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An *Aspergillus nidulans* bZIP response pathway hardwired for defensive secondary metabolism operates through *aflR*

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Summary

The eukaryotic bZIP transcription factors are critical players in organismal response to environmental challenges. In fungi, the production of secondary metabolites (SMs) is hypothesized as one of the responses to environmental insults, e.g. attack by fungivorous insects, yet little data to support this hypothesis exists. Here we establish a mechanism of bZIP regulation of SMs through RsmA, a recently discovered YAP-like bZIP protein. RsmA greatly increases SM production by binding to two sites in the *A. nidulans* AflR promoter region, a C6 transcription factor known for activating production of the carcinogenic and anti-predation SM, sterigmatocystin (ST). Deletion of *aflR* in an overexpression *rsmA* (*OE::rsmA*) background not only eliminates ST production but also significantly reduces asperthecin synthesis. Furthermore, the fungivore, *Folsomia candida*, exhibited a distinct preference for feeding on wild type rather than an *OE::rsmA* strain. RsmA may thus have a critical function in mediating direct chemical resistance against predation. Taken together, these results suggest RsmA represents a bZIP pathway hardwired for defensive SM production.

Keywords

sterigmatocystin; asperthecin; fungivory; Yap; *rsmA*

Introduction

The fungal kingdom is remarkable for the production of bioactive molecules commonly referred to as secondary metabolites (SMs). In general these metabolites are considered to provide fitness attributes to the producing organism, often as a defense against environmental insult. For example, stress responses have been linked to increased aflatoxin level in *A. parasiticus* (Roze *et al.* 2011; Chang *et al.* 2008) and a series of recent works

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have definitively shown that SM production is associated with increased fungal fitness in confrontations with insects where both LaeA (a global regulator of fungal secondary metabolism, Bok *et al.* 2004) and AflR (the C6 transcription factor responsible for aflatoxin and ST synthesis in Aspergilli, Fernandes *et al.* 1998; Chang *et al.* 1995) are required for protection from fungivores (Rohlfs *et al.* 2007; Trienens *et al.* 2010, Staaden *et al.* 2010).

In fungi, developmental processes in response to various abiotic or biotic external triggers are commonly associated with secondary metabolism (Calvo *et al.* 2002; Braus *et al.* 2010). A breakthrough in fungal biology was the discovery of heterotrimeric VelB/VeA/LaeA transcriptional complex (known as the Velvet complex) which connects SM with light signal (Bayram *et al.* 2008). Different members of the velvet protein family partner with each other, coordinating SM biosynthesis with fungal development through various signal transduction pathways (reviewed in Bayram and Braus, 2011). However, how Velvet controls SM production is unknown. Because many genes for the synthesis of secondary metabolites are arranged in gene clusters (Keller and Hohn, 1997), the considerable evidence for SM gene regulation can be in part explained by transcriptional control through hierarchical levels of transcriptional regulatory elements including cluster specific regulatory elements, global regulators as well as transcriptional complexes like Velvet (reviewed in Yin and Keller, 2011).

Identification of SM regulatory elements could potentially provide a means of increasing production of beneficial metabolites, aid in the identification of “silent” natural products, and, importantly, also contribute to a broader understanding of the molecular mechanisms by which SM are produced. The case for a fungal SM stress response pathway was recently strengthened by the finding of RsmA (restorer of secondary metabolism A), a putative YAP-like bZIP protein identified in a multicopy suppressor screen for restoration of sterigmatocystin (a carcinogenic and anti-predation SM), in Velvet complex mutants (Shaaban *et al.* 2010). Overexpression of RsmA conferred a remarkable ability to greatly increase ST in multiple genetic backgrounds of *A. nidulans*. YAP proteins are well known mediators of stress response pathways throughout the spectrum of life from yeast to humankind (Rodrigues-Pousada *et al.* 2010). Several bZIP proteins have been characterized in *Aspergillus* spp. as responding to oxidative, osmotic, drug, nutrient and iron stress (Roze *et al.* 2011; Asano *et al.* 2007; Hagiwara *et al.* 2008; Balazs *et al.* 2010; Qiao *et al.* 2008). Here we uncover the mechanism by which RsmA regulates SM production. We find that RsmA operates through activation of *aflR*, encoding the C6 transcription factor embedded in the ST gene cluster. RsmA binds to two sites in the *aflR* promoter and elimination of either site or *aflR* eliminates not only enhanced ST synthesis by overexpression of *rsmA* but also reduces asperthecin production, a metabolite produced by a SM cluster containing no transcription factor. Furthermore, when given the choice between wild type (WT) and a *rsmA* overexpression strain, fungivorous insects overwhelmingly refuse to feed on the latter strain in accordance with the role of ST as a metabolite mediating resistance against predation.

Results

RsmA is involved in SM regulation

The initial assessment of RsmA as a multicopy suppressor of loss of *laeA* and *veA* expression focused on the enhanced sterigmatocystin synthesis in the overexpression RsmA (*OE::rsmA*) strain (Shaaban *et al.*, 2010). To examine impact of *OE::rsmA* on other aspects of *Aspergillus* biology, we first made a new version of the overexpression allele. To minimize impact of possible ectopic affects on RsmA function, we replaced the native promoter with the constitutive glyceraldehyde-3-phosphate dehydrogenase gene *(gpdA*) promoter, rather than placing the allele ectopically as described in Shaaban *et al.* (2010).

Assessment of this new strain showed that it looked identical to the original strain and also produced enhanced quantities of ST (Fig. 1A and B). LC-MS analysis of this strain grown on solid medium revealed greatly increased production of not only ST but also asperthecin (Fig. 1B), a recently characterized anthraquinone (Szewczyk *et al.* 2009).

Role for RsmA in fungivore resistance but not canonical stress response

Several studies have shown VeA and LaeA driven secondary metabolites, particularly ST, to be involved in resistance against fungivores (Rohlfs *et al.* 2007, Staaden *et al.* 2011, Trienens and Rohlfs, in press). To assess any impact of *OE::rsmA* on fungivore selectivity, we presented both the WT and the *OE::rsmA* strain to fungivorous collembolans, *Folsomia candida*, in a food choice assay. While patch choice was inconsistent during the first two days, collembolans showed an increasing, statistically significant tendency to stay on the WT patches during the following three days (Fig. 2A). Even though animals were moving between the WT and *OE::rsmA* patches they were almost exclusively feeding on the WT colonies. The latter is indicated by extensive feeding damage and accumulation of fecal pellets around the WT colonies but not at the *OE::rsmA* colonies (Fig. 2B and C). As this food choice test was conducted from fungus grown on malt extract agar, we assessed SM on this medium as well and again found *OE::rsmA* greatly increased ST over WT (Fig. S1).

In addition to the impact on SM, we reasoned that RsmA, which bears sequence similarity to a *Candida* bZIP with enhanced resistance to antifungal drugs (Alarco *et al.* 1999), could be involved in some drug or canonical YAP-like stress response. However, an examination of the *OE::rsmA* strain in comparison to WT revealed no altered response to antifungals, oxidative stressors or heavy metals (data not shown).

RsmA is a bZIP that binds two sites in the divergent aflR aflJ promoter

To address the hypothesis that RsmA is a bZIP protein that regulates SM genes through transcriptional activation, microarrays comparing an *OE::rsmA* with WT were assessed for common motifs in promoters of differentially regulated genes. Quality of the microarrays was confirmed by examining the expression of ST cluster genes. As expected, the majority of the ST cluster genes in *OE::rsmA* have a higher expression level in comparison with WT (Fig. 3). The two genes downregulated in the *OE::rsmA* strain, *stcC* and *stcD*, have not been found to have a role in ST biosynthesis.

Bioinformatic assessment of the microarray coupled with predictions from yeast YAP binding sites identified two candidate binding sites, TGACTCA and TT(G)ACTAA (Fig. S2). Two of these sites, TGACACA (R) with one base variation (underlined letter) and TTAGTAA(Y), were identified in the bidirectional promoter region of *aflR* and *aflJ* (Fig. 4A). AflR is necessary for activation of ST and aflatoxin cluster genes (Fernandes *et al.* 1998; Chang *et al.* 1995). AflJ, which shares some similarities to methyltransferases, is required for and enhances AflR activity (Du *et al.* 2007). RsmA bound to both candidate binding sites, and both *aflR* and *aflJ* are overexpressed in the *OE::rsmA* strain (Fig. 4B and C).

The requirement for these sites was confirmed *in vivo* by comparing the expression of ectopic copies of *aflR* with either an intact promoter sequence or with one or both RsmA site mutation sequences in a strain lacking native *aflR* but containing an *OE::rsmA* allele at the *trpC* locus. Mutations at either site significantly reduced *aflR* and *stcU* (a biosynthetic gene in the ST pathway) expression (Fig. 4D) as well as ST synthesis (Fig. 4E and Fig. S3). As expected, *aflJ* expression remained constant as this gene remained at its native locus.

AflR is required for RsmA mediated SM production

We reasoned AflR could be primarily responsible for the SM phenotype of *OE::rsmA.* An *OE::aflR* strain was created with *A. nidulans gpdA* as promoter at the native locus. Examination of this strain and an *OE::rsmA*, Δ*aflR* strain yielded the expected increased and decreased ST phenotype respectively (Fig. 5 and Fig. S4). Unexpectedly, however, we found that asperthecin synthesis was also reduced in the *OE::rsmA*, Δ*aflR* double mutant as compared to the *OE::rsmA* strain despite no increase in the *OE::aflR* strain alone.

Discussion

The study of fungal secondary metabolism (SM) has recently garnered significant interest, and the advent of fungal genomics has lead to new insights into SM regulatory mechanisms. Much of this activity is driven by interests in drug discovery due to the biological properties of these metabolites. Several elegant studies have characterized metabolites from ‘silent’ or ‘repressed’ SM clusters via activation of cluster specific transcription factors (Bergmann *et al.*, 2007; Chiang *et al.*, 2010) and global SM regulators such as LaeA (Bok *et al.* 2006) or through chromatin remodeling studies (Bok *et al.* 2009). However, at the heart of these discoveries lies the largely unexplored topic of why these compounds are generated by the producing fungus.

Studies ranging from Flemings’ serendipitous discovery of penicillin synthesis by *Penicillium notatum* to recent findings of bacterial induction of a silent *A. nidulans* gene cluster (Schroeckh *et al.* 2009) serve to illustrate a possible evolutionary development of protective SM by fungi. In support of a direct conduit from environment stress to SM production is our finding of RsmA regulation of SM, particularly the anti-predation metabolite ST. RsmA belongs to a sub-family of bZIP transcription factors commonly referred to as YAP proteins in *Saccharomyces cerevisiae* based on their homology and similar DNA binding sites as the mammalian AP-1 factor complex (Rodrigues-Pousada *et al.* 2010; Fernandes *et al.* 1997). Specific YAP proteins are activated when exposed to environmental challenges ranging from oxidative to heavy metal stress or iron imbalances. RsmA shares the greatest homology with YAP3 of unknown function in yeast. Because the *C. albicans* homolog of YAP3, FCR3, exhibits resistance to antifungals when overexpressed (Yang *et al.* 2001), the *A. nidulans OE::rsmA* strain was originally assessed for a similar activity but was found to show no difference in susceptibility to antifungals as WT (Shabaan *et al.* 2010 and data not shown). Indeed, this strain exhibited no stress response typical of the YAP activities in yeast. The most striking phenotype was the greatly elevated production of ST and, to a lesser degree, the anthraquinone asperthecin.

No where has a role for ST been more clearly established than in a recent series of fungivore-*Aspergillus* confrontation studies. Loss of either *laeA* or *aflR* or even various enzymatic genes in the ST cluster yields a fungal strain less resistant to fungivore feeding (Rohlfs *et al.* 2007; Staaden *et al.* 2010). In these studies, insect preference is consistently towards the SM deletion mutants with distinct repulsive behavior demonstrated towards WT *A. nidulans*. Here we saw the opposite behavior where fungivores, *F. candida*, avoided the *OE::rsmA* strain. Moreover, as indicated by the appearance of insect fecal pellets and damage caused to the colonies, the insects were almost exclusively feeding on the WT strain. This suggests an important role of RsmA in mediating resistance to fungal predators.

Chemical resistance against predation or competition for food substrates is a common phenomenon in nature. Both ST and aflatoxin (produced from ST through two enzyme conversions in *A. flavus* and *A. parasiticus*, Chang *et al.* 1995) have often been cited for their toxicity towards certain insects (Niu *et al.* 2009; Labrousse and Matile 1996; Llewellyn *et al.* 1988, Matasyoh *et al.* 2011, Gunst *et al.* 1982). These metabolites are not

constitutively produced but require exposure to undefined environmental ligands for expression. We propose that RsmA, a bZIP apparently dedicated to SM, represents a defensive response to a specific set of environmental insults requiring up-regulation of ST and other SMs. Specifically, the environmental distresses which could trigger an RsmA mediated SM response include fungivory and confrontations with competitive microbes.

Finally, we found that RsmA works primarily through induction of AflR, a C6 transcription factor required for ST and aflatoxin biosynthesis (Brown *et al.*, 1996; Chang 2003). We show that RsmA operates through binding to the shared *aflR aflJ* promoter region in the ST gene cluster resulting in both *aflR* and *aflJ* expression. Loss of RsmA binding sites negates this regulation as does *aflR* loss itself. Interestingly, while both overexpression of *rsmA* and *aflR* greatly increased ST synthesis over WT (57 and 43 fold respectively), only overexpression of the former increased asperthecin synthesis (15 fold). Nevertheless, loss of *aflR* in an *OE::rsmA* background not only resulted in cessation of ST synthesis as expected but also reduced asperthecin levels comparable to that of WT. Such interactions could possibly be reflective of SM cluster cross talk recently described in *Aspergillus* (Bergmann *et al.* 2010). We note that the asperthecin gene cluster does not contain an in-cluster transcription factor but does contain at least one AflR and one RsmA binding motif, either in an asperthecin gene promoter or a nearby gene (Table S2), possibly reflective of a need for both factors in up regulation of this metabolite.

Experimental procedures

Strains, media and growth conditions

The fungal strains used in this study are listed in Table 1. All strains were grown at 37 °C on glucose minimum medium (GMM) (Shimizu *et al.* 2001) and when appropriate were supplemented with 0.56 g uracil l^{-1}, 1.26 g uridine l^{-1}, 1.0 g tryptophan l^{-1}, 0.5 μM pyridoxine HCl, 0.01 μM biotin, 100 μM arginine and maintained as glycerol stocks at −80 °C. For preparing RNA used for microarray, WT and *OE::rsmA* strains were grown in a liquid minimal media at 37 °C for 48 h in dark, with shaking at 250 rpm, three replicates each. *Escherichia coli* strains DH5α and BL21 (DE3) (Novagen, Madison, WI) were propagated in LB medium with appropriate antibiotics for plasmid DNA and protein expression, respectively.

Fungivore food choice

Circular glass fiber filters discs (MN85/70 BF), 1.0 cm in diameter, provided the matrix for the experimental patches. Autoclaved discs were soaked in liquid malt extract agar (30 g l^{-1} standard malt extract, 5 g l^{-1} soy peptone, 20 g l^{-1} agar). After hardening, each disc was inoculated with 2 μl conidia suspension (1000 conidia per μl). The discs were transferred to non-vented Petri dishes and incubated at 20 °C and constant darkness for four days prior to food choice experiments. Choice assays were conducted in non-vented 9 cm Petri dishes. Four days after inoculation WT and *OE::rsmA* patches were placed in each Petri dish (n = 20). Fungal patches were connected by a wet piece of filter paper (length: 5 cm, width: 1.5 cm). Both the position of the patches relative to one another (left/right) in each arena and the position of the arenas in a 2 × 10 grid were randomized. Then 25 *F. candida* per arena were released in the middle of the filter paper. Subsequently, fungivores that were found to stay on the WT or the *OE::rsmA* patch were counted every 24 hours after the release of the collembolans into the arenas. The experiment was stopped after the five days and the number of fecal pellets per patch was counted to provide a measure of feeding intensity.

By subtracting the proportion of fungivores found on *OE::rsmA* patches from the proportion of animals on WT patches for each arena we obtained “Δ patch choice” values. Values

around zero indicate no preferences, whereas positive (max. +1) or negative (min. −1) values indicate preference for the WT or the *OE::rsmA* respectively. We ran a time series mixed model using the MIXED procedure provided by SAS 9.2 (see Trienens *et al.* 2010). As a *post hoc* procedure we tested for significant deviation from "Δ patch choice" values = 0, i.e. no preference for either patch. Variation in the number of fecal pellets was analyzed by means of a paired t-test as data were found to be normally distributed.

Microarray analysis

Sample labeling was performed as previously described (Gasch, 2002) using cyaninedyes (Amersham), Superscript III (Invitrogen, Carlsbad, CA), and amino-allyl-dUTP (Ambion, Austin, TX). Custom *A. nidulans* FGSC A4 4X expression arrays were designed by NimbleGen. Arrays were hybridized in a NimbleGen hybridization system 12 (BioMicro, Salt Lake City, UT), washed, and scanned using a scanning laser (GenePix 4000B, Molecular Devices). Hybridization, washing and scanning were performed according to NimbleGen protocols (http://www.nimblegen.com/). Data normalization and statistical analyses on biological triplicates were performed using Bioconductor (Gentleman *et al.* 2004) and custom perl scripts. The *affy* package (Gautier *et al.* 2004) was used to apply probe-level quantile normalization to the $\log_2$ signal of RNA versus genomic DNA control. Gene-level expression changes were summarized with the median value of each probe set contained completely within each predicted ORF (open reading frame). Genes with significant expression differences in *OE::rsmA* as compared to WT were identified by performing paired t-tests using the Bioconductor package Limma v2.9.8 (Smyth, 2004) with a false discovery rate (FDR) correction of 0.05 (Storey and Tibshirani, 2003). The group of genes significantly induced in *OE:rsmA* was analyzed for enrichment of upstream sequence motifs using multiple em for motif elicitation (MEME; Bailey and Elkan 1994). MEME parameters were motif distribution, zoops; minimum width, 6; maximum width, 12; maximum number of motifs, 5.

Gene cloning, plasmid construction and genetic manipulation

The plasmids utilized in this work are listed in Table 1. The oligonucleotide sequences for PCR primers and gel shift assay are given in Table S1 and Table 2, respectively. PCR amplification was carried out on a C1000™ Thermal Cycler from Bio-Rad (Hercules, CA). For creation of *rsmA* overexpression (OE) strain at native locus, the OE cassette was constructed by using single and double joint PCR procedures (Yu *et al.* 2004). Single joint PCR reaction was set up for fusion of the maker gene *A. fumigatus pyrG* (1.97 kb) which was amplified from genomic DNA of *A. fumigatus* and *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene *(gpdA*) promoter (1.5 kb) which was amplified from pTMH44.2 (See Table S1 for primer sequences). Then, the 1.03 kb fragment upstream of *rsmA* and 1.1 kb fragment containing *rsmA* ORF and downstream were amplified from genomic DNA of *A. nidulans* using designated primers (Table S1), respectively. These three amplified PCR products were purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA), quantified, and fused using double joint PCR procedures. The final PCR product was amplified with the primer pairs OErsmA_NEST_for and _rev, confirmed with endonuclease digestion and purified for fungal transformation. All PCR steps were performed using an Expand long template PCR system (Roche, Indianapolis, IN) according to the manufacturer's instructions. Using the same strategy as creation of *OE::rsmA* cassette, *OE::aflR* cassette was constructed with *A. fumigatus pyroA* as select maker gene and *A. nidulans gpdA* as promoter. To construct the bacterial overexpression plasmid, the entire coding region of *rsmA* was amplified from cDNA library of *A. nidulans* by using primers HisMBPRsmA_for and rev. The *rsmA* fragment was then integrated into plasmid pKLD116 (Rocco *et al.* 2008) (Table 2) to create pWY20 by using QuickChange strategy (Stratagene, Wilmington, DE). This construct was confirmed by PCR with primers pKLD116_for and

_rev and then sequenced. RsmA has in-frame codons for six histidines maltose binding protein (MBP) at the N-terminus as well as a TEV (tobacco etch virus) site for cleaving fusion protein. To detect RsmA binding DNA activity *in vivo*, we created auxotrophic strain RWY16.76 (*A. fumigatus pyrG::gpdA(p)::rsmA, ΔaflR::argB, trpC801, veA*) by crossing TWY5.2 (*OE::rsmA*) with RJH256 (*ΔaflR*) according to standard methods (Pontecorvo *et al.* 1953). Similarly, prototrophic strain was constructed by crossing RAMB38 to TWY5.2 to construct RSA15.2 (*OE::rsmA, ΔaflR*). Crossing TWY16.12 with RDIT55.37 created RWY20.3 (*OE::aflR*) prototroph. The progeny's genotypes were determined by growth on select media and PCR confirmation with designated primers (Table S1). The plasmids which were used for *ΔaflR* complementation in the *OE::rsmA* background were constructed in two steps. Firstly, a 2.4-kb fragment including the original promoter of *aflR*, ORF and 0.5 kb downstream of the stop codon was amplified by using designated primers (Table S1) and digested by using restriction enzymes *BamH I* and *EcoR I*. Then, the *BamH I-EcoR I* fragment of *aflR* was cloned into *BamH I* and *EcoR I* sites of the half *trpC*-containing plasmid pSH96 to create pWY43.23. Secondly, plasmids pWY44.6, pWY45.2 and pWY46.9 were constructed by multiple-site mutation in the promoter of *aflR* using designated primers (Table S1) according to QuickChange Multi Site-directed Mutagenesis approach (Stratagene, Wilmington, DE). All plasmids were confirmed by sequencing. Fungal protoplast preparation and transformation were carried out as described by Bok and Keller (2004). Each five micrograms of the double-joint cassette was used to overexpress *rsmA* and *aflR* by using *A. nidulans* strain RJMP1.49 as the recipient host. For RsmA binding assay *in vivo*, each ten micrograms of plasmids pWY43.23, pWY44.6, pWY45.2 and pWY46.9 was used for complementation of *ΔaflR* using *A. nidulans* strain RWY16.76 as the recipient host, respectively. Overexpression and complementation strains were verified by PCR and Southernblot analysis.

Nucleic acid analysis

Plasmid preparation, digestion with restriction enzyme, gel electrophoresis, blotting, hybridization, and probe preparation were performed by standard methods (Sambrook *et al.* 1989). *Aspergillus* DNA for diagnostic PCR was isolated using the described method previously (Lee and Taylor, 1990). Sequence data were analyzed using the LASERGENE software package from DNASTAR.

Expression and purification of RsmA

E. coli BL21(DE3) cells were transformed with plasmid pWY20. Cells were grown at 37°C in LB medium containing 25 mg ml^{-1} kanamycin to an OD_{600} of 0.6. Then, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into media for induction at 4 time points (1.5 h, 3 h, 4.5 h and 16 h) under two different temperatures (25 °C and 37 °C). The bacterial cells were harvested and lysed by freeze-thawing and cell debris removed by centrifugation. Cell lysates were examined for recombinant RsmA expression by SDS-PAGE followed by Coomassie blue staining. The best expression condition (3 h induction at 25 °C) was used for RsmA protein preparation (Fig. S5). Recombinant RsmA was purified by using the Ni-NTA resin (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Gel shift assay

The complementary oligonucleotide pairs (Table 2) were annealed in a Thermal Cycler (Bio-Rad, Hercules, CA) following heating at 95 °C for 15 min by cooling 1 °C per minute until the temperature dropped to 25 °C. MBP protein was obtained by cleavage of recombinant RsmA with TEV protease (10127-017) according to the protocol (Invitrogen, Carlsbad, CA) and used as control. The DNA-protein binding reaction was conducted in a 24 μl reaction mixture including 1 μg of poly (dI:dC), 3 μg of purified protein, 3 μg of

bovine serum albumin (BSA) and [λ-^{32}P]-ATP oligonucleotide probes with T4 polynucleotide kinase (NEB, Ipswich, MA). The binding buffer contained 8 mM Tris (pH 8.0), 24 mM HEPES, 12 % glycerol, 2 mM EDTA and 1 mM DTT (ditheiothreitol). This mixture was incubated for 20 min at room temperature in the presence of the radiolabeled probe and resolved on a 5% acrylamide gel (Bio-Rad, Hercules, CA) that had been prerun at 110 V for 40 min with 1% Tris-borate-EDTA buffer. The loaded gel was run at 140 V for 60–90 min and then wrapped with plastic and placed on Storage Phosphor screen cassette (GE, Madison, WI) for exposure. After 2 h, the cassette was scanned at Storm 860 phosphoimager (Molecular Dynamics, Sunnyvale, CA) for imaging.

Northern analysis

We examined expression of ST cluster gene transcripts by Northern analysis. Fifty milliliter of liquid GMM were inoculated with 10^6 spores/ml of *OE::rsmA* (RWY2.12) and WT (RDIT9.32) strains and incubated with shaking at 250 rpm at 37 C. After 24, 36, and 48 hours, the mycelium was collected and total RNA was extracted using the TRIzol reagent according to the instructions (Invitrogen, Carlsbad, CA). For expression assessment of strains of *aflR* at *trpC* locus (TWY18.14, TWY19.15, TWY20.4 and TWY21.20), only the time point of 48 hours was used. Blots were hybridized with fragment from each of of *rsmA, aflR, aflJ* and *stcU* which were individually amplified from RDIT9.32 genomic DNA with appropriate primers (Table S1). The rest of the cultures were extracted for ST analysis (See below). All experiments were performed in duplicate. Detection of signals was carried out with a Storm 860 phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Sterigmatocystin (ST) analysis

Five microliter of 10^5 spores of *A. nidulans* strains were point inoculated onto solid glucose minimal medium (GMM) and incubated for 4 days at 37°C (Shimizu *et al.* 2001). An equal size agar plug, 7 mm in diameter, was removed from the center of each plate culture, homogenized in 3 ml dd. water and extracted with an equal amount of chloroform by agitation for 30 minutes at room temperature. The chloroform extracts were then dried completely at room temperature and resuspended in 100 μl of chloroform. Metabolites were separated in the developing solvent toluene:ethyl acetate:acetic acid (TEA, 8:1:1) on silica-coated thin-layer chromatography (TLC) plates (Shwab *et al.* 2007) and photographs were taken following exposure to UV radiation at 254 and 366 nm wavelengths.

Fermentation and LC/MS analysis

A. nidulans strains were cultivated at 37°C on solid YAG (5 g/l yeast extract, 15 g/l agar and 20 g/l *d*-glucose supplemented with 1 ml/l of a trace element solution) at 1.0×10^5 spores per 10 cm plate in the dark or under lights. After 7 days, three 7 mm diameter agar plugs were taken from each strain and transferred to a 10 ml vial. The plugs were extracted with 2 ml of MeOH followed by 2 ml of 1:1 CH_2Cl_2/MeOH each with 1 h sonication. The organic extract was transferred to a 7 ml new vial, in which the organic solvents were evaporated by TurboVap LV (Caliper Life Sciences) to dryness. The crude extract was then re-dissolved in 0.2 ml of DMSO:MeOH (1:4). After filtration, 10 μl of DMSO/MeOH extract was injected for high performance liquid chromatography-photodiode array detection- mass spectrometry (HPLC-DAD-MS) analysis as described previously (Bok *et al.*, 2009).

For determining fold differences, negative ion electrospray ionization (ESI) was used for the detection of asperthecin and positive mode was used for the detection of ST by using extracted ion chromatogram (EIC) at m/z 317 and 325, respectively. The fold differences were calculated according to the following formula: $[\text{Area}_{(\text{Sample})} - \text{Area}_{(\text{Blank})}]/[\text{Area}_{(\text{WT})} - \text{Area}_{(\text{Blank})}]$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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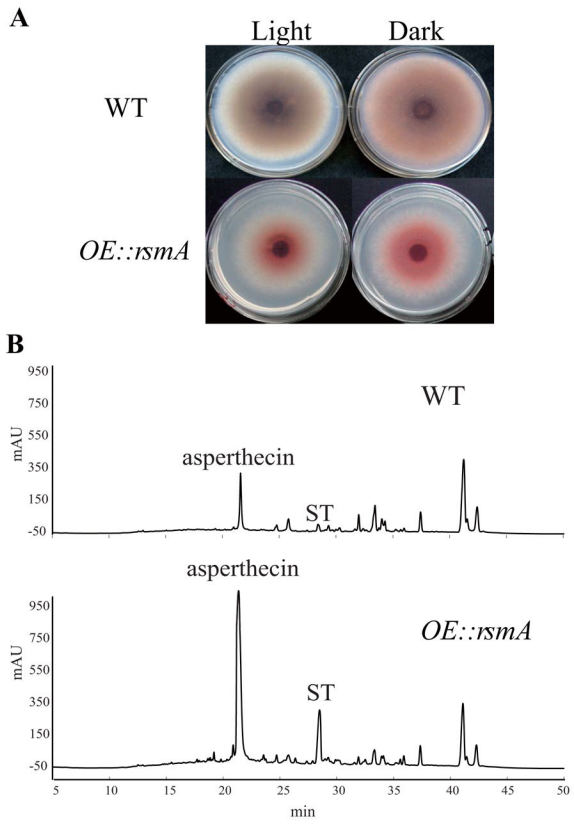

Figure 1. RsmA overexpression increases secondary metabolite production

Panel A Bottom side of plates of WT (RDIT9.32) and RsmA overexpression (*OE::rsmA*, RWY2.12) strains grown on solid GMM media under light and dark conditions. These strains were incubated with point inoculation at 37 °C for 4 days.

Panel B. LC-MS analysis of secondary metabolite production by WT and *OE::rsmA* respectively.

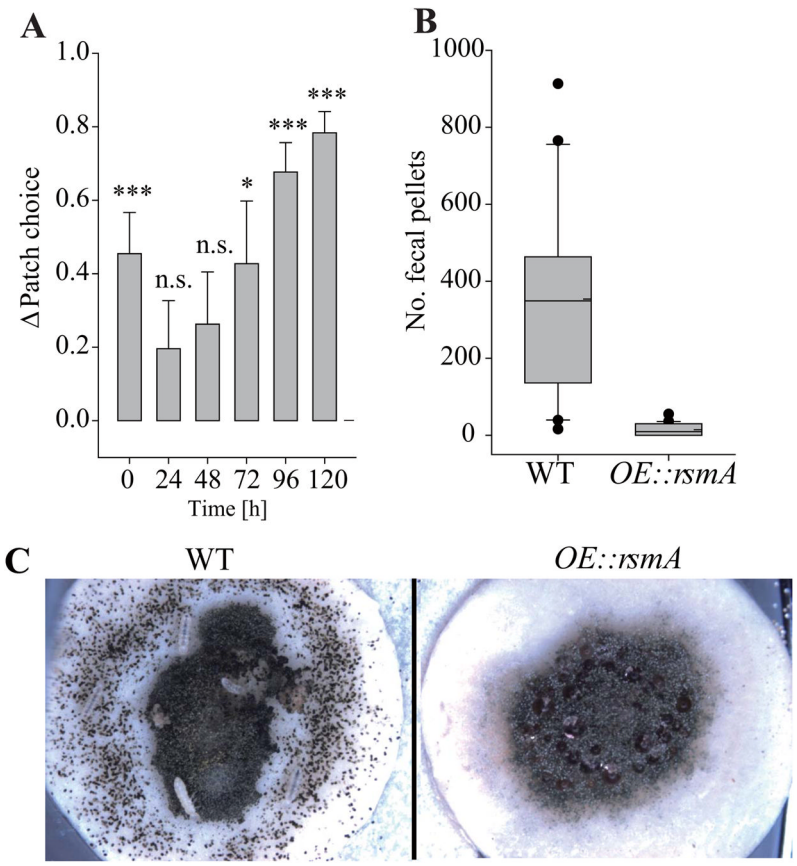

Figure 2. Fungivorous collembolans, *Folsomia candida*, display strong avoidance of the *OE::rsmA* strain

Panel A The tendency of fungivores to stay on WT or *OE::rsmA* fungal patches measured as Δ patch choice (± SE). Values around zero indicate no preference for either patch, whereas positive values indicate preference for the WT and negative ones preference for the *OE::rsmA* strain (see Experimental procedures for details). Patch choice behavior varied with time (time series mixed model: $F_{5,94} = 3.06$, $P = 0.0132$); yet with increasing time fungivores showed an increasing preference for the WT strain (n.s. = not significant, * $P \leq 0.05$, *** $P < 0.001$; for deviation from Δ patch choice = 0).

Panel B. Box plots depicting the number of fecal pellets as an indicator of local fungivores feeding activity. Box plots display the sample minimum and maximum, the upper and lower quartile, and the median (solid line) as well as the mean (dashed line). Filled circles may be considered as outlier. Paired t-test on the number of fecal pellets ($P < 0.0001$) indicates strong feeding activity on WT versus only very little feeding on the *OE::rsmA* strain.

Panel C. Representative images of colonies showing the differences in feeding activity (note all the fecal pellets surrounding the left colony) and damage caused to the WT (left) and the *OE::rsmA* strain (right).

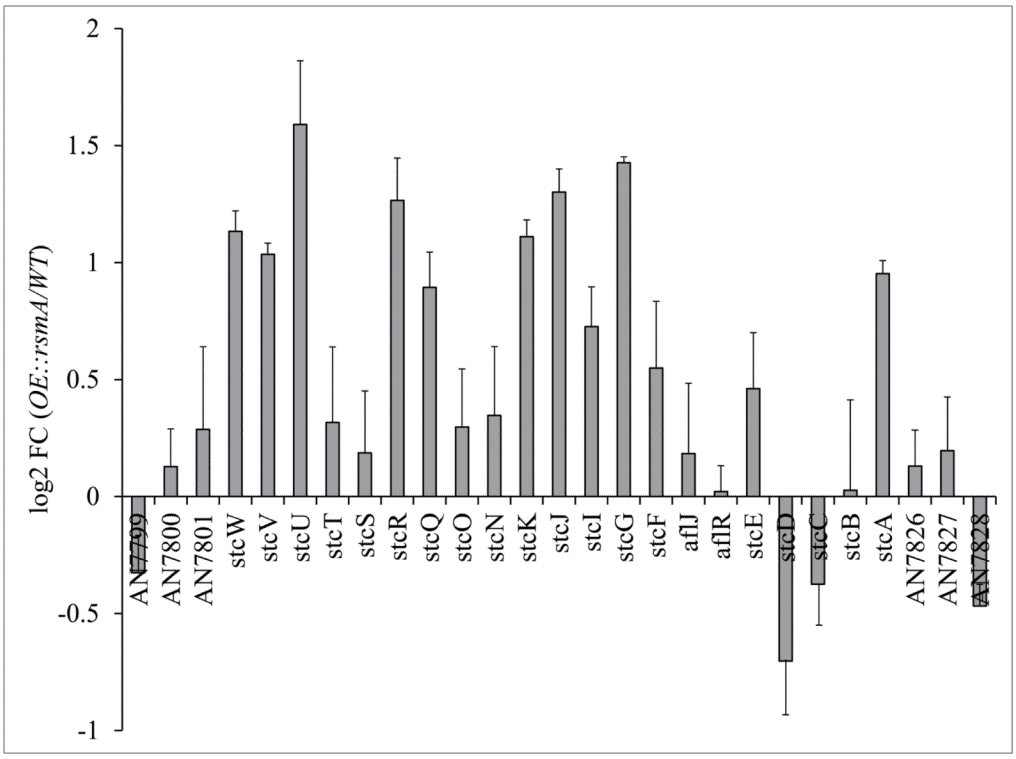

Figure 3. Microarray analysis of sterigmatocystin (ST) gene cluster

Mean (n = 3) expression ratios (log_2, *OE::rsmA* vs. WT) for genes on Chromosome IV in the region including the ST cluster (AN7899.4 AN7828.4), as measured by microarray.

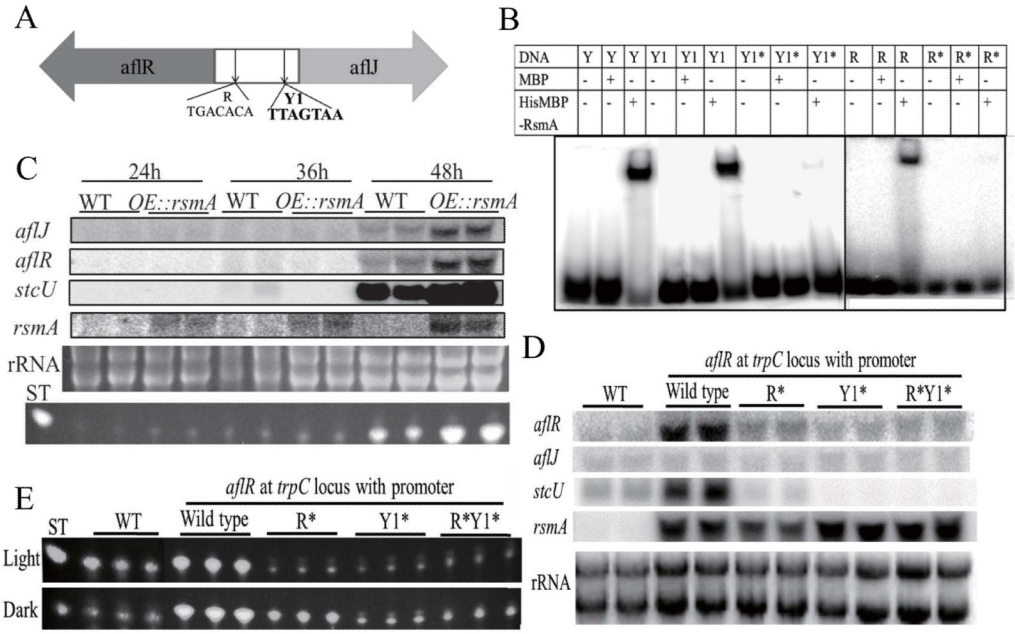

Figure 4. RsmA activates *aflR* and *aflJ* expression by specific DNA-binding in the *aflR*/*aflJ* divergent promoter

Panel A RsmA binding sites in the promoter region of *aflR*/*aflJ*. Y1: yeast Yap 1 site, R: RsmA site.

Panel B. Specific binding of RsmA to the *aflR/aflJ* promoter. The purified maltose binding protein (MBP)-tagged RsmA or MBP alone was incubated with the following ^{32}P-labeled sequences: Yap 1 binding site (Y: TTACTAA), Yap 1 binding site in the promoter region of *aflR/aflJ* (Y1: TTAGTAA), a mutation in this Y1 site (Y1* = TAAGTTA) and RsmA site (R = TGACACA and R*=AAACAGG). *=mutated sequences.

Panel C. *rsmA* overexpression results in increased *aflR*, *aflJ* and *stcU* expression. RNA was extracted after 24, 36 and 48 h of growth in liquid shake cultures. ST production from the same samples, as analyzed by TLC, is shown below rRNA. Ethidium bromide-stained rRNA is shown as a loading control.

Panel D. RsmA binding sites are required for *aflR* expression *in vivo*. *aflR*, *aflJ*, *rsmA* and *stcU* expression in five strains: WT (RDIT9.32), *aflR* with WT promoter at *trpC* locus (TWY18.14), *aflR* and promoter with R* mutation at *trpC* locus (TWY19.15), *aflR* and promoter with Y1* mutation at *trpC* locus (TWY20.4) and *aflR* and promoter with R* and Y1* mutations at *trpC* locus (TWY21.20). RNA was extracted after 48 h of growth in liquid shake cultures. Ethidium bromide-stained rRNA is shown as a loading control.

Panel E. ST production from strains shown in Panel D.

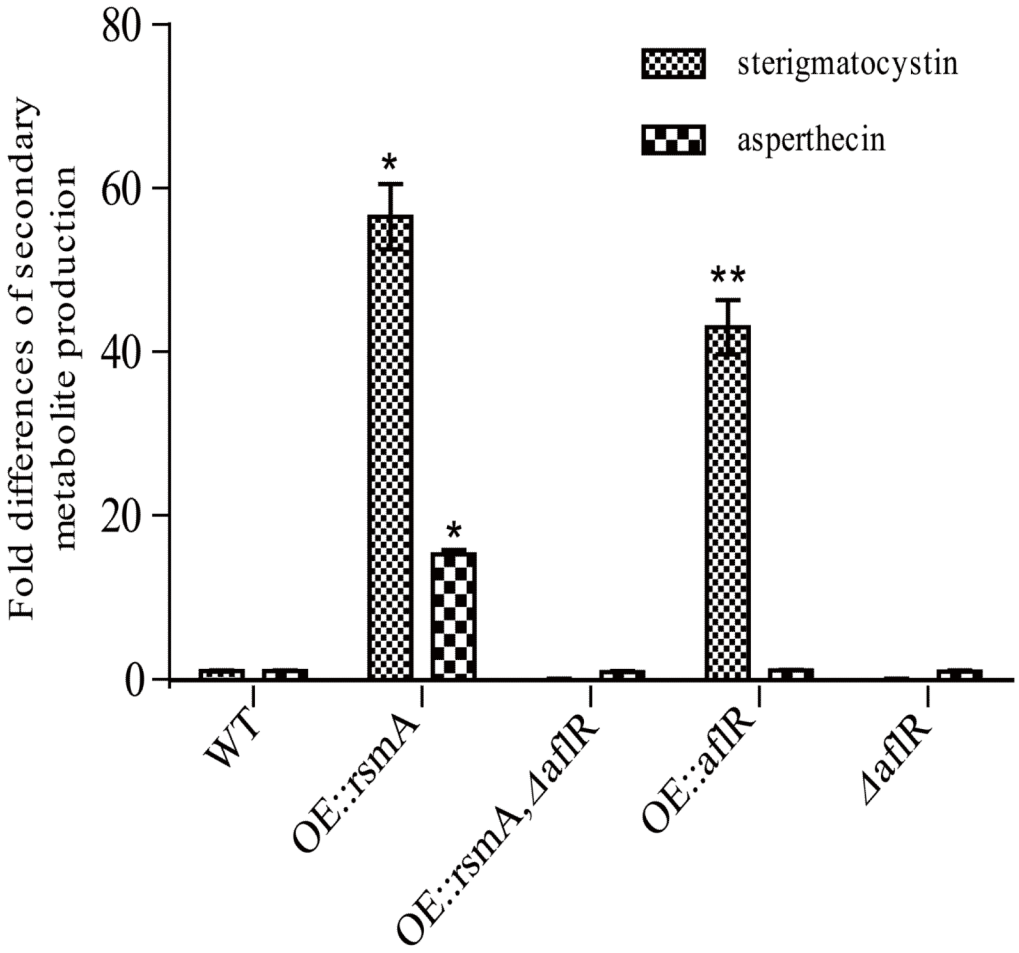

Figure 5. Sterigmatocystin and asperthecin over production require AflR in the *OE::rsmA* strain

Both metabolites require the presence of *aflR.* Statistical differences were analyzed in each secondary metabolite group by using the JMP software package, version 3.2.6 (SAS Institute, Inc., Cary, NC). Statistically significant mean values, indicated with different asterisks in the figures, are significant at $P < 0.0001$.

Table 1

Plasmids and fungal strains used in this study

Strain/plasmid	Descrption	Reference
RDIT9.32	*veA*	Tsitsigiannis *et al.* (2004a)
RDIT2.3	*veA1*	Tsitsigiannis *et al.* (2004a)
RDIT55.37	*pyroA4, veA*	Tsitsigiannis *et al.* (2004b)
RMS4.25	*gpdAp::rsmA::pyroA4::pyroA, veA*	Shaaban *et al.* (2010)
RJH256	*biA1; argB2; ΔaflR::argB; trpC801; veA1*	Bok *et al.* (2006)
RSCS3	*ΔaflR::argB, veA*	Wilkinson *et. al.* (2004)
RAMB38	*biA1; methG1; ΔaflR::argB; trpC801; veA1*	Bok *et al.* (2004)
RJMP1.49	*pyrG89, pyroA4, ΔnkuA::argB, veA*	Shaaban *et al.* (2010)
TWY5.2	*pyrG89, A. fumigatus pyrG::gpdA(p)::rsmA, pyroA4, ΔnkuA::argB, veA*	This study
TWY18.14	*A. fumigatus pyrG::gpdA(p)::rsmA, ΔaflR::argB, trpC::aflR, veA*	This study
TWY19.15	*A. fumigatus pyrG::gpdA(p)::rsmA, ΔaflR::argB, trpC::aflR^{R}, veA*	This study
TWY20.4	*A. fumigatus pyrG::gpdA(p)::rsmA, ΔaflR::argB, trpC::aflR^{Y1}, veA*	This study
TWY21.20	*A. fumigatus pyrG::gpdA(p)::rsmA, ΔaflR::argB, trpC::aflR^{R,Y1}, veA*	This study
RWY2.12	*A. fumigatus pyrG::gpdA(p)::rsmA, veA*	This study
RSA15.2	*A. fumigatus pyrG::gpdA(p)::rsmA, ΔaflR::argB, veA*	This study
RWY16.76	*A. fumigatus pyrG::gpdA(p)::rsmA, ΔaflR::argB, trpC801, veA*	This study
TWY16.12	*pyrG89, A. fumigatus pyroA::gpdA(p)::aflR, pyroA4, ΔnkuA::argB, veA*	This study
RWY20.3	*A. fumigatus pyroA::gpdA(p)::aflR, veA*	This study
pSH96	½ *trpC* in pBSSK-	Wieser and Adams (1995)
pTMH44.2	*gpdA(p)::gfp* frag::*trpC(t)*	McDonald *et al.* (2005)
pKLD116	6XHis, MBP in pET21a(+)	Rocco *et al.* (2008)
pWY20	*rsmA* in pKLD116	This study
pWY43.23	*aflR* and promoter in pSH96	This study
pWY44.6	*aflR* and *aflR(p)^{R}* in pSH96	This study
pWY45.2	*aflR* and *aflR(p)^{Y1}* in pSH96	This study
pWY46.9	*aflR* and *aflR(p)^{R,Y1}* in pSH96	This study
pWY25.16	*A.fumigatus pyroA::gpdA* in pGEMT easy vector	This study

R: TGACACA site mutated to AAACAGG; Y1: TTACTAA site mutated to TAAGTTA

RXX = ascospore recombinant, TXX = original transformant

Table 2

Oligonucleotides utilized for gel shift assay

Name	Oligonucleotide sequence (5′-3′)	Uses
AflR EMSA_F	CGACTGACACAAGAAATAACAATTC	RsmA site probe
AflR EMSA_R	GAATTGTTATTTCTTGTGTCAGTCG	RsmA site probe
AflR EMSA mu_F	CGACAAACAGGAGAAATAACAATTC	Mutated RsmA site probe
AflR EMSA mu_R	GAATTGTTATTTCTCCTGTTTGTCG	Mutated RsmA site probe
TRX2 site2_for	GATCCTCTTTTCTTACTAAGCGCG	Yeast Yap 1 probe
TRX2 site2_rev	GATCCGCGCTTAGTAAGAAAAGAG	Yeast Yap 1 probe
aflR_Yap_for	ACAGACTTTTTAGTAATGGCGACTC	*A. nidulans* Yap 1 probe
aflR_Yap_rev	GAGTCGCCATTACTAAAAAGTCTGT	*A. nidulans* Yap 1 probe
aflR_Yapmu2_for	ACAGACTTTTAAGTTATGGCGACTC	*A. nidulans* mutated Yap 1 probe
aflR_Yapmu2_rev	GAGTCGCCATAACTTAAAAGTCTGT	*A. nidulans* mutated Yap 1 probe