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bZIP transcription factors affecting secondary metabolism, sexual development and stress responses in *Aspergillus nidulans*

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The eukaryotic basic leucine zipper (bZIP) transcription factors play critical roles in the organismal response to the environment. Recently, a novel YAP-like bZIP, restorer of secondary metabolism A (RsmA), was found in a suppressor screen of an *Aspergillus nidulans* secondary metabolism (SM) mutant in which overexpression of *rsmA* was found to partially remediate loss of SM in Velvet Complex mutants. The Velvet Complex is a conserved fungal transcriptional heteromer that couples SM with sexual development in fungi. Here we characterized and contrasted SM in mutants of RsmA and four other *A. nidulans* bZIP proteins (NapA, ZipA, ZipB and ZipC) with predicted DNA binding motifs similar to RsmA. Only two overexpression mutants exhibited both SM and sexual abnormalities that were noteworthy: *OE*::*rsmA* resulted in a 100-fold increase in sterigmatocystin and a near loss of meiotic spore production. *OE*::*napA* displayed decreased production of sterigmatocystin, emericellin, asperthecin, shamixanthone and epishamixanthone, coupled with a shift from sexual to asexual development. Quantification of bZIP homodimer and heterodimer formation using fluorescence resonance energy transfer (FRET) suggested that these proteins preferentially self-associate.

INTRODUCTION

Dimeric basic leucine zipper (bZIP) proteins are conserved transcriptional enhancers found in all eukaryotes. These proteins play critical roles, often species-specific, in many aspects of organismal development. A distinct subset of proteins within the bZIP family is referred to as yeast activator protein (Yap) transcription factors, which have been well defined in yeast (Rodrigues-Pousada *et al.*, 2010). Members of the Yap family, eight proteins in yeast, contain a conserved set of residues in their basic region conferring distinct DNA-binding properties that overlap and differ from those of the canonical Gcn4 factor. The Yap family binding site was characterized as TTAC/GTAA and the terms 'Yap site' or 'Yap response element' (YRE) were coined. Yap proteins frequently mediate stress responses, and are often associated with resistance to reactive oxygen species (ROS), osmotic imbalances, drugs or heavy metals.

Since the finding of the first Yap protein in *Saccharomyces cerevisiae* (Moye-Rowley *et al.*, 1989), several orthologues have been characterized in other fungi. Similar to the reported roles in yeast, these proteins typically are associated with resistance to ROS or antifungals. The Yap1 orthologues Afyap1, Aoyap1, Apyap1 and MoAP1 in the human pathogen *Aspergillus fumigatus*, the mycotoxic pathogens *Aspergillus ochraceus* and *Aspergillus parasiticus*, and the rice pathogen *Magnaporthe oryzae*, respectively, all mediate the oxidative stress response, and in the latter pathogen, MoAP1 is critical for pathogenicity (Guo *et al.*, 2011; Lessing *et al.*, 2007; Qiao *et al.*, 2008; Reverberi *et al.*, 2007, 2012). Yap-like proteins in *Aspergillus nidulans* and *Neurospora crassa* have also been characterized in ROS and

Abbreviations: DCF, dichlorofluorescein; FRET, fluorescence resonance energy transfer; MSB, menadione sodium bisulphite; ROS, reactive oxygen species; RS, reactive species; SM, secondary metabolism; TAMRA, carboxytetramethylrhodamine; *t*BOOH, *tert*-butylhydroperoxide.

Two supplementary tables and five supplementary figures are available with the online version of this paper.

heavy metal resistance (Asano *et al.*, 2007; Tian *et al.*, 2011).

A recently reported and novel function for bZIPs is association of these proteins with secondary metabolite production in filamentous fungi. In a mutagenesis screen for secondary metabolism (SM) activation in the filamentous fungus *A. nidulans*, a Yap-like bZIP, termed restorer of secondary metabolism A (RsmA), has been identified (Shaaban *et al.*, 2010). Overexpression of *rsmA* was able to partially compensate for loss of LaeA and VeA, two members of the fungal-specific Velvet Complex required for global regulation of SM and sexual development in *A. nidulans* (Bayram *et al.*, 2008). Yap-like bZIPs have also been associated with negative regulation of secondary metabolites. For example, deletion of Aoyap1 and Apyap1 in *A. ochraceus* and *A. parasiticus* resulted in increases of ochratoxin and aflatoxin, respectively (Reverberi *et al.*, 2007, 2012). These latter two studies led to the hypothesis that fungal SM can be triggered by stress-response pathways.

To further explore this linkage of bZIP response pathways to SM in the model aspergillus *A. nidulans*, we assessed the effects of deletion and overexpression of five Yap-like proteins, including RsmA. We found that both RsmA and NapA, previously identified as having a major role in the fungal stress response, have significant and distinct effects on SM and sexual development, a linkage previously characterized by the Velvet Complex (Bayram & Braus, 2012).

METHODS

Fungal strains and culture conditions. The fungal strains and plasmids used in this study are listed in Table 1. All strains were grown at 37 °C on glucose minimum medium (GMM) (Shimizu & Keller, 2001) with appropriate supplements corresponding to the auxotrophic markers, and were maintained as glycerol stocks at −80 °C. *Escherichia coli* strain DH5α was propagated in Luria–Bertani (LB) medium with appropriate antibiotics for plasmid DNA.

Creation of fungal strains. The oligonucleotide sequences for PCR primers are given in Table S1 available with the online version of this paper. PCR amplification was carried out on a C1000 Thermal Cycler from Bio-Rad. For creation of overexpression strains of *napA* (*AN7513*), *zipA* (*AN11891*), *zipB* (*AN8772*) and *zipC* (*AN10378*) at the native locus, double joint PCR procedures (Yu *et al.*, 2004) were carried out. Briefly, the marker gene cassette (*A. fumigatus pyroA* and a constitutive *A. nidulans gpdA* promoter) was amplified by using pWY25.16 (Table 1) as template and used as marker gene for all overexpression bZIP mutants. Approximately 1 kb fragments upstream and downstream of the target genes were amplified from genomic DNA of *A. nidulans* using the designated primers (Table S1), respectively. These three amplified PCR fragments were then purified with a QIAquick gel extraction kit (Qiagen), quantified, and fused using double-joint PCR procedures. The final PCR product was amplified with the primer pairs NEST_for and _rev or the end primers of each flanking region, confirmed with endonuclease digestion and purified for fungal transformation. Except for the first-round PCR with *Pfu* Ultra II DNA polymerases (Agilent), all PCR steps were performed by using an Expand Long Template PCR system (Roche) according to the manufacturer's instructions. Using the same double-joint PCR strategy (Yu *et al.*, 2004), *napA*, *zipA* and *zipB* deletion strains were created with *A. fumigatus pyroA* as the selectable maker. Fungal protoplast preparation and transformation were carried out as described by Bok & Keller (2004). Samples (5 μg) of the double-joint cassette were used to overexpress/delete bZIPs by using *A. nidulans* strain RJMP1.49 (*pyrG89*, *pyroA4*, Δ*nkuA*::*argB*, *veA*) as the recipient host. Overexpression and deletion strains were verified by PCR and Southern blot analysis.

Prototrophic strains, other than the bZIP mutation, were generated by sexual crossing for physiological studies, according to standard methods (Pontecorvo *et al.*, 1953). Briefly, crossing TMS6.30 with HZS189 yielded RWY8.5 (Δ*rsmA*). Crossing RDIT55.37 with TWY6.2, TWY7.3, TWY8.3, TWY13.15, TWY14.3, TWY15.5 and TWY17.10 created RWY9.2 (Δ*zipA*), RWY10.3 (Δ*napA*), RWY11.1 (Δ*zipB*), RWY17.3 (*OE*::*napA*), RWY18.4 (*OE*::*zipA*), RWY19.1 (*OE*::*zipB*) and RWY22.1 (*OE*::*zipC*), respectively. The genotypes of the progeny were determined by growth on selection media and PCR confirmation with designated primers (Table S1). Possession of the *veA* or *veA1* allele was determined by using the primers veA + For and veA + Rev and veA1For and veA + Rev, respectively (Table S1).

Northern analysis. Extractions were made from mycelia of cultures where 10^6 spores ml^{-1} were grown in 50 ml liquid GMM at 37 °C for 48 h with shaking at 250 r.p.m. Mycelia were harvested and lyophilized. RNA was extracted by using Isol-RNA Lysis Reagent according to the manufacturer's instructions (5 Prime). About 30 μg of total RNA was used for RNA blot analysis. RNA blots were hybridized with designated DNA fragments, which were generated by PCR using gene-specific primers as shown in Table S1. All experiments were performed in duplicate.

Sterigmatocystin examination. Samples (5 μl) of 10^5 spores of *A. nidulans* strains were point- or overlay-inoculated onto YAG medium (5 g yeast extract l^{-1}, 15 g agar l^{-1} and 20 g D-glucose l^{-1}, supplemented with 1 ml of a trace element solution l^{-1} and 200 μg pyridoxine l^{-1}) or GMM (Shimizu & Keller, 2001) with pyridoxine at a concentration of 200 μg l^{-1}, and incubated for 4 or 7 days at 37 °C under both light and dark conditions. An equal-size agar plug, 7 mm in diameter, was removed from the centre of each plate culture, homogenized in 3 ml nanopure water and extracted with an equal amount of chloroform by agitation for 30 min at room temperature. The chloroform extracts were then dried completely at room temperature and resuspended in 100 μl chloroform. Metabolites were separated in the developing solvent toluene : ethyl acetate : acetic acid (TEA; 8 : 1 : 1) on silica-coated TLC plates (Shwab *et al.*, 2007), and photographs were taken following exposure to UV radiation at 254 and 366 nm wavelengths.

LC–MS analysis. The same cultures from 7-day YAG plates were used for secondary metabolite analysis by LC-MS. Briefly, 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 methanol followed by 2 ml CH_2Cl_2/methanol (1 : 1) each with 1 h sonication. The organic extract was transferred to a fresh 7 ml vial, in which the organic solvents were evaporated by TurboVap LV (Caliper Life Sciences) to dryness. The crude extract was then redissolved in 0.2 ml DMSO/methanol (1 : 4). After filtration, 10 μl of DMSO/methanol extract was injected for HPLC-photodiode array detection-MS (HPLC-DAD-MS) analysis as described previously (Bok *et al.*, 2009).

For determining fold differences, negative ion electrospray ionization (ESI) at *m*/*z* 317 was used for the detection of asperthecin by using an extracted ion chromatogram (EIC). Positive mode at *m*/*z* 325, 409, 389 and 389 was used for the detection of sterigmatoycstin, emericellin, shamixanthone and epishamixanthone, respectively. The fold differences were calculated according to the following formula: $(area_{(sample)} - area_{(blank)})/(area_{(WT)} - area_{(blank)})$.

Table 1. Plasmids and fungal strains used in this study

pXX, Plasmid; RXX, ascospore recombinant; TXX, original transformant.

Strain or plasmid	Description	Reference or source
Strains		
RDIT9.32	*veA*	Tsitsigiannis *et al.* (2004b)
RDIT55.37	*pyroA4*, *veA*	Tsitsigiannis *et al.* (2004a)
RWY2.12	*gpdA*(*p*)::*rsmA*::*A. fumigatus pyrG*, *veA*	Yin *et al.* (2012)
RJMP1.49	*pyrG89*, *pyroA4*, Δ*nkuA*::*argB*, *veA*	Shaaban *et al.* (2010)
HZS189	*paba1*, *ribo2B*, Δ*atfA*::*riboB*, *veA1*	Balázs *et al.* (2010)
TMS6.30	*pyrG89*::Δ*rsmA*::*A. parasiticus pyrG*, *pyroA4*, *veA*	Shaaban *et al.* (2010)
TWY6.2	*pyrG89*; Δ*zipA*::*pyroA A. fumigatus*, *pyroA4*, Δ*nkuA*::*argB*; *veA*	This study
TWY7.3	*pyrG89*; Δ*napA*::*pyroA A. fumigatus*, *pyroA4*, Δ*nkuA*::*argB*; *veA*	This study
TWY8.3	*pyrG89*; Δ*zipB*::*pyroA A. fumigatus*, *pyroA4*, Δ*nkuA*::*argB*; *veA*	This study
TWY13.15	*pyrG89*; *A. fumigatus pyroA*::*gpdA*(*p*)::*napA*, *pyroA4*, Δ*nkuA*::*argB*; *veA*	This study
TWY14.3	*pyrG89*; *A. fumigatus pyroA*::*gpdA*(*p*)::*zipA*, *pyroA4*, Δ*nkuA*::*argB*; *veA*	This study
TWY15.5	*pyrG89*; *A. fumigatus pyroA*::*gpdA*(*p*)::*zipB*, *pyroA4*, Δ*nkuA*::*argB*; *veA*	This study
TWY17.10	*pyrG89*; *A. fumigatus pyroA*::*gpdA*(*p*)::*zipC*, *pyroA4*, Δ*nkuA*::*argB*; *veA*	This study
RWY8.5	Δ*rsmA*::*pyrG A. parasiticus*, *veA*	This study
RWY9.2	Δ*zipA*::*pyroA A. fumigatus*, *veA*	This study
RWY10.3	Δ*napA*::*pyroA A. fumigatus*, *veA*	This study
RWY11.1	Δ*zipB*::*pyroA A. fumigatus*, *veA*	This study
RWY17.3	*A. fumigatus pyroA*::*gpdA*(*p*)::*napA*, *pyroA4*, *veA*	This study
RWY18.4	*A. fumigatus pyroA*::*gpdA*(*p*)::*zipA*, *pyroA4*, *veA*	This study
RWY19.1	*A. fumigatus pyroA*::*gpdA*(*p*)::*zipB*, *pyroA4*, *veA*	This study
RWY22.1	*A. fumigatus pyroA*::*gpdA*(*p*)::*zipC*, *pyroA4*, *veA*	This study
Plasmids		
pTlex3	Yeast two-hybrid bait vector	Cho *et al.* (2003)
pWY25.16	*A. fumigatus pyroA*::*gpdA* in pGEM-T Easy vector	Yin *et al.* (2012)
pWY16	*zipA* cDNA in pTlex3	This study
pWY17	*napA* cDNA in pTlex3	This study
pWY18	*zipB* cDNA in pTlex3	This study
pWY19	*zipC* cDNA in pTlex3	This study

Analysis of spore production. Before adding chloroform for secondary metabolite extractions of YAG overlay samples, 100 µl of spore-agar suspension described above was collected for each strain to use for ascospore (sexual spore) and conidia (asexual spore) quantification. Serial dilutions were carried out in double-distilled water, and spores were quantified with a haemocytometer.

Stress sensitivity assays on nutrient agar plates. To estimate the stress sensitivity of the mutants, 10^5 freshly grown (6 days) conidia washed and suspended in 5 µl 0.9 % NaCl, 0.01 % Tween 80 (Eigentler *et al.*, 2012) were spotted on minimal-nitrate medium agar plates (Balázs *et al.*, 2010; Hagiwara *et al.*, 2007, 2008), which were also supplemented with one of the following stress-generating agents (concentrations and mechanisms of actions are given in parentheses): diamide (2 mmol l^{-1}, triggers glutathione redox imbalance), menadione sodium bisulphite (MSB; 0.12 mmol l^{-1}, increases intracellular superoxide concentrations), *tert*-butylhydroperoxide (*t*BOOH; 0.8 mmol l^{-1}, accelerates lipid peroxidation) and H_2O_2 (6.0 mmol l^{-1}, increases intracellular peroxide concentrations). The surface cultures of RWY17.3 (*OE*::*napA*), RWY18.4 (*OE*::*zipA*) and RWY19.1 (*OE*::*zipB*) were always supplemented with pyridoxine at a concentration of 200 µg l^{-1}. All stress plates were incubated at 37 °C for 5 days (Balázs *et al.*, 2010), the colony diameters were measured and the percentage growth inhibition was calculated. The growth inhibition of the mutant strains was always compared with that of the RDIT9.32 reference strain.

Determination of physiological parameters in submerged cultures of *A. nidulans*. *A. nidulans* strains (RDIT9.32: wild-type, RWY10.3 Δ*napA*, RWY17.3 *OE*::*napA*) were pre-grown in Erlenmeyer flasks (500 ml) containing 100 ml minimal-nitrate medium (pH 6.5) also supplemented with 200 µg pyridoxine l^{-1} (wild-type, *OE*::*napA*) as required. Culture media were inoculated with 5×10^7 spores and incubated at 37 °C and at 3.7 Hz shaking frequency. Oxidative stress was induced by the addition of 0.8 mmol *t*BOOH l^{-1} to exponential growth phase (18 h) cultures, and samples were taken at 28 h (10 h stress exposure) and 42 h (24 h stress exposure) incubation times for the determination of selected physiological parameters.

The intracellular reactive species (RS) levels were characterized by the formation of 2′,7′-dichlorofluorescein (DCF) from 2′,7′-dichlorofluorescin diacetate (Halliwell & Gutteridge, 2007). RS includes all ROS and reactive nitrogen species, which oxidize 2′,7′-dichlorofluorescin to DCF (Halliwell & Gutteridge, 2007). At the incubation times tested, 10 µmol 2′,7′-dichlorofluorescin diacetate ml^{-1} was added to 20 ml aliquots of the cultures, and after incubating further for 1 h in 100 ml culture flasks, the mycelia were harvested by centrifugation. The production of DCF was determined spectrofluorimetrically (Emri *et al.*, 1997, 1999).

Changes in the specific activities of certain antioxidant enzymes were also recorded in separate experiments. In these cases, cell-free extracts were prepared by X-pressing and centrifugation (Emri *et al.*, 1997). Specific catalase (Roggenkamp *et al.*, 1974) and glutathione peroxidase (GPx; Chiu *et al.*, 1976) activities were measured. Briefly, catalase and

GPx activities were determined spectrophotometrically, measuring H_2O_2 decomposition and NADPH diminution rates, respectively (Roggenkamp *et al.*, 1974; Chiu *et al.*, 1976). In the GPx assay, cumene hydroperoxide was used as substrate and the glutathione disulphide formed was reduced by glutathione reductase auxiliary enzyme, which oxidizes NADPH cofactor (Chiu *et al.*, 1976).

In sterigmatocystin determinations, mycelia from 24 h cultures were filtered and washed. After lyophilization, sterigmatocystin was extracted by 70 % (v/v) acetone from 20 mg quantities of the freeze-dried mycelial powder. The sterigmatocystin content of the solutions was quantified on silica gel according to Klich *et al.* (2001).

Plasmid construction. bZIP ORFs were amplified from an *A. nidulans* cDNA library with designated primers (Table S1). Then, each bZIP fragment was cloned into pTlex3 using the Quick-change method (Bok & Keller, 2012) to obtain pWY16 to pWY19. All plasmids were sequenced for confirmation before use.

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 as described previously (Shaaban *et al.*, 2010). Sequence data were analysed in the SeqBuilder (v. 7.0) of the Lasergene software package from DNASTAR.

Cloning, expression, purification and labelling of proteins for FRET. bZIP domains were cloned from plasmids pWY16 to pWY19 (Table 1) or *A. nidulans* genomic DNA into a modified pTXB1 (NEB) plasmid using the sequence and ligation-independent cloning (SLIC) method (Li & Elledge, 2007), or were restriction-digested with *Xho*I and *Nsi*I. All constructs were verified by sequencing. Sequences for the proteins used are listed in Table 2. Proteins were expressed as intein–chitin binding domain fusions in RP3098 *E. coli* by growing 1 l LB cultures at 37 °C to OD_{600} 0.4–0.8, at which point expression was induced with 0.5 mM IPTG. Cultures were then incubated for 3–4 h and cells pelleted. Cells were resuspended in buffer (20 mM HEPES, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1 M guanidine-HCl, 0.2 mM PMSF and 0.1 % Triton X-100) and lysed using sonication. The lysate was then split and each half was poured over a column containing 1 ml chitin beads (NEB). The column was washed and then equilibrated with EPL buffer [50 mM HEPES, pH 8.0, 500 mM NaCl, 200 mM 2-mercaptoethane sulfonate (MESNA), 1 M guanidine-HCl]. To cleave the intein and label the proteins with a fluorescent dye, the columns were incubated with EPL buffer containing 1 mg cysteine-lysine-dye ml^{-1}, where the dye was either fluorescein or carboxytetramethylrhodamine (TAMRA; Celtek). Columns were capped and incubated for 16 h. Cleaved and labelled proteins were eluted and diluted fivefold into denaturing buffer (6 M guanidine-HCl, 5 mM imidazole, 0.5 M NaCl, 20 mM Tris, 1 mM DTT, pH 7.9). This solution was flowed over columns containing 1 ml nickel-nitrilotriacetic acid (Ni-NTA) resin. After washing, proteins were eluted with 60 % acetonitrile/0.1 % trifluoroacetic acid. Labelled proteins were lyophilized, resuspended and desalted using spin-columns (Bio-Rad). Proteins were stored in 10 mM potassium phosphate, pH 4.5, at −80 °C. Peptide concentrations were measured in 6 M guanidine-HCl/100 mM sodium phosphate, pH 7.4, using the absorbance of the dye with an absorption coefficient of 68 000 M^{-1} cm^{-1} at 499 nm for fluorescein and 86 000 M^{-1} cm^{-1} at 560 nm for TAMRA. Molecular masses of *A. nidulans* fluorescein-labelled proteins were determined by MS to be within 0.1 % of the expected mass.

FRET measurements. TAMRA-labelled proteins diluted in 1 mM tris (2-carboxyethyl)phosphine (TCEP) were serially diluted twofold in black 384-well non-binding surface plates (Corning). This resulted in 12 concentrations of each TAMRA protein from 1000 to 0 nM with each well containing 30 µl. Fluorescein-labelled proteins were diluted to 80 nM in 1 mM TCEP and 10 µl of each was transferred to the 384-well plate. A 40 µl volume of 2 × binding buffer (100 mM potassium phosphate, pH 7.4, 300 mM KCl, 0.2 % BSA, 0.2 % Tween-20) was then added to each well and mixed. Binding reactions were set up using a Tecan Freedom EVO liquid-handling robot. Plates were incubated for 105 min at 37 °C. Following incubation, plates were read using a fluorescence plate reader (Molecular Devices) with excitation at 480 nm and emission at 525 nm. Plates were then transferred to 21 °C and incubated for 60 min and measured again. Plates were then transferred to 4 °C and incubated for 60 min and measured again.

To fit equilibrium disassociation constants, a system of ordinary differential equations (ODEs) was integrated in MATLAB as described previously (Ashenberg *et al.*, 2011). The K_d of each homodimer along with an upper and lower baseline was fitted. Homodimer K_ds were used subsequently to determine the K_ds of each heterodimer. When fitting heterodimers, the ODEs describing donor or acceptor homodimer formation were only included in the system when their respective K_ds were less than 5000 nM (otherwise, the system was modelled with no homodimer species). Each curve was manually inspected. For several curves with bad upper or lower baselines, one or more points were removed to allow for an improved fit. All interactions identified were also observed in a replicate experiment.

Statistical analysis. For statistical analyses, data were analysed with the GraphPad Instate software package, version 5.01 using the Tukey–Kramer multiple comparison test at $P \leqslant 0.05$.

RESULTS

Selection of bZIPs

RsmA, identified as a suppressor in a Δ*laeA* screen, was found to activate sterigmatocystin through binding to the

Table 2. Protein sequences utilized for FRET

Protein	Sequence*
NapA	KKPGRKPLTSEPTSKRKAQNRAAQRAFRERKEKHLKDLEAKVEELQKASDSANQENGLLKAQVERLQVELREYRKRLSWVT
RsmA	EKDKDGIGITPAQSKRKAQNRAAQRAFRERKERHVRDLEEKVSNLQQESSNLLADNERLKREIARYSTENEILR
ZipA	LLASEEGKKLSSKERRQLRNKVSARAFRSRRKEYIGQLENEVAQKTNEAHELRQQNRALCDENARLTDLVRQLL
ZipB	STKENASEPTDPGLRRKEQVRRAQQTYRLRKESYIKSLEREILHLRTAKSDLTGETRKLRAEVRRLRQVIEQHG
ZipC	ETPKTYGKRPLSTSKRAAQNRAAQRAFRQRKESYIRKLEEQVKEYEVMSQEYKALQAENYQLREYVINLQSRLL
JUN	SPIDMESQERIKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNHV
FOS	KVEQLSPEEEEKRRIRRERNKMAAAKCRNRRRELTDTLQAETDQLEDEKSALQTEIANLLKEKEKLEFILAAHR

*All sequences contained the linkers SHHHHHHDWKGSS at the N terminus and GA at the C terminus.

Yap-like site TTAGTAA (Yin *et al.*, 2012). In yeast, Yap proteins often exhibit overlapping roles, so we reasoned that other *A. nidulans* Yap-like bZIPs would also affect SM. We examined this possibility by characterizing four other bZIP proteins that bore residues conserved with respect to RsmA/Yap proteins (as assessed by CLUSTAL W analysis) in the DNA binding motif and also showed differential expression in microarray profiles of *laeA* mutants (Bok *et al.*, 2006; data not shown). These proteins included the already characterized NapA (AN7513) (Asano *et al.*, 2007), as well as three uncharacterized proteins (AN11891, AN8772 and AN10378, hereinafter called ZipA, ZipB and ZipC). Fig. 1 shows the alignment of the DNA binding and leucine zipper motifs of these five proteins in comparison with Yap-family proteins and the human Yap-like bZIPs JUN and FOS (Ferguson & Goodrich, 2001).

Deletion and overexpression of RsmA has already been reported (Shaaban *et al.*, 2010), and similar procedures were conducted to create the other bZIP deletion and overexpression mutants (Table 1). The only mutant that we failed to create was the *zipC* deletion, perhaps due to a near-lethal effect of loss of this gene on fungal growth. Genomic DNA was extracted from all transformants and analysed by PCR followed by Southern blot analysis (Figs S1 and S2). Overexpression strains were also confirmed by Northern analysis (Fig. S3). Prototrophic strains were created according to standard sexual crossing methods (Pontecorvo *et al.*, 1953) and were always PCR-confirmed with designated primers (Table S1). We found that several of the overexpression mutants displayed a pyridoxine marker gene effect, and thus added pyridoxine to the media of all strains for secondary metabolite and sexual spore assessment, and for selected stress test assessments as described below.

Secondary metabolite assessment

As production of secondary metabolites is greatly dependent on culture conditions, secondary metabolite production of the bZIP mutants was assessed under a variety of conditions. We first assessed sterigmatocystin production, as this metabolite is produced in all growth media. The effect of overexpression or deletion of the different bZIP genes on sterigmatocystin production was dependent on growth medium and light conditions. As reported earlier, sterigmatocystin was always increased in the *OE*: : *rsmA* (RMY2.12) strain, but its levels varied depending on the growth medium in the other bZIP mutant backgrounds (Fig. S4). For comparison's sake, we quantified production of five secondary metabolites, asperthecin, sterigmatocystin, emericellin, shamixanthone and epishamixanthone, under the same solid-state culture conditions (Fig. 2).

The most dramatic effect on secondary metabolite production was that of *OE*: : *rsmA*, which increased sterigmatocystin production by 100-fold (Fig. 2a). Although several other mutants showed increased sterigmatocystin production under these conditions (Fig. 2b), the increases were modest compared with that of the *OE*: : *rsmA* strain. *OE*: : *rsmA* also showed a significant increase in asperthecin, as reported previously (Yin *et al.*, 2012). Conversely, *OE*: : *napA* presented a phenotype of significant decreases in emericellin, asperthecin, shamixanthone and epishamixanthone production (Fig. 2c–f), and, dependent on growth conditions, decreased sterigmatocystin production (Fig. S4).

Sexual development

Because secondary metabolite synthesis has been genetically linked with sexual development, we also assessed

Fig. 1. Comparison of the Yap bZIP domains with *A. nidulans* bZIPs. Sequences of the eight *S. cerevisiae* Yap bZIP domains (Yap1 to Yap8) compared with corresponding regions from five *A. nidulans* bZIPs (RsmA, NapA, ZipA, ZipB and ZipC) and two human bZIPs (JUN and FOS). In the basic region, residues that directly interact with base pairs and Yap-specific residues are indicated in bold type; in the leucine zipper region, the conserved leucines (or other residues) are also indicated in bold type.

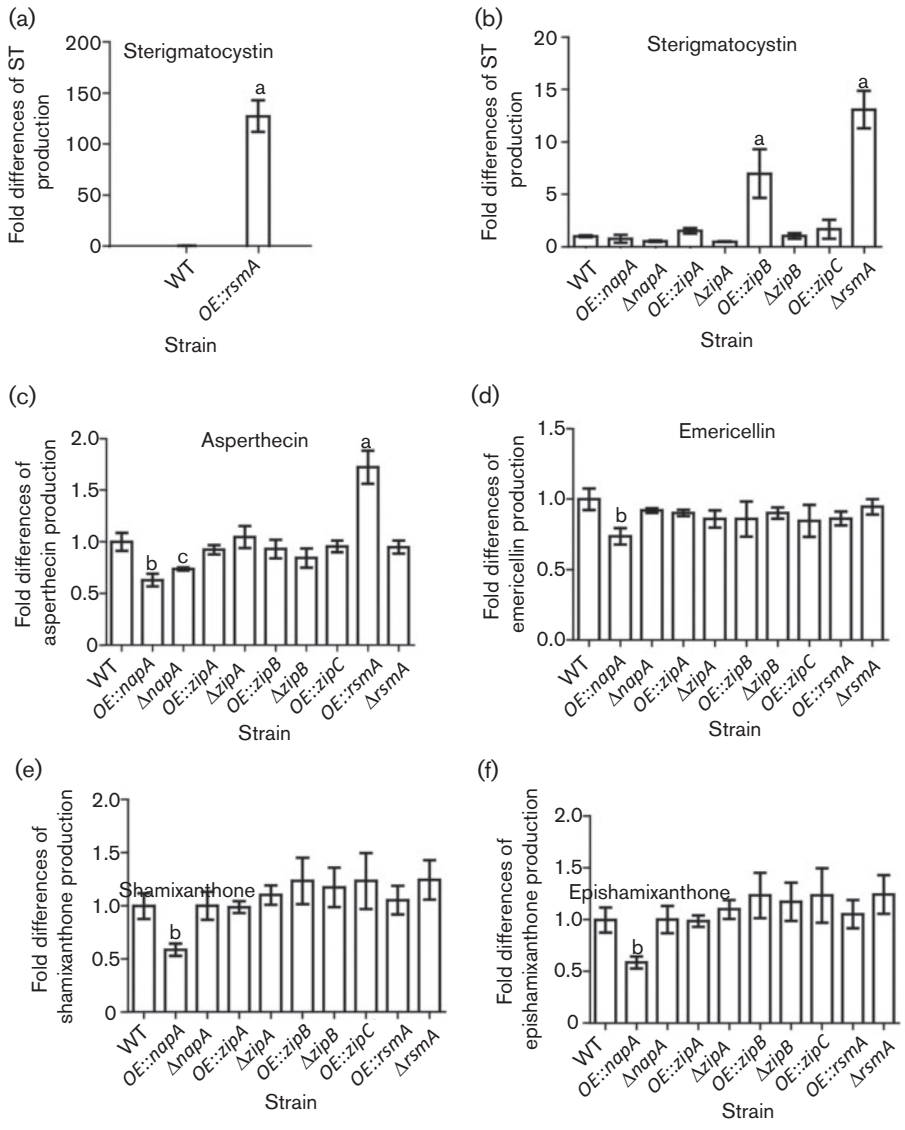

Fig. 2. LC-MS analysis of secondary metabolite production of bZIP strains. Strains were grown on YAG medium with overlay inoculation at 37 °C for 7 days under dark conditions before extraction. Sterigmatocystin (ST) (a, b), asperthecin (c), emericellin (d), shamixanthone (e) and epishamixanthone (f) were analysed by LC-MS. Data were analysed with the GraphPad Instate software package, version 5.01, using the Tukey–Kramer multiple comparison test at $P \leqslant 0.05$. Mean values with different letters show significant differences.

production of the meiotic spore, the ascospore, in all mutants. Ascospore production was significantly reduced in three overexpression strains: *OE*::*napA*, *OE*::*rsmA* and *OE*::*zipA* (Fig. 3a). Macroscopic examination of these three strains showed that decreased sexual spore production was correlated with increases in asexual spore production in *OE*::*napA* and *OE*::*zipA* (Fig. 3b); this was verified upon quantification of the asexual conidia (Fig. 3c). In contrast, *OE*::*rsmA* developed only sexually, but the sexual fruiting bodies, the cleistothecia, contained few ascospores (Fig. 3b, c).

Stress challenge

The oxidative stress tolerances of all bZIP mutants were tested on nutrient agar stress plates (Fig. 4 and Fig. S5). As expected, the deletion of *napA* resulted in increased oxidative stress sensitivity in the presence of a series of well-characterized oxidants (diamide, MSB, *t*BOOH, H_2O_2), which is in accordance with previous reports (Asano *et al.*, 2007). The overexpression of *napA* increased moderately the diamide and *t*BOOH tolerance of the fungus. In general, the other bZIP proteins had little effect on stress sensitivity as determined by growth of mutants on

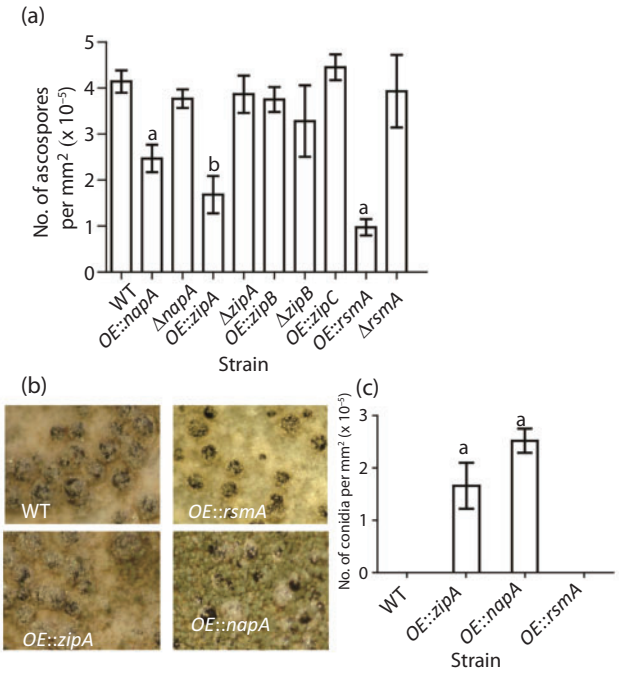

Fig. 3. Assessment of asexual and sexual spore production by bZIP strains. (a) Ascospore numbers of bZIP strains which were grown on YAG plates for 7 days. (b) Cleistothecia are shown for four bZIP strains which have decreased ascospore counts in (a). (c) Conidia numbers of selected bZIP strains from (a). Statistical differences were analysed in each strain group with Prism 5 software, version 5.01 (GraphPad software), using the Tukey–Kramer multiple comparison tests at $P \leqslant 0.05$. Mean values with different letters are significantly different.

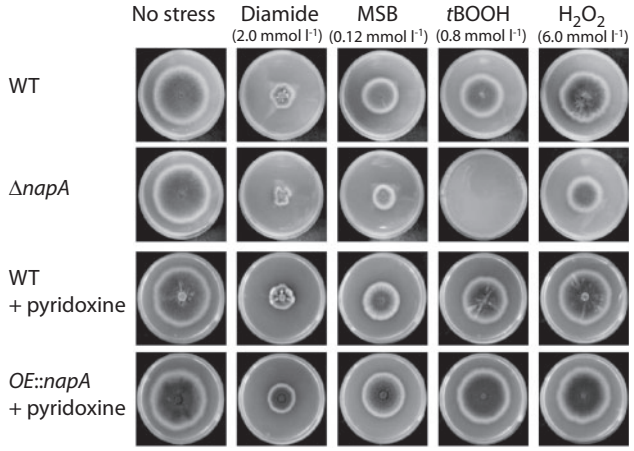

Fig. 4. Comparison of the oxidative stress tolerances of *napA* mutants. RDIT9.32 (wild-type), RWY10.3 (Δ*napA*) and RWY17.3 (*OE*::*napA*) strains were incubated at 37 °C for 5 days, and selected culture media were supplemented with 200 µg pyridoxine l^{-1}, as required. Stress-initiated percentage changes (ΔA) in growth (colony diameters) were determined for each strain and stress-generating agent, and differences between the percentage changes recorded for the mutants and the RDIT9.32 control strain ($\Delta\Delta A = \Delta A_{mutant} - \Delta A_{RDIT9.32}$) were calculated. Significantly decreased stress tolerances were found in oxidative stress (MSB, *t*BOOH and H_2O_2)-exposed RWY10.3 (Δ*napA*) cultures ($-80\% < \Delta\Delta A < -20\%$); meanwhile, growth stimulation caused by the overexpression of *napA* (RWY17.3) was $\Delta\Delta A \leqslant 15\%$ under different oxidative stress treatments. Pyridoxine supplementation increased slightly the oxidative stress resistance of RDIT9.32 ($\Delta\Delta A \leqslant 5\%$), which was similar to previous findings (Balázs *et al.*, 2010).

common stress media; however, *OE*::*zipA* and *OE*::*zipC* were more sensitive to MSB and diamide, respectively, and Δ*zipA* showed an increased tolerance to hydrogen peroxide (Fig. S5).

NapA involvement in SM is stress-related

The above studies clearly demonstrated that NapA had a unique role in the fungal response to stress, SM and sexual development that, in many respects, was opposite to that of RsmA. Whereas RsmA regulation of sterigmatocystin is positive via DNA binding of *aflR* (Yin *et al.*, 2012), studies of NapA deletants in *A. ochraceus* and *A. parasiticus* (Aoyap1 and Apyap1, respectively) suggested that the effect of this bZIP factor on SM was indirect. Specifically, a hypothesis was proposed that the loss of Aoyap1/Apyap1 disabled the redox balance in fungi, leading to activation of secondary metabolite synthesis (Reverberi *et al.*, 2007, 2008, 2012). We therefore set out to examine the relationship of oxidative stress regulation and sterigmatocystin production more exhaustively in the *napA* mutants. To do this, biomass, sterigmatocystin and RS (Halliwell & Gutteridge, 2007) production as well as changes in the specific catalase and glutathione peroxidase activities were measured in exponential growth phase cultures of wild-type, Δ*napA* and *OE*::*napA* exposed to *t*BOOH, a lipid peroxidation-inducing agent (Table 3).

First, we compared wild-type with Δ*napA*. There were no statistical differences in biomass accumulation in these two strains, regardless of *t*BOOH treatment, and in both strains the addition of *t*BOOH elicited sterigmatocystin overproduction. Differences were observed in RS production and antioxidant enzyme activity in the Δ*napA* strain as compared with wild-type with or without *t*BOOH addition. In general, RS production was higher in the deletion mutants, with decreases in catalase activity at early time points and higher glutathione peroxidase activity at 24 h for the *t*BOOH treatment.

The comparison of wild-type with *OE*::*napA* required pyridoxine supplementation as described above. As expected, the addition of pyridoxine ameliorated the slow growth normally associated with *t*BOOH treatment. However, this compound still had the effect of inducing sterigmatocystin overproduction in the wild-type. Notably, sterigmatocystin levels in both treatments were statistically decreased in the *OE*::*napA* strain compared with wild-type.

Table 3. Biomass, sterigmatocystin, RS and antioxidant enzyme production in *A. nidulans* Δ*napA* and *OE*::*napA* mutants

WT, wild-type.

Strain and treatment	Physiological parameter*									
	Biomass (DCM†) production (g l^{-1})			Sterigmatocystin production [mg (g DCM)$^{-1}$]	RS‡ production [pmol DCF (g DCM)$^{-1}$]		Specific antioxidant enzyme activities			
							Catalase [kat (kg protein^{-1})]		Glutathione peroxidase (GPx) [mkat (kg protein)$^{-1}$]	
	0 h	10 h	24 h	24 h	10 h	24 h	10 h	24 h	10 h	24 h
WT	2.0 ± 0.3	4.5 ± 0.6	4.2 ± 0.5	1.5 ± 0.5	0.25 ± 0.05	0.19 ± 0.05	0.21 ± 0.02	0.28 ± 0.03§	0.54 ± 0.05	0.31 ± 0.03§
WT + *t*BOOH	2.1 ± 0.3	2.2 ± 0.6¶	4.4 ± 0.5§	3.1 ± 1¶	27 ± 5¶	0.52 ± 0.06§¶	0.56 ± 0.05¶	0.31 ± 0.04§	0.68 ± 0.05¶	0.28 ± 0.03§
Δ*napA*	2.1 ± 0.3	4.4 ± 0.3	3.6 ± 0.4§	1.4 ± 0.5	0.41 ± 0.06#	0.47 ± 0.07#	0.12 ± 0.02#	0.20 ± 0.03§#	0.68 ± 0.07#	0.33 ± 0.03§
Δ*napA* + *t*BOOH	2.1 ± 0.2	1.8 ± 0.3¶	3.4 ± 0.4§#	3.3 ± 1¶	32 ± 7¶	1.8 ± 0.5§¶#	0.31 ± 0.03¶#	0.28 ± 0.03¶	0.76 ± 0.08	0.65 ± 0.07¶#
Pyridoxine supplementation										
WT	2.2 ± 0.3	4.5 ± 0.6	4.2 ± 0.5	1.6 ± 0.5	0.24 ± 0.05	0.17 ± 0.05	0.22 ± 0.02	0.27 ± 0.04	0.51 ± 0.05	0.21 ± 0.03§‖
WT + *t*BOOH	2.1 ± 0.2	4.6 ± 0.6‖	4.0 ± 0.5	3.0 ± 1¶	19 ± 4‖¶	0.44 ± 0.05§¶	0.39 ± 0.02‖¶	0.28 ± 0.03§	0.63 ± 0.05¶	0.23 ± 0.03§
OE::*napA*	2.3 ± 0.3	4.8 ± 0.5	4.0 ± 0.5	0.7 ± 0.3**	0.3 ± 0.05	0.19 ± 0.04§	0.19 ± 0.02	0.26 ± 0.03§	0.62 ± 0.06**	0.53 ± 0.07**
OE::*napA* + *t*BOOH	2.2 ± 0.3	4.8 ± 0.5	4.2 ± 0.5	1.0 ± 0.3¶**	1.1 ± 0.2¶**	0.18 ± 0.05§**	0.21 ± 0.02**	0.25 ± 0.03	0.68 ± 0.06	0.47 ± 0.07§**

*Mean ± SD values calculated from three independent experiments are presented.

†DCM, dry cell mass.

‡RS includes all intracellular oxidants which react to give rise to 2′,7′-dichlorofluorescein from from 2′,7′-dichlorofluorescin diacetate (Halliwell & Gutteridge, 2007).

§‖¶#**Significant differences ($P<0.05$, where P values were calculated using Student's t test) between 10 and 24 h incubations (§), caused by pyridoxine (‖) supplementation or *t*BOOH treatment (¶) or initiated by Δ*napA* (#) or *OE*::*napA* (**), respectively.

Also, unlike in the wild-type, addition of *t*BOOH did not increase sterigmatocystin in this strain (0.7 ± 0.3 versus 1.0 ± 0.3). Most striking was the large decrease of RS production in the *t*BOOH-treated *OE*::*napA* strain. This appeared largely independent of catalase and glutathione peroxidase activity, which showed little change compared with the wild-type.

FRET analysis of *A. nidulans* bZIP proteins

bZIP proteins function as either homo- or heterodimers, and the same bZIP can exist as a homodimer in some instances and a heterodimer in others (Vinson *et al.*, 2006). To examine the likelihood of homodimer and heterodimer interactions of seven *A. nidulans* bZIP proteins, we quantified the interactions of 15 pairs of leucine-zipper dimerization domains using a solution FRET assay. We also included the human bZIP proteins FOS and JUN as controls, because they are known to form very stable heterodimers, with weaker homo-association. The assay measured protein–protein association in the absence of DNA. FRET analysis at three temperatures supported preferential homo-association of all five *A. nidulans* bZIP proteins, with strong self-association of ZipC. Heterodimer formation was also observed between ZipA and ZipB (at 4 °C) and ZipA and ZipC (at 21 °C, Fig. 5). Although binding to DNA is expected to stabilize all complexes, the differential protein–protein affinities that we observed may be important for competitive binding to transcriptional regulatory elements. Interestingly, several *A. nidulans* bZIPs were able to bind to human FOS and JUN leucine-zipper domains, emphasizing the conserved sequence and structure of this ancient protein family.

DISCUSSION

This study was motivated by a recent finding that a newly discovered *A. nidulans* Yap-like bZIP called RsmA was instrumental in regulating the production of the anti-predation metabolite sterigmatocystin (Shaaban *et al.*, 2010; Yin *et al.*, 2012), a compound known to show insecticidal activities towards several organisms (Gunst *et al.*, 1982; Matasyoh *et al.*, 2011). However, no obvious role for this protein was observed in canonical stress responses, in contrast to those reported for other Yap-like bZIPs including those of *Aspergillus* species (Asano *et al.*, 2007; Hagiwara *et al.*, 2008; Reverberi *et al.*, 2012; Sakamoto *et al.*, 2009). We hypothesized that Yap-like bZIPs might preferentially govern different defensive responses in *A. nidulans*, and thus created a series of deletion and overexpression strains of a subset of Yap-like bZIPs to examine this hypothesis.

We found that only two of the five bZIPs examined had a large effect on SM. As reported previously, *OE*::*rsmA* increased production of sterigmatocystin and asperthecin (Yin *et al.*, 2012). In contrast, *OE*::*napA* produced lower amounts of shamixanthone, asperthecin, emericellin, epishamixanthone and sterigmatocystin (dependent on culture conditions, Table 3, Fig. S4) than the wild-type. NapA orthologues in *A. ochraceus* and *A. parasiticus* have been reported to be negative regulators of ochratoxin and aflatoxin in these two species, thus fitting with our observations (Reverberi *et al.*, 2007, 2012). In solid-state cultivation, *OE*::*zipA* also showed some increase in sterigmatocystin (Fig. 2), but the effect of this allele on sterigmatocystin was minimal under other culture conditions (Fig. S4). As noted previously (Shaaban *et al.*, 2010), loss of *rsmA* resulted in some increase in sterigmatocystin as well (Fig. 2). In the previous study, it was suggested that *rsmA* loss may allow for another bZIP protein to activate sterigmatocystin; perhaps ZipA fulfils this role.

Several studies have reported a linkage between secondary metabolite production and sexual development. Overexpression of both *rsmA* and *napA* negatively affected sexual development but through different mechanisms. Although increases in SM are generally associated with sexual development (Bayram *et al.*, 2008; Lee *et al.*, 2012), the data do not always distinguish between fruiting body formation (cleistothecia in *A. nidulans*) and ascospore production. Here the *OE*::*rsmA* strains produced cleistothecia but few ascospores were generated in the cleistothecia. Possibly the extreme increase in sterigmatocystin

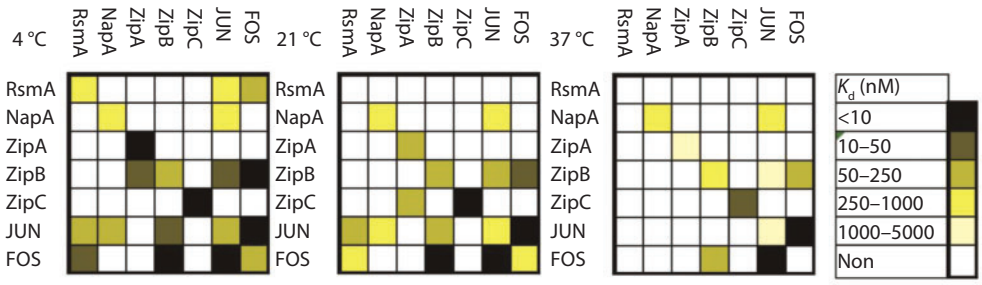

Fig. 5. FRET analysis of *A. nidulans* bZIP proteins. Dissociation constants at 4, 21 and 37 °C for selected *A. nidulans* bZIPs and human JUN and FOS are presented as a heat map, with the strength of each interaction indicated by the scale at the right. Fluorescein-labelled proteins are in rows and TAMRA-labelled proteins are in columns.

production could come at a cost to sexual spore production, and initial data from an *OE*::*rsmA* Δ*aflR* strain suggest that this could be the case (our unpublished data). On the other hand, the decrease of ascospore numbers in the *OE*::*napA* strain was associated with a shift to increased asexual growth. Whereas the cleistothecia produced in this strain produced abundant ascospores, there were simply fewer cleistothecia. The one other bZIP to show a sexual developmental phenotype, *OE*::*zipA*, was similar to the *OE*::*napA* strain. Considered together, our data here support the noted linkage of SM and sexual development.

An assessment of stress sensitivities in the bZIP mutants confirmed that NapA plays a major role in resistance to oxidative stress, with *napA* loss yielding a more sensitive oxidative stress phenotype and *OE*::*napA* a more resistant phenotype. The effect of NapA loss has been examined in several fungi, including *A. nidulans*, with all studies showing this protein to be important in protection from oxidative and other stressors (Asano *et al.*, 2007; Thön *et al.*, 2010). However, to our knowledge, this is the first examination of *OE*::*napA* in *A. nidulans.*

bZIP-type transcription factors may regulate secondary metabolite production, either directly by binding at promoters of genes encoding key elements of the biosynthetic machinery, as shown for RsmA and other bZIPs (Roze *et al.*, 2011; Yin *et al.*, 2012), or via modulating intracellular ROS levels through fine-tuning the activity of the antioxidative defence system, as demonstrated for NapA orthologues in *A. ochraceus* and *A. parasiticus* (Reverberi *et al.*, 2007, 2008, 2012). This theory has also been supported by observations that the inhibition of oxidation processes including lipid peroxidation hinders biosynthesis of mycotoxins, including aflatoxin (Jayashree & Subramanyam, 1999, 2000), and the addition of lipid peroxidation-stimulating agents to fungal cultures increases the yields of certain metabolites (e.g. ochratoxin A; Reverberi *et al.*, 2012).

To further assess this possible relationship of oxidative stress and SM, the effect of NapA on sterigmatocystin and antioxidative defence was examined in greater depth. This bZIP seems to be important in combating *t*BOOH-induced stress, and also in the regulation of cellular recovery processes from this stressor (Table 3). For instance, both Δ*napA* and OE::*napA* mutants showed aberrancies in either growth recovery or RS concentrations with the *t*BOOH treatment as compared with the wild-type strain; these were in part associated with changes in catalase and glutathione peroxidase activities. Thus it would seem that like ApYapA and AoYap1, *A. nidulans* NapA is an important element in modulating intracellular RS levels through the regulation of antioxidative enzyme activities. Although the deletion of *napA* did not increase sterigmatocystin yields under the conditions tested here (unlike in the cases of *ApyapA* and *Aoyap1* loss and its effect on aflatoxin and ochratoxin), overexpression of this gene resulted in a strain unable to increase sterigmatocystin in response to *t*BOOH, thus supporting a ROS involvement in SM in this *Aspergillus* species.

Finally, it is unclear what role(s) ZipA, ZipB and ZipC play in fungal biology. As seen in Fig. 5, the FRET data did not support a high probability of heterodimer formation among these five proteins, with the possible exception of ZipC/ZipA and ZipA/ZipB. One of our future goals will be to examine double mutants (e.g. Δ*zipA*Δ*zipB*) and the use of RNA interference technology to downregulate *zipC* for a further understanding of any effect of these proteins on *Aspergillus* development.

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