical filamentous ascomycete frequently recovered from herbivore dung, where many other species are present that feed on partially degraded plant material. It has been reported that *P. anserina* exhibits specific antifungal (Silar, 2013) and antibacterial (Lamacchia *et al.*, 2016) properties. *Podospora anserina* also has a long history of use as an efficient laboratory model because of its ease of molecular genetic manipulations, especially in the construction of multiple deletion strains (Silar, 2013). Sequence analysis of the *P. anserina* genome revealed its ability to produce 40 putative SMs including 18 PKS (Espagne *et al.*, 2008). However, until now, only a few chemical investigations have been conducted on this species and the contributions of SMs during its life cycle remain unclear. Two benzoquinones, anserinones A and B, isolated from *P. anserina* exhibited antifungal, antibacterial and cytotoxic activities (Wang *et al.*, 1997). Other *Podospora* spp. are known to produce diverse bioactive polyketide or furanone compounds in large amounts, such as podosporin A, sordarins or decipinin A, some of them exhibiting antibacterial or antifungal activities (Weber *et al.*, 1988; Che *et al.*, 2002; Webar *et al.*, 2005).

Slot and Rokas reported the existence of an intact 24-gene cluster, dispersed across 57 kb on chromosome II, in *P. anserina* that had a striking similarity to the *A. nidulans* ST cluster, suggesting the possibility of horizontal gene transfer between these species (2011). It was also shown that ST was detected in cultures of a freshly isolated *P. anserina* strain (Bills and Gloer, 2016). Nevertheless, the functional characterization of a fungal ST cluster in *P. anserina* has never been undertaken and the contribution of ST during its life cycle still remains unclear. There was a report of ST being involved in larvicidal activity against the malaria vector, *Anopheles*, after being isolated with other molecules from an endophytic *Podospora* species that is closely related, if not identical, to *P. anserina* (Matasyoh *et al.*, 2011).

In the present study, the gene deletion of *Pa_2_7410* (*PaStcA*; potentially encoding the ST-core PKS PaStcA) and gene overexpression of *Pa_2_7360* (*PaAflR*; potentially encoding the ST-specific transcription factor PaAflR) were performed, in order to better understand the role of ST production in the *P. anserina* life cycle. Mutants were assayed for defects in mycelial growth, sexual reproduction (e.g. perithecia and ascospore production), resistance to oxidative stresses, and their behaviour during interspecific confrontations. Our results show that deletion of *PaStcA* led to a clear sensitivity to the oxidant agent methylglyoxal (MG), reported to deplete intracellular glutathione inducing the production of reactive oxygen species (Du *et al.*, 2001), while the overexpression of *PaAflR* showed a little inhibition to oxidative stress, thus indicating that ST production may enhance oxidative stress tolerance in *P. anserina.* Moreover, we showed that overexpression of *PaAflR* led to hyperpigmentation of the thallus, to a female sterility phenotype and to a higher competitiveness in some interspecific confrontations. Finally, metabolite profiling of the ST mutant strains compared to the wild type allowed us to follow the production of ST in all these strains. We thus contribute to a better characterization of ST, especially in link with its involvement in the physiology of *P. anserina.*

Results

Generation of deletion mutants, overexpression and complementation

It was previously shown that the complete ST gene cluster in *P. anserina* could be horizontally transferred from *Aspergillus* sp and was located on chromosome II, as a 24 gene cluster dispersed across 57 kb (Slot and Rokas, 2011). The complete CDS of *Pa_2_7410* (named here *PaStcA*) and *Pa_2_7360* (named here *PaAflR*), potentially encoding the core ST PKS and the ST specific transcription factor, respectively, were retrieved from the annotated genome sequence of *P. anserina.* The open reading frame of *PaStcA* was interrupted by three putative introns and consisted of 2263 amino acids and share nearly 70% identity to the orthologous protein (PksA/AflC/StcA) found in *Aspergillus* species. Besides, *PaAflR* encodes a putative transcription factor orthologous to that of *A. nidulans* and shared about 37% identity. In addition, the *P. anserina* ST gene cluster organization is highly conserved and showed

Table 1. *Podospora anserina* strains used in this study.

Strains	Description	Source or reference
S	Wild type (WT)	Rizet (1952)
△*mus51:: hygro*^{R}	Full-length deletion of *Pa_6_6180*, WT background, hygromycin^R	El-Khoury *et al*. (2008)
PaPks1-193	Single mutation in *Pa_2_510*, WT background	Coppin and Silar (2007)
PaStcA$^△$	Full-length deletion of *Pa_2_7410*, WT background, geneticin^R	This study
PaStcA$^△$*comp*	Complementation of *PaStcA*$^△$, hygromycin^R	This study
OE-PaAflR	Overexpression of *Pa_2_7360*, WT background, hygromycin^R	This study
OE-PaAflR/*PaStcA*$^△$	Overexpression of *Pa_2_7360*, *PaStcA*$^△$ background, hygromycin^R, geneticin^R	This study
OE-PaAflR/*193*	Overexpression of *Pa_2_7360*, *PaPks1-193* background, hygromycin^R	This study

striking similarity in gene order and orientation when compared to that of *A. nidulans.*

In order to better understand the role of ST in fungal life cycle, the functional characterization of mutant lacking *PaStcA* (*PaStcA*$^△$) and strain overexpressing the ST specific transcription factor *PaAflR* in the wild-type background (*OE-PaAflR*) was then assayed. To verify that phenotypes observed in the *PaStcA* mutant was linked to the inactivation of the relevant gene, the wild-type allele of *PaStcA* was reintroduced into the corresponding mutant by transformation. Resistant transformants were then examined for growth and the development on M2 medium, supplemented or not by oxidative stress compounds, according to the phenotype of the single mutant, as described in the relevant section about oxidative stress response. Overall, three transformants out of four regained a wild-type phenotype in complementation experiments, confirming that phenotypes observed in the mutant was due to the deletion of *PaStcA.* One complemented strain was selected in relevant following experiments and was named *PaStcA*$^△$*comp.*

Overexpression strains were achieved by locating the *PaAflR* CDS under the control of the AS4 constitutive promoter. Three hygromycin resistant transformants were selected independently and crossed with the wild type. Progeny was recovered and F1-resistant transformants were selected. Thus purified, the three independent transformants were then characterized and displayed similar phenotypes. One of them was presented in the relevant following experiments and designated *OE-PaAflR.* By appropriate genetic crosses, overexpression of *PaAflR* was also introduced in *PaStcA*$^△$ background and the selected transformants were designated as *OE-PaAflR*/*PaStcA*$^△$. All the mutant strains constructed in this study were listed in Table 1.

Impacts of targeted gene studies on growth and morphology

To investigate the impacts of target genes on the vegetative growth of *P. anserina*, mutants and the wild-type strain were submitted to phenotypic analysis by incubating them on solid and liquid M2 media at 27°C which are the optimal condition for growth (Fig. 1). After 8-days incubation, we did not detect any difference in colony size, morphology and hyphal pigmentation, among *PaStcA*$^△$, *PaStcA*$^△$*comp* and the wild-type strain on solid M2 plates (Fig. 1A). Moreover, in liquid culture conditions, these mentioned strains behaved as the wild type, namely displaying an abundant dark-green pigmented mycelium and colourless culture filtrate that was similar to M2 control (Fig. 1B). In contrast, overexpression of *PaAflR* resulted in a significant overpigmentation of the *OE-PaAflR* thallus, compared to wild type on solid M2 plates (Fig. 1A). Besides, the *OE-PaAflR* liquid culture and filtrate presented a red-orange coloration (Fig. 1B). It should be also noticed that vegetative growth of *OE-PaAflR*/*PaStcA*$^△$ was similar to that of the wild type, indicating that this pigmented phenotype depends on ST synthesis and is probably not related to unspecific regulation of *PaAflR* on others BGCs. These results suggested that the lack of ST has no effect of vegetative fungal growth in our experimental conditions, and that overexpression of *PaAflR* is involved, directly or indirectly, in the pigmentation process of the thallus and in the production of coloured metabolites in liquid cultures.

In *P. anserina*, the main pigment is melanin, a polyketide whose biosynthesis requires a PKS encoded by the *PaPks1* gene. In order to better characterize which pigment is accumulated in our strains, we introduced the albino *PaPks1-193* strain that possesses a melanin-deficient background in our study (Coppin and Silar, 2007). By appropriate genetic crosses, overexpression of *PaAflR* was then introduced in *PaPks1-193* background and the selected strains were designated as *OE-PaAflR*/*193.* Interestingly, on agar plates, *OE-PaAflR*/*193* do not present this dark greenish coloration as the *OE-PaAflR* do, suggesting that the pigmentation is due to melanin accumulation. Additionally, *OE-PaAflR*/*193* showed an unprecedented colony morphology with a deep pink colour, when compared to the unpigmented *PaPks1-193* strain that was previously characterized (Coppin and Silar, 2007) (Fig. 1A). In liquid culture conditions, a yellow coloration was also evident in fungal culture and filtrate from *OE-PaAflR*/*193* (Fig. 1B and, C), whereas *PaPks1-193* displayed a colourless transparent filtrate, comparable to the M2 liquid medium, used as control.

Fig. 1. Vegetative growth of wild type and mutants.
A. Colony morphology of *P. anserina* strains on solid M2 medium.
B.Growth of *P. anserina* strains on M2 liquid culture.
C. Culture filtrate without mycelium.
Pictures were taken after 8-days incubation at 27°C. WT: wild-type strain. [Color figure can be viewed at wileyonlinelibrary.com]

Overall, these data imply that overexpression of *PaAflR* in both wild type and *PaPks1-193* contexts enhances accumulation of non-identified pigments that are secreted in the liquid medium. In contrast, the overpigmentation phenotype of the thallus was only observed when *PaAflR* was overexpressed in a wild-type background, suggesting that ST overexpression in *OE-PaAflR* could mediate overpigmentation via the melanin pathway.

During vegetative growth analyses, we observed that the edge of the wild-type strain displayed a dark pigmented line when meet with *OE-PaAflR* or *OE-PaAflR/193* strains (Fig. 2A). In order to better characterize this interesting point about the localized accumulation of pigments, confrontation experiments between different strains were realized (Fig. 2B). For that, two strains possessing the same mating type were spotted on a M2 plate. The distance between the two opposite inoculated points was gradually increased. The observed confrontation types included pigment formation and diffusion. We observed that, regardless the distance between the two partners, a dark pigmented line was found at the edge of the wild-type strain when it was confronted to *OE-PaAflR.* To decipher whether the pigment was able to diffuse from the hyperpigmented *OE-PaAflR* strain or was synthetized in the competitor, the wild-type strain was replaced by the melanin-deficient strain *PaPks1-193.* In this case, as shown in Fig. 2B, the localized pigment accumulation was not observed at the confrontation zone, suggesting that the pigment was not diffusible from the *OE-PaAflR* strain. Moreover, when wild type was replaced by the *PaStcA*$^{\triangle}$ deleted strain, we observed that the localized pigmentation was also present in *PaStcA*$^{\triangle}$, suggesting that ST production was not a prerequisite for pigment accumulation in the competitor. Lastly, in confrontations involving *OE-PaAflR/PaStcA*$^{\triangle}$ and wild-type strain, the localized accumulation of pigments was not observed, suggesting that ST production could be involved in this process. Taken together, our results suggested that wild type and *PaStcA*$^{\triangle}$ respond by a strong enhancement of melanin biosynthesis that could be due to a ST overproduction.

Impacts of targeted gene studies on sexual development

To determine whether our targeted gene studies could affect sexual development, fertility of wild type and mutant strains was tested by inoculating a mixture of fragmented *mat* + and *mat*-mycelia on solid M2 medium (Fig. 3A). As expected, a ring of perithecia was observed after 1 week of incubation in crosses involving *PaStcA*$^{\triangle}$ as in wild type, meaning that deletion of *PaStcA* had no effect on sexual development. In contrast, the *OE-PaAflR* strains developed a radial growth that almost covered the whole plate, and no perithecia were produced even though they were subjected to a longer incubation time (up to 1 month). To test whether overpigmentation could be the cause of this sterility, the same experiment was performed with the unpigmented *OE-PaAflR/193* strain. Results showed that this strain was also sterile, as *OE-PaAflR*, demonstrating that inability to produce perithecia was not linked to hyperpigmentation. In contrast, the *OE-PaAflR/PaStcA*$^{\triangle}$ strain could differentiate perithecia with the same pattern as wild type. Moreover, in crosses involving *PaStcA*$^{\triangle}$ and *OE-PaAflR/PaStcA*$^{\triangle}$, expelled ascospores are able to germinate as wild-type

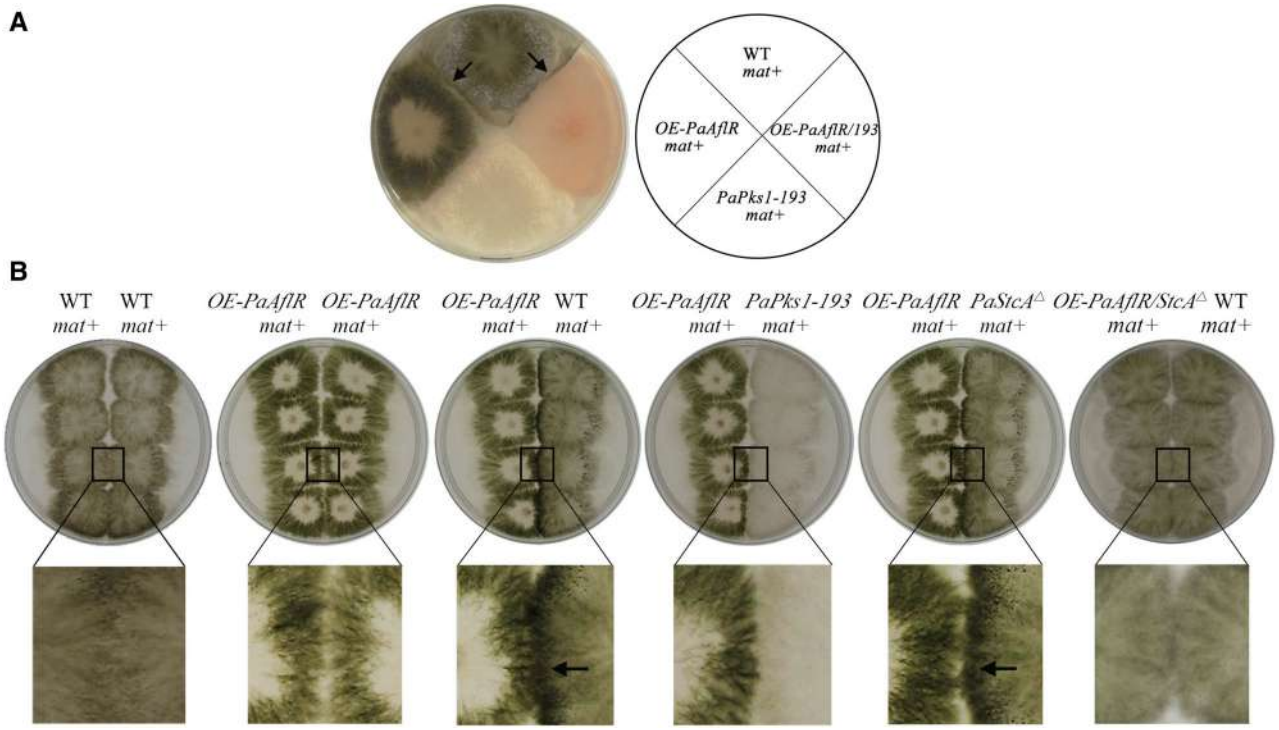

Fig. 2. Pigment accumulation caused by overexpression of *PaAflR*.
A. Strains were set up on M2 plate as indicated. Both *OE-PaAflR* and *OE-PaAflR/193* strains showed altered pigmentation. Arrows indicate the induced dark pigment in the edge of wild-type strain.
B. Two strains possessing the same mating type were spotted on a M2 plate, spanning increasingly gradient distance. Plates were incubated at 27°C for 5 days. Arrows indicate the hyperpigmentation in the interaction zone. [Color figure can be viewed at wileyonlinelibrary.com]

ascospores (results not shown). Our results suggested that the sterility could be due to ST overproduction.

Because the *OE-PaAflR* and *OE-PaAflR/193* strains were unable to produce perithecia, we investigated the male and female fertility of both by crossing them with the wild-type strain in spreading experiments, as indicated in Fig. 3B. When *OE-PaAflR* strain acts as the paternal partner, and the wild-type strain as the maternal partner, perithecia were observed only on the wild-type thallus, and to a lesser extent when compared with a strictly wild-type cross. Numbering of fertilized perithecia on three independent WT thalli of identical size, allowed us to estimate the production of spermatia by each partner; about 200 perithecia were formed on the WT (*mat-*) thallus when fertilized by *OE-PaAflR* (*mat+*) spermatia, and about 600 perithecia were counted when fertilized by WT (*mat+*) spermatia. So the production of *OE-PaAflR* spermatia seems to be reduced by 30%. While ascogonia were observed, no perithecia were found on the surface of the *OE-PaAflR* thalli, indicating that their ascogonia failed to be fertilized by wild-type spermatia. Reciprocal crosses with opposite mating type strain demonstrated similar results, indicating that the related phenotype was not dependent on mating type. Similar observations were noted with crosses involving *OE-PaAflR/193* and wild type. Overall, for both *OE-PaAflR* and *OE-PaAflR/193*, we showed that overexpression of *PaAflR* led to a clear female sterile phenotype. This result implies that overexpression of the transcription factor *PaAflR*, and then the overproduction of ST, either directly or indirectly, affects sexual development process and represses sexual differentiation of female gametes in *P. anserina.* Finally, we noticed that perithecia could be observed in the overpigmented zone of the wild-type strain, corresponding to the tiny area where mycelia of wild type and *OE-PaAflR* or *OE-PaAflR/193* strains converged, as described earlier (Fig. 3B). These observations confirm that the defect in fertility in *OE-PaAflR* or *OE-PaAflR/193* was not directly linked to a pigment accumulation.

Role of ST production in oxidative stress response

Oxidative stress assays were performed on the wild-type strain, the *PaStcA*$^{\triangle}$, *OE-PaAflR* and *OE-PaAflR/PaStcA*$^{\triangle}$ mutant strains, and the *PaStcA*$^{\triangle}$*comp* strain (Table 2; Fig. 4). All the constructed strains showed no apparent effect on growth rate compared to wild type on M2 medium. MG is mainly generated as a cytotoxic by-product of glycolysis, it has been reported that MG depletes intracellular glutathione inducing the production of reactive oxygen species

(Du *et al.*, 2001). An excess of MG can increase ROS production and cause oxidative stress (Desai *et al.*, 2010) and thereby is commonly used in phenotypic assays. *PaStcA*$^{\triangle}$ mutant showed a high sensitivity to oxidative stress generated by MG at 2.5 mM, as its growth was inhibited of about 30% on this medium when compared to wild type. The complementation strain restored tolerance to MG indicating that the observed effects could be attributed to the deletion of *PaStcA.* The *OE-PaAflR* strain also displayed resistance to oxidative stresses, as this strain is able to grow on MG at 10 mM and to H_2O_2 at 1 mM (Table 2). Our findings suggest that ST might protect *P. anserina*, either directly or indirectly, from oxidative stresses.

Role of ST production in interspecific confrontations

The fungal species used in this experiment as challengers were selected according to their different biological characteristics. The basidiomycete *Trametes versicolor* is a common saprophytic polypore generally found on fallen trees. The leotiomycete *Botrytis cinerea* and the eurotiomycete *Penicillium chrysogenum* belongs to the ascomycete lineage. *Botrytis cinerea* is a necrotrophic fungus that is at one stage of its life cycle plant pathogen. *Penicillium chrysogenum* is described as a saprophytic fungus, commonly found in soil and decaying matter (Silar, 2005).

It was shown that interspecific confrontations may be associated with peroxides production by one or both competitors (Silar, 2005). We investigated the ability of the *P. anserina* wild-type strain and mutants to produce peroxides. Firstly, without challengers, we showed that the *OE-PaAflR* strain was able to produce high level of peroxide over all of the thallus (Fig. 5A). When *P. anserina* is challenged with competitors, we noticed that high levels of peroxides were specifically accumulated at the confrontation zone as previously described (Silar, 2005). For each challenger species tested, the level of peroxide accumulation in the confrontation zone was consistent, across all the *P. anserina* strains. Moreover, when *OE-PaAflR* was confronted with *T. versicolor* or *B. cinerea*, peroxide accumulation was highly enhanced in the thallus of ST-overexpressed strains, as well as at the confrontation zone (Fig. 5A).

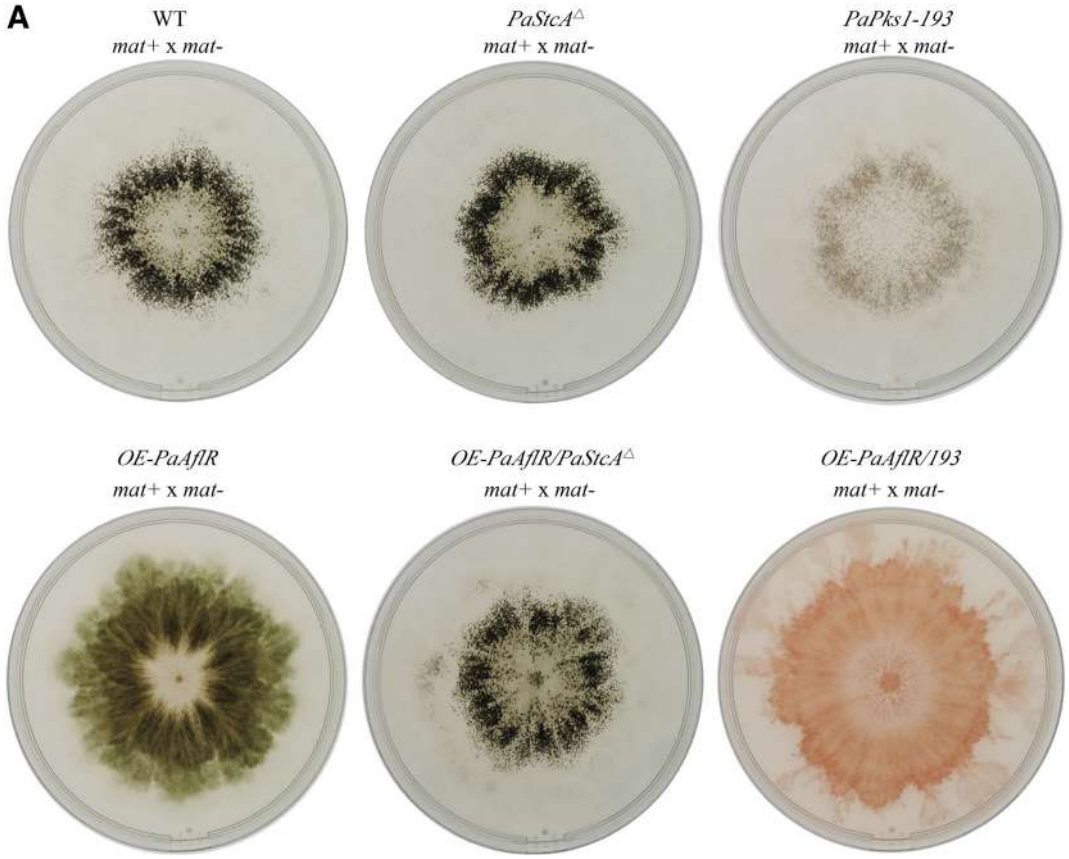

Fig. 3. Sexual development analysis.
A. Fertility of wild-type and mutant strains on M2 medium. Fragmented mycelia from *mat* + and *mat*-strains were mixed and then inoculated in the centre of plate under constant light illumination. Photographs were taken after 8 days of incubation.
B. Spreading experiments. Crosses were made by inoculating the strains 1.5 cm apart. After 3 days of growth, 1.5 ml of water was added and spread all over the plate. The pictures were taken 5 days after fertilization, at which time fully mature perithecia expel ascospores. Perithecia are visible as small dots in the close-up view. [Color figure can be viewed at wileyonlinelibrary.com]

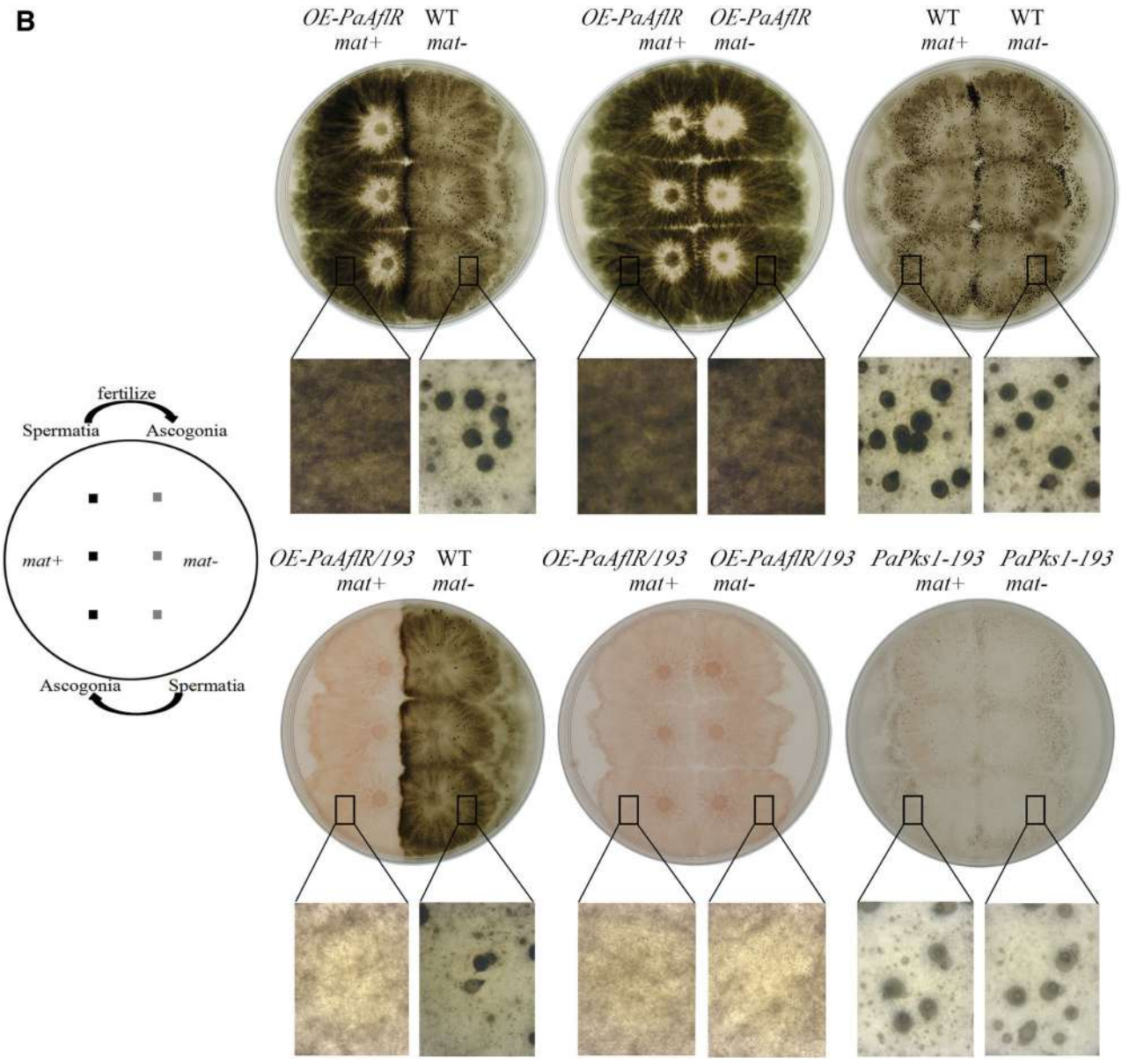

Fig. 3. (Continued). [Color figure can be viewed at wileyonlinelibrary.com]

Secondly, we evaluated the role of ST production in the cell death reaction, specifically at the confrontation zone, when the *P. anserina* wild-type strain and mutants were confronted with competitors. This was observed as the accumulation of dead cells specifically stained by Trypan blue at their confrontation zones and specifically on the thalli of each *P. anserina* strain. The *T. versicolor* challenger was able to kill *P. anserina* wild-type, *PaStcA*$^{\triangle}$ and *OE-PaAflR*/*PaStcA*$^{\triangle}$ strains. However, when *OE-PaAflR* was confronted with *T. versicolor*, dead cells accumulated at the confrontation zone, and specifically on the thallus of *T. versicolor* (Fig. 5B). Such results mean that overexpression of *PaAflR* prevents cell death on the *P. anserina* thallus by first killing *T. versicolor* cells. When *B. cinerea* was tested against our *P. anserina* wild type, *PaStcA*$^{\triangle}$ and *OE-PaAflR*/*PaStcA*$^{\triangle}$ strains, no cell death was observed. Yet when *OE-PaAflR* was confronted with *B. cinerea*, dead cells accumulated at the confrontation zone and specifically on the thallus of *B. cinerea* (Fig. 5B). The *P. chrysogenum* tests showed complete susceptibility to all of our *P. anserina* strains, evidenced by the accumulation of dead cells at each respective zone of confrontation and on each thallus of *P. chrysogenum* (data not shown). Our findings that *P. anserina* is a successful antagonist to *P. chrysogenum* supports those described by Silar (2005). We also showed that *OE-PaAflR*/*193* behave as *OE-PaAflR*

Table 2. Growth of strains exposed to oxidative stress.

	Control	MG 2.5 mM	MG 5 mM	MG 10 mM	H_2O_2 100 µM	H_2O_2 500 µM	H_2O_2 1 mM	Men 10 µM	Men 25 µM	Men 50 µM	TBY 10 µM	TBY 50 µM	TBY 75 µM
WT	3.55 ± 0.05	2.95 ± 0.09	1.62 ± 0.05	0	3.25 ± 0.07	1.86 ± 0.08	0	2.91 ± 0.08	2.37 ± 0.08	0	3.24 ± 0.05	2.88 ± 0.04	0
PaStcA$^{\triangle}$	3.54 ± 0.05	**0.96 ± 0.05**	**0.33 ± 0.02**	0	3.21 ± 0.07	1.86 ± 0.08	0	2.94 ± 0.06	2.27 ± 0.05	0	3.21 ± 0.06	2.93 ± 0.05	0
PaStcA$^{\triangle}$ *comp*	3.56 ± 0.05	2.94 ± 0.05	1.59 ± 0.05	0	3.22 ± 0.08	1.83 ± 0.1	0	2.92 ± 0.14	2.35 ± 0.05	0	3.25 ± 0.07	2.88 ± 0.1	0
OE-PaAflR/ *PaStcA*$^{\triangle}$	3.52 ± 0.06	2.91 ± 0.10	1.65 ± 0.10	0	3.21 ± 0.07	1.87 ± 0.06	0	2.94 ± 0.07	2.33 ± 0.08	0	3.19 ± 0.06	2.94 ± 0.05	0
OE-PaAflR	3.53 ± 0.04	2.99 ± 0.05	**2.11 ± 0.08**	**1.50 ± 0.04**	3.25 ± 0.07	1.87 ± 0.07	**1.88 ± 0.06**	2.94 ± 0.06	2.29 ± 0.05	0	3.24 ± 0.05	2.93 ± 0.04	0

Table gives the colony diameter (cm) of each strain after 5 days growth. Depicted values are means ±SD of three independent experiments. Bold values ($P < 0.001$) indicate significant differences of each strain relative to the wild type (WT) as determined by Student's *T-test*. MG = methylglyoxal; Men = menadione; TBY = t-butyl hydroxyperoxide.

throughout confrontation experiments (results not shown). Overall, we evidenced here that overexpression of *PaAflR* could trigger high level accumulation of peroxides in ST-overpressed strains and led to cell death reaction when confronted to some challengers.

Involvement of PaStcA *and* PaAflR *in ST biosynthesis*

In addition, monitoring of the qualitative ST production was performed by high-performance liquid chromatography-UV analysis. The reference ST was isolated as the major compound from a liquid cultivation medium of the WT strain and its NMR and MS spectral data were compared with the literature. Its retention time was defined at 27.3 min (Fig. 6). It is worth mentioning here that all the extracts were dissolved in the same volume of MeOH. Therefore, direct comparison of the peak intensity (mAU) allows a semi-quantitative analysis of the chromatograms. Interestingly, at 254 nm, the HPLC-UV profile of the WT strain evidenced three major peaks at 16.1, 19.1 and 27.3 min respectively. Deletion of the *PaStcA* gene dramatically impacted the ST production, with the two other major peaks remaining and a similar HPLC profile. The chromatogram of the complemented strain confirmed the reactivation of ST production albeit in a moderate intensity compared to WT. In addition, the peak at 16.1 min has disappeared indicating that other metabolites pathways were also impacted. As expected, the chromatogram of the *OE-PaAflR* mutant revealed the accumulation of ST with a mAU intensity higher than 2000. It is reasonable to assume that the overexpression of ST gene cluster resulted in a higher production of the corresponding metabolite. Moreover, the chromatogram is more complex with the presence of new peaks in the baseline together with a new peak at 19.8 min. Interestingly, the peak at 16.1 min which was the most intense in the WT strain and was detected in the other mutants is totally absent in this strain. In addition, it was clearly detected as the sole interesting peak in the chromatogram of the *OE-PaAflR*/*PaStcA*$^{\triangle}$ mutant. These results suggest that the ST transcription factor could also regulate other metabolites pathways.

Discussion

Given the abundance of predicted metabolic gene clusters present in the genome of *P. anserina*, and the fact that, to the best of our knowledge, only melanin biosynthesis genes, which do not form a cluster, have been functionally characterized to date (Coppin and Silar, 2007; Xie *et al.*, 2018), it is clear that there is still a plethora of SMs yet to be discovered. In the present work, we focused on the role of ST production in physiology of *P. anserina* by the functional characterization of the polyketide core gene *PaStcA* and the specific transcription

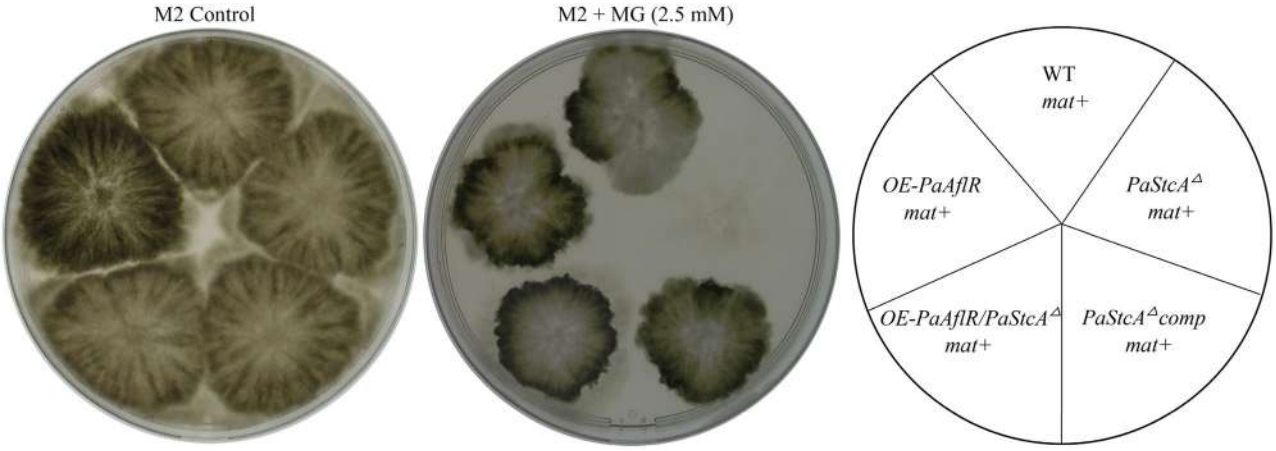

Fig. 4. Growth of *P. anserina* strains on M2 plates exposed to oxidative stressors.
Pictures were taken after 5 days incubation at 27°C. The *PaStcA*$^{\triangle}$ mutant strain displayed almost no growth on M2 medium supplemented with MG at 2.5 mM. MG: methylglyoxal, WT: wild-type strain. [Color figure can be viewed at wileyonlinelibrary.com]

factor *PaAflR* of the ST gene cluster. Genes and enzymes involved in ST metabolic pathways have been known for many years, but their molecular characterizations were mainly related to *Aspergillus* species, despite its discovery in several different fungal genera (Brown *et al.*, 1996; Rank *et al.*, 2011; Kjærbølling *et al.*, 2018). Previous work has established that *stcA* and *aflR* are required for the biosynthesis of ST in *A. nidulans* and AF in *A. parasiticus* and *A. flavus* respectively. Inactivation of *stcA* leads to the blockage of this pathway and disruption or mutation of *aflR* inhibits expression of AF/ST clustered genes. Conversely, overexpression of *aflR* in the genome increases biosynthetic gene expression and SM production (Woloshuk *et al.*, 1994; Yin and Keller, 2011). The transferred BGCs might undergo marked changes during evolution, including gene loss and/or gain, gene rearrangement, and divergence of gene function (Cardoza *et al.*, 2011). The red pigment bikaverin gene cluster was horizontally transferred from *Fusarium* to *Botrytis*, even though several *Botrytis* species have lost this cluster but still retained one of its six genes, the non-pathway-specific regulator bik4 (Campbell *et al.*, 2012, 2013; Rokas *et al.*, 2018). Fusarin C gene cluster was consisted of nine co-regulated genes (*FUS1-FUS9*) in genus *Fusarium* (Niehaus *et al.*, 2013; Wiemann *et al.*, 2013); however, a similar gene cluster existed in *Metarhizium species*, which was able to produce NG-391 and NG-393 (analogs of Fusarin C) (Krasnoff *et al.*, 2006; Donzelli *et al.*, 2010). This cluster was partially conserved in *Trichoderma reesei*, which has only three genes (*FUS1*, *FUS2* and *FUS3*) left, until now no fusarin-like products has been characterized (Martín *et al.*, 2014).

In *P. anserina*, even if little is known about the role of ST in fungal physiology, the related gene cluster has been well characterized and reported as likely obtained through horizontal gene transfer (Slot and Rokas, 2011). Our deletion of the core PKS gene *PaStcA* in *P. anserina*, as expected, blocked the ST pathway and therefore ST production. Moreover, according to the universal strategies to activate silent gene clusters (or to strengthen low-expressed clusters), overexpression of the encoding activator (*PaAflR*) of the ST gene cluster was performed and resulted in the excessive accumulation of ST. Interestingly, we observed red-orange and yellow-colorations in culture filtrates from strains overexpressing the *PaAflR* transcription factor in both wild-type and *PaPks1-193* backgrounds. However, it is well known that other pigmented components, as norsolorinic acid, averantin and versicolorin are intermediate compounds in the AF/ST pathway (Butchko *et al.*, 1999; Trail *et al.*, 1995). So, further investigations should be undertaken to isolate and precisely identify which pigments are involved in the coloration of culture filtrates of ST-overproduced strains. Nevertheless, we observed that not only ST was accumulated but also some additional unknown compounds were accumulated, and a major peak (16.1 min) was clearly absent in the *OE-PaAflR* strain. Moreover, we showed here that *PaAflR* is not only involves in the ST regulation but also regulates the melanin production. Our findings were in accordance with previous published works in relation with the occurrence of cross pathway regulation by BGC transcription factor. Although a pathway-specific transcription factor was originally thought to solely regulate the genes within a given BGC (Fernandes *et al.*, 1998; Ehrlich *et al.*, 1999), it has been shown that some specific transcription factors also regulate genes within other BGCs and several metabolic pathway (Keller, 2019). Price *et al.* (2006) indicated that the so-called pathway-specific regulator *aflR* not only regulated genes within the aflatoxin (AF) cluster but also induced additional three genes (*nadA*, *hlyC* and *niiA*) expression outside the AF cluster in *A. parasiticus.* And they also pointed that the number of such genes outside as being associated with *aflR* expression is likely underestimated. In

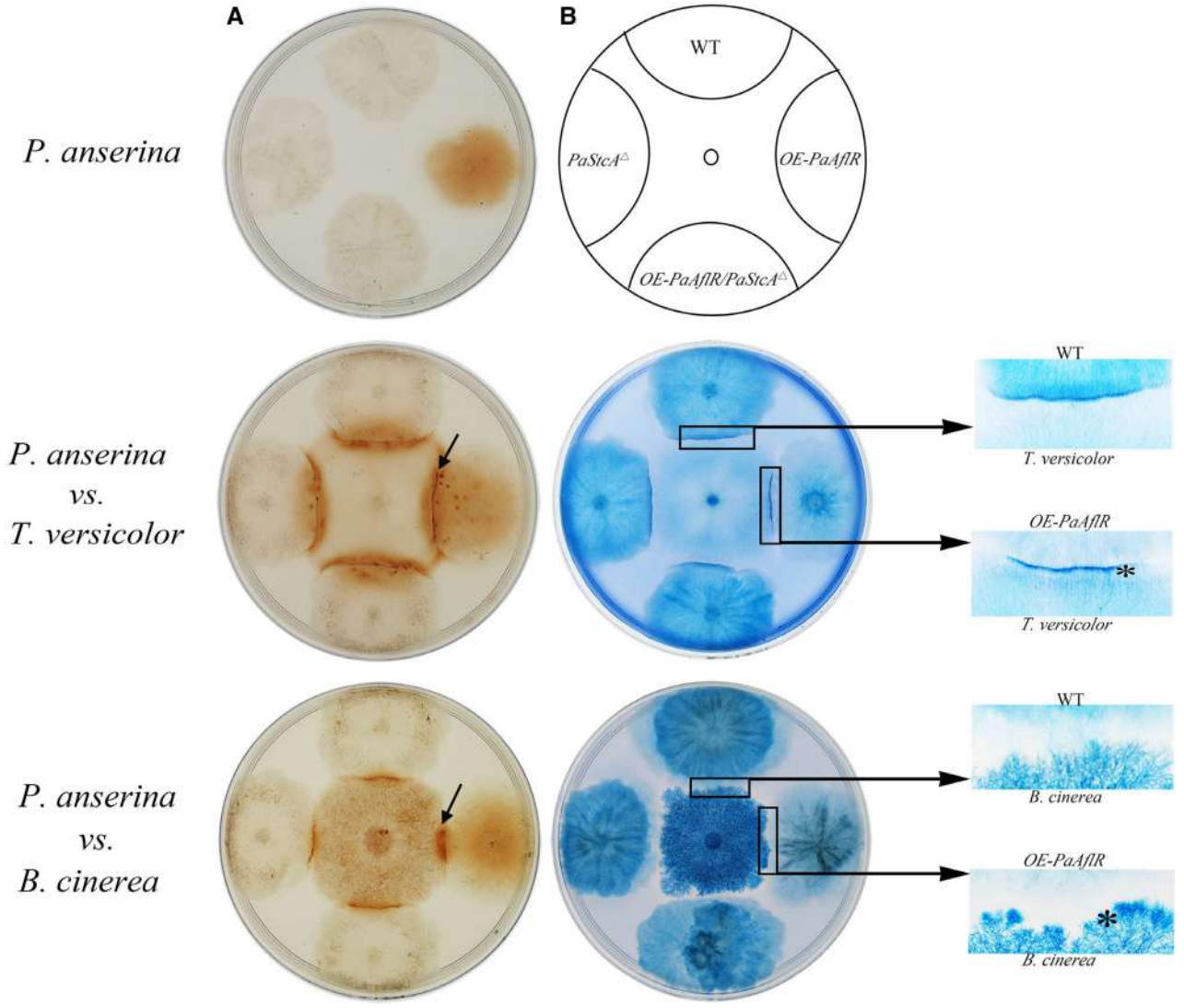

Fig. 5. Confrontation of *P. anserina* strains against other fungal species. Peroxide detection (A) and cell death assay (B) were conducted as previously described (Silar, 2005).
A. Peroxide detection. Arrows indicate accumulation of peroxide that is enhanced when *OE-PaAflR* is involved.
B. In close up views, asterisks indicate the Trypan blue stained dead cells triggered by *OE-PaAflR* in the contestant strains. [Color figure can be viewed at wileyonlinelibrary.com]

addition, Bergmann *et al.* (2010) unequivocally demonstrated an example of crosstalk regulation between SM gene clusters in fungi. The *inp* pathway-specific transcription factor *scpR* played a dual role in *A. nidulans*, induction of *scpR* triggers expression of not only NRPS-encoding *inp* gene cluster but also physically unrealated PKS-encoding asperfuranone (*afo*) gene cluster on the other chromosome. Meanwhile, four genes (*easA*, *easB*, *easC* and *easD*) involved in emericellamide biosynthesis, which do not form a cluster, were also upregulated under *scpR*-overexpressing conditions. Combined with the complexity of interconnecting SM networks in fungi as mentioned earlier, we speculated that our studies indicating that the transcription factor *PaAflR* may have the potential to directly regulate several distant metabolite pathway genes, or the accumulated ST product induced by *PaAflR* would indirectly interact with the regulation of other unknown SM gene locus.

We showed that deletion of the core PKS gene *PaStcA* in *P. anserina*, and thus the lack of ST production, has no visible effect on fungal morphology or in sexual reproduction. These observations are in accordance with the general definition of SMs that is now largely consensual, and which is based on the fact, that SMs are not involved in global fungal physiology but are more specifically crucial players in defence mechanisms as a response against the challenging environment (Keller, 2015). However, we found that our *OE-PaAflR* and *OE-PaAflR/193* strains were female sterile, probably due to female

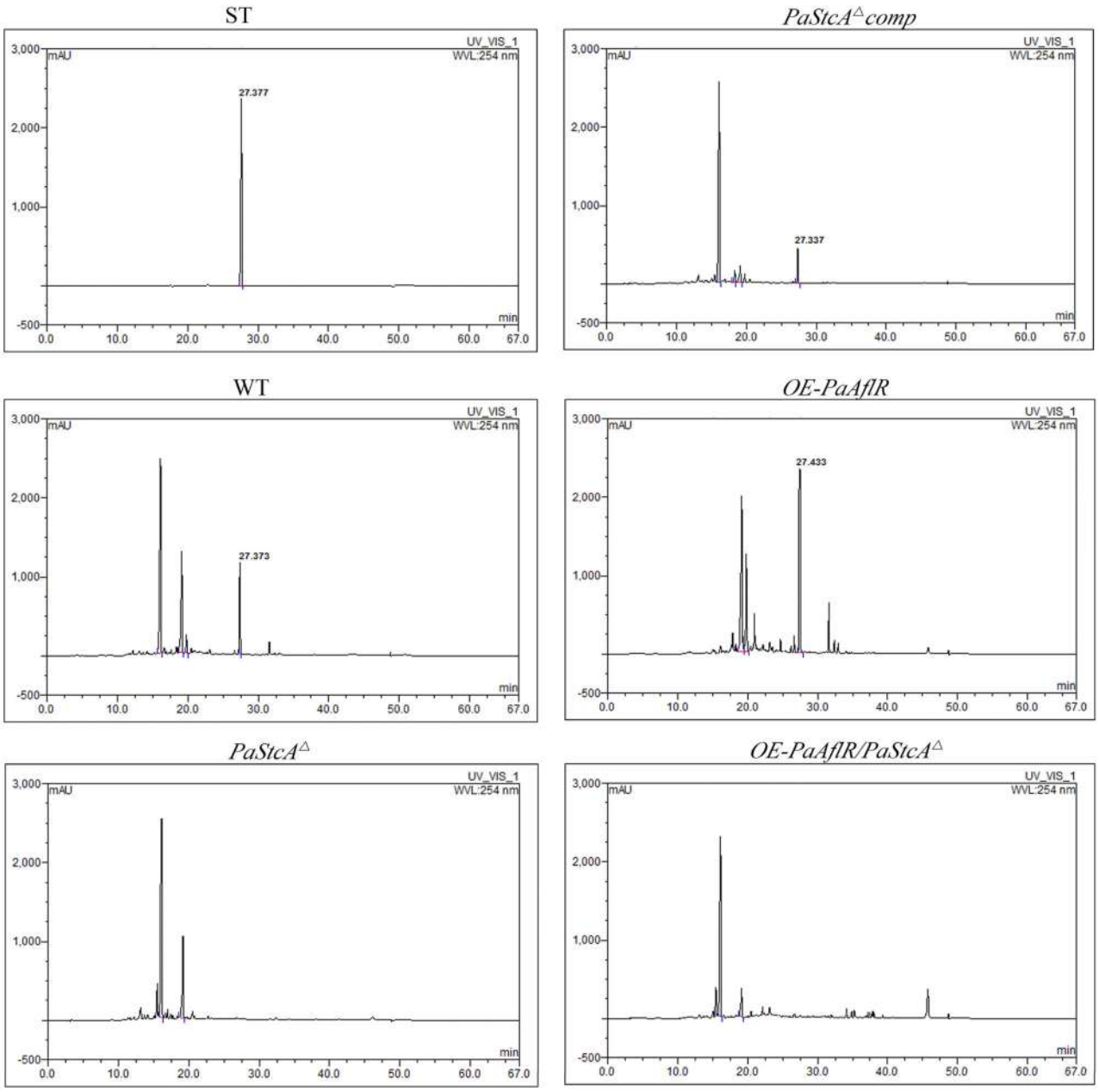

Fig. 6. HPLC-UV detection of ST in wild type and mutants strains. The chromatograms display the peaks intensity (mAU) according to the retention time (rt). ST presents a rt of 27.3 min. WT: wild-type strain. X-axis (min): retention time. Y-axis (mAU): signal intensity. [Color figure can be viewed at wileyonlinelibrary.com]

gametes were undifferentiated or remained some defects after differentiation, as well as the impaired maturation of the fruiting bodies. As sterility occurred in both ST-overexpressed strains, it was not linked to overpigmentation. So, sterility could be due to ST overproduction, supporting a link between the coordination of sexual development and secondary metabolism, as in *A. nidulans*, where ST was described as a protector of its reproductive structure (Ámon *et al*., 2018). In this case, sexual development and ST processes share common regulation, allowing them to be coupled in environmental conditions (Kato *et al.*, 2003). Further experiments need to be conducted in order to better understand this point.

Herein, we also found that *PaStcA*$^{\triangle}$ displayed a decrease in resistance to oxidative stress, whereas overexpression of *PaAflR* led to an increased tolerance to its stress. So, these data provide evidence that response to oxidative stress was clearly correlate with ST production in *P. anserina.* It has been generally assumed that secondary metabolism evolved as a response of the fungal cell to oxidative stress

(Roze *et al.*, 2011). In *A. parasiticus*, it has been shown that oxidative stress may be a prerequisite for AF production (Jayashree and Subramanyam, 2000). In *A. flavus*, SMs could function as a supplemental source of antioxidant protection and then enhance its stress tolerance along with antioxidant enzyme gene expression and developmental regulation (Fountain *et al.*, 2016a,2016b). So, in *P. anserina*, ST may be produced as part of coordinated oxidative stress responses. Nevertheless, further investigations will be necessary to highlight the role of ST in its stress tolerance.

Additionally, we showed that overexpression of *PaAflR* in the wild-type background resulted in the accumulation of ST along with a hyperpigmentation of the thallus after 3-days incubation in normal growth condition. Mycelial pigmentation in fungi is commonly considered as the output of secondary protective mechanisms against oxidative stress and light, which is controlled by a complex regulatory network (Gmoser *et al.*, 2017). Then, melanin, a complex aggregate of polyketides produced by many fungal species, is required for the survival and pathogenic virulence. Diverse fungi are able to produce melanin and the production of these black or brown pigments may protect the organisms from environmental insults (Nosanchuk and Casadevall, 2003). In *P. anserina*, it has been previously shown that dihydroxynaphtalene (DHN)-mediated pathway orchestrates the melanin biosynthesis (Coppin and Silar, 2007; Pal *et al.*, 2014; Xie *et al.*, 2018). Experiments conducted with the albino *PaPks1-193* mutant allowed us to show that the accumulated pigment was (i) not diffusible from the *OE-PaAflR* when confronted to *PaPks1-193* and (ii) was synthesized via the melanin pathway, as overexpression of *PaAflR* in this melanin-deficient background did not lead to an overpigmentation of the thallus. Overall, we could then hypothesize that the mycelial melanization in the *OE-PaAflR* strain could be linked to the level of production of ST in related strains. Moreover, when confronted to ST-overexpressed strains, the wild-type strain (as well as *PaStcA*$^{\triangle}$) responded by a strong enhancement of melanin biosynthesis, at the edge of its thallus. It is then possible that a localized diffusion of ST, from *OE-PaAflR* or *OE-PaAflR/193* towards the edge of the thallus of the confronted strain, could locally trigger accumulation of melanin in wild type, where mycelium converged. Such a phenomenon might also occur in confrontation involving two *OE-PaAflR*. In this case, as both thalli are overpigmented, it is probably not possible to evidence an additional melanin accumulation. This implies that ST and melanin biosynthesis pathways could be linked, either directly or indirectly. Referring to the structural similarities between the first stable intermediate of melanin biosynthesis and AF biosynthesis in *Aspergillus* species, some researchers proposed that AF and melanin biosynthetic pathway share common initial stages, later the polyketide biosynthetic chain may branch toward different pathways resulting in the production of either AF or melanin (Brown and Salvo, 1994; Dzhavakhiya *et al.*, 2016).

We clearly demonstrated in this study that ST production was involved in interspecific interactions. It has been previously shown that *P. anserina* was able to produce peroxide when confronted with a filamentous fungus, only in non-self confrontations (Silar, 2005). In this case, peroxide accumulation, which was strikingly similar to an oxidative burst, has been described as a signalling component in interspecific confrontations, rather than playing a direct toxic role. In some interspecific confrontations, *P. anserina* was then able to trigger cell death of the challenger hyphae. Here, we showed that ST-overexpressed strains were able to produce a large amount of peroxides overall the thallus, which could be linked to a higher competitiveness in some interspecific confrontations. The most striking result was obtained when *T. versicolor* was tested as challenger. In this case, ST-overexpressed strains won the battle against *T. versicolor* by triggering cell death in the challenger thallus, whereas other strains of *P. anserina*, including wild type, were killed. Such results are consistent with the fact that fungal SMs confer plenty of survival functions during the competition process with other microbes, which can induce biosynthetic gene cluster expression and metabolite production (Künzler, 2018). Concerning ST, in *A. nidulans*, it has been shown that this compound acted as a protective agent against arthropod fungivores (Döll *et al.*, 2013). In a previous study, *P. anserina* transcriptional responses revealed that core PKS gene of ST cluster was upregulated under confrontation condition against bacterial species, *Serratia fonticola* and *S. marcescens* (Lamacchia *et al.*, 2016). Beyond larvicidal activity of ST against *Anopheles*, which was previously reported (Matasyoh *et al.*, 2011), we showed here that *P. anserina* ST was also clearly involved in the fight against some other fungal species. Thus, the overproduction of ST could benefit to *P. anserina* by conferring its ability to outcompete other competitor in the environment. Considering that ST could function as a supplemental source of antioxidant protection, as hypothesized previously, it remains to better understand physiological processes underlying ST and peroxide overproductions, both occurring in strains overexpressing *PaAflR*. An alternative explanation is that ST overexpression constitutively activates stress-protecting genes, allowing to cope with higher concentration of oxidative product. Finally, we showed in this study that *P. anserina* strains overexpressing *PaAflR* possess a higher competitiveness in some interspecific confrontations, but we have to keep in mind that this selective advantage might be countered by the lack of sexual reproduction observed in ST-overexpressed strains.

In summary, we described here the role of ST production in physiology of *P. anserina* via the functional characterization of the PKS gene *PaStcA* and the specific transcription factor *PaAflR* from the ST cluster. We then evidenced that

ST production in *P. anserina* was involved in the response to oxidative stress, sexual development, thallus pigmentation and interspecific competitions, which constitute new areas of investigations to better understand the role of SM in fungal physiology. This work displayed the complexity of regulatory network for SM biosynthesis and enriched our knowledges about the functional involvement of ST in fungal physiology.

Experimental procedures

Strains and growth conditions

The *P. anserina* strains used in this study are listed in Table 1. They are all derived from the 'S' (big S) wild-type strain that was the reference strain for sequencing of the *P. anserina* genome (Rizet, 1952; Espagne *et al.*, 2008). All the protocols about standard culture conditions, media composition and genetic methods for this organism can be accessed at http://podospora.i2bc.paris-saclay.fr. The *PaPks1-193* mutant is affected in the gene *PaPKS1* coding the PKS acting at the first step in melanin biosynthesis (Coppin and Silar, 2007). For deletion experiments, the *Δmus51::hygroR* strain was used. This mutant strain differed from the S strain by a single deletion of the *mus-51 gene*, which led to an increased frequency of targeted gene replacement (El-Khoury *et al.*, 2008).

Trametes versicolor ATCC 32745 and *B. cinerea* B05.10 were kindly provided by Dr MC Soulié (INRA Versailles). *Penicillium chrysogenum peniC* (Silar, 2005) was retrieved from the fungal collection of the lab. Theses strains were used as challengers in interspecific confrontation assays. These strains are able to correctly grow on M2 medium at 27°C, allowing us to use this medium for confrontation experiments in order to standardize the procedure.

Deletion of PaStcA *and complementation*

Targeted gene deletion in *P. anserina* was performed via protoplast-mediated fungal transformation, as previously described (Xie *et al.*, 2014). Briefly, the geneticin resistance cassette of *PaStcA* was constructed as follow. Two fragments (approx. 800 bp) of the *PaStcA* flanking sequences were amplified by PCR from genomic DNA with ad *hoc* primer pairs (Supporting Information Table S1) and then were, respectively, fused to geneticin cassette, which was amplified from plasmid pBC-genetic containing the geneticin selective marker (Chan Ho Tong *et al.*, 2014). Protoplasts of *Δmus51::hygroR* strain were transformed with the mixture of two purified fusion PCR products (Lambou *et al.*, 2008). All geneticin-resistant transformants were firstly screened by PCR with two primers pairs that located within the geneticin cassette and outside the flanking region (Supporting Information Table S1 and Fig. S1). Two candidate transformants were then crossed with the wild-type strain to eliminate potential untransformed nuclei and to segregate out the Δ*mus51* mutation. Several *mat* + and *mat* − strains carrying the *PaStcA* deletion were obtained and further confirmed by Southern blot analysis (Supporting Information Fig. S1).

To ensure that the phenotypes observed for the *PaStcA*$^{\Delta}$ strain were actually linked to inactivation of the relevant gene, the complementation involved ectopic insertion of the corresponding gene under control of its native promoter. A 7.8 kb fragment encompassing the wild-type complete CDS along with its own constitutive promoter and terminator, was amplified with a high-fidelity polymerase (Phusion High-Fidelity DNA Polymerase, Thermo Scientific), using the adequate pair of primers (Supporting Information Table S1). The amplified DNA fragment was cloned into the *EcoR*V site of pBC-hygro plasmid to yield a new vector pBC-hygro-PaStcA. This plasmid was sequenced and then introduced into the protoplasts of the *PaStcA*$^{\triangle}$ mutant strain. Transformants were selected on medium containing 40 μg/ml hygromycin B. The presence of the wild-type allele was verified by PCR.

Overexpression of the transcription factor PaAflR

In order to overexpress *PaAflR*, the plasmid pBC-hygro-OE-PaAflR was constructed. We used the pBC-HA vector (Kicka and Silar, 2004) that contains the *AS4* promoter (Silar and Picard, 1994) and the ribP2 terminator cloned into the *NotI* site of pBC-hygro. A 750 bp fragment carrying the AS4 promotor, a 2000 bp fragment carrying the complete CDS of *PaAflR* and a 216 bp fragment carrying the were amplified using adequate primer pairs (Supporting Information Table S1). These three amplicons were fused by PCR and then inserted into the *EcoR*V-digested pBC-hygro plasmid generating the pBC-hygro-OE-PaAflR. All the PCR reactions were performed with a high-fidelity polymerase (Phusion High-Fidelity DNA Polymerase). Candidate plasmids were then validated by sequencing (Genewiz, Germany) and transformations were performed as described previously using protoplasts from the S strain.

Growth and development in standard conditions

To determine the role of *PaStcA* and *PaAflR* in fungal physiology, the mutants and the wild-type strain were incubated separately on solid M2 medium at 27°C for an 8-day period. Colony morphology, pigmentation, perithecium formation, ascospore production, ascospore dispersal and germination were observed during their vegetative growth and their sexual cycle. Similarly, M2 liquid cultures were also realized at 27°C for 8 days in order to check the fungal pigmentation of the thallus and the coloration of culture filtrate after the removal of the mycelial suspension.

Sensitivity to oxidative stresses

Fungal growth was assessed on M2 plates in the presence of various concentrations of H_2O_2 (0.1–1 mM), methylglyoxal (MG; 2.5–10 mM), menadione (10–50 μM) or t-butyl hydroxyperoxide (TBY; 10–75 μM). Wild-type and mutant strains were inoculated on the media by spotting 5 μl of a standardized fragmented mycelium suspension and incubated at 27°C for 5 days. Colony diameters were determined daily. All experiments were performed in a triplicate.

Peroxide detection and cell death assay during interspecific confrontations

Peroxide detection and cell death assays were conducted as previously described (Silar, 2005). Briefly, the *T. versicolor* and *B. cinerea* strains were pre-grown in the centre of M2 plates at optimal temperature for 4 days, and then the *P. anserina* wild-type and mutant strains were set up around them. The *P. chrysogenum* strain was simultaneously inoculated around *P. anserina* wild-type and mutant strains on M2 plates, and then incubated at optimal temperature for 3 days. Peroxide detection and cell death assay were performed after incubation for three additional days to obtain the appropriate grown thalli, meaning that the central fungus was in contact with other fungal thalli. Diaminobenzidine (DAB) staining is classical assay to detect peroxide that triggers the accumulation of a red precipitate (Munkres, 1990; Malagnac *et al.*, 2004). Detection of cell death in the contact zone was performed by the Trypan blue exclusion method according to Silar (2005).

Metabolite extraction and HPLC-UV analysis

In order to monitor the ST production in fungal cultures, metabolite extraction and HPLC-UV analysis were performed in different strains of *P. anserina.* Briefly, liquid M2 stationary cultures of *P. anserina* strains were incubated at 27°C for 8 days. Then, the content (mycelium and liquid medium) was transferred to a blender and crushed for 2 min. An equal volume of ethyl acetate was added and the aqueous phase was extracted under ultrasonic irradiations for 20 min. After separation of the layers, this step was repeated twice on the aqueous phase. The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo.* The residue was then dissolved in 2 ml of methanol, filtered over a 0.2 μM filter and subjected to reversed-phase high-performance liquid chromatography coupled to UV detection (HPLC-UV) analysis. HPLC-UV analysis was performed on Dionex UltiMate 3000 HPLC Systems using a column (X-bridge C18, 4.6 × 250 mm, 5 μm, Waters, Ireland) with a flow rate of 1 ml/min. Fresh extracts of all strains were detected with a linear gradient of ACN: H_2O from 0% to 100%.

ST purification and assays

ST was isolated from a wild-type strain culture following the general process given above. The residue was subjected to chromatography on silica gel to give pure ST as an off-white solid. Analysis of its ^1H and ^{13}C NMR spectra together with its Mass Spectra allowed to clearly identify it. The data were identical to those reported in the literature (Zhu and Lin, 2007).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Primers used in this study

Fig. S1. Southern blot validation of *PaStcA* deletion mutant. Genomic DNA was isolated from wild type (WT) and two transformants and then digested with *Sac*I. The blots were probed either with a sequence containing the relevant flanking region. A restriction map of the wild-type and mutant locus is presented. The sizes of the expected fragments are indicated on the maps and are reported close to the corresponding fragment on the southern blot. Primers for junction verification are also indicated.