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1 **Homeobox Transcription Factor HbxA Influences Expression of over One Thousand Genes**
2 **in the Model Fungus *Aspergillus nidulans***

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4

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15 **ABSTRACT**

16 In fungi, conserved homeobox-domain (HD) proteins are transcriptional regulators governing
17 development. In *Aspergillus* species, several HD transcription factor genes have been identified,
18 among them, *hbxA/hbxI*. For instance, in the opportunistic human pathogen *Aspergillus*
19 *fumigatus*, *hbxA* is involved in conidial production and germination, as well as virulence and

20 secondary metabolism (SM), including production of fumigaclavines, fumiquinazolines, and
21 chaetominine. In the agriculturally important fungus *Aspergillus flavus*, disruption of *hbx1*
22 results in fluffy aconidial colonies unable to produce sclerotia. *hbx1* also regulates production of
23 aflatoxins, cyclopiazonic acid and aflatrem. Furthermore, transcriptome studies revealed that
24 *hbx1* has a broad effect on the *A. flavus* genome, including numerous genes involved in SM.
25 These studies underline the importance of the HbxA/Hbx1 regulator, not only in developmental
26 processes but also in the biosynthesis of a broad number of fungal natural products, including
27 potential medical drugs and mycotoxins. To gain further insight into the regulatory scope of
28 HbxA in *Aspergilli*, we studied its role in the model fungus *Aspergillus nidulans*. Our present
29 study of the *A. nidulans hbxA*-dependent transcriptome revealed that more than one thousand
30 genes are differentially expressed when this regulator was not transcribed at wild-type levels,
31 among them numerous transcription factors, including those involved in development as well as
32 in SM regulation. Furthermore, our metabolomics analyses revealed that production of several
33 secondary metabolites, some of them associated with *A. nidulans hbxA*-dependent gene clusters,
34 was also altered in deletion and overexpression *hbxA* strains compared to the wild type,
35 including synthesis of nidulanins A, B and D, versicolorin A, sterigmatocystin, austinol,
36 dehydroaustinol, and three unknown novel compounds.

37 **INTRODUCTION**

38 Developmental studies of the model filamentous fungus *Aspergillus nidulans* have provided
39 broad valuable insight into the genetic regulatory mechanisms of morphogenesis in fungi (1–3).
40 *A. nidulans* efficiently disseminates by asexual reproduction, forming specialized structures
41 called conidiophores, which bares large numbers of air-borne conidia. Activation of
42 conidiogenesis is mediated by several transcription factor genes, including *flb* genes, such as

43 *flbB*, *flbC*, *flbD*, and *flbE*, which activate the central regulatory pathway comprised of *brlA*, *abaA*
44 and *wetA* (4). This model organism is also able to reproduce sexually by producing cleistothecia,
45 fruiting bodies containing meiospores called ascospores. Cleistothecia form by aggregation of
46 vegetative mycelia, surrounded by nursing Hülle cells. This results in the formation of
47 cleistothecial primordia, which later mature into melanized cleistothecia (5,6). Several genes are
48 involved in the regulation of these processes, including *nsdD*, *medA*, *phoA*, *stuA*, *lsdA* and *tubB*
49 (7–12).

50 Other developmental regulators include Homeobox-domain transcription factors (HD-TFs).
51 These are global regulators governing developmental processes in many eukaryotic organisms
52 (13–15). The HD contains approximately 66 conserved amino acid that bind to the promoter of
53 genes governing development and other cellular processes in fungi, plants, and animals. In
54 general, fungi possess 6–12 HD-TF genes in their genome (13,14,16,17). The first reported HD-
55 TF gene is *pah1* in *Podospora anserina*, where it controls microconidiation as well as mycelial
56 branching (18). Loss-of-function of seven HD-TF genes in this fungus revealed their role in
57 sexual development (19). Another study showed that several HD-TFs in the rice
58 pathogen *Magnaporthe oryzae* are necessary for proper hyphal growth, asexual development,
59 and appressorium formation (20,21). In three species of *Fusarium*, loss-of-function of
60 the *htf1* homeobox gene leads to alteration of phialides during conidiophore formation,
61 accompanied by a drastic reduction in conidial production (22). In the fungus *Botrytis cinerea*,
62 the BcHOX8 gene has been shown to regulate growth, conidiation, and virulence in different
63 host plants (16). Also, lack of the *GRF10* HD-TF gene in the human pathogen *Candida albicans*
64 resulted in a decrease in growth, defects in chlamydospore morphology, alterations in biofilm
65 production, and a reduction of virulence (23).

66 HD-TFs are also key regulators in species of the genus *Aspergillus*. In the agriculturally relevant
67 fungus *Aspergillus flavus*, deletion of eight HD-FT genes revealed that *hbx1* in particular, was
68 required for normal vegetative growth and production of conidia and sclerotia. The regulation of
69 morphological development as well as regulation of SM, are often genetically linked (24–26).
70 Interestingly, in this case also, the production of secondary metabolites, including mycotoxins
71 (aflatoxins, cyclopiazonic acid and aflatrem), was under the regulation of *hbx1* (17).
72 Furthermore, study of the *hbx1*-dependent transcriptome indicated its importance in
73 morphological development and in regulation of secondary metabolite production (27).
74 Remarkably, the gene category corresponding to SM was the most affected by *hbx1*.
75 Additionally, in our previous study of the *hbx1* homolog in the opportunistic human pathogen
76 *Aspergillus fumigatus*, *hbxA*, showed that this gene is necessary for proper spore formation,
77 regulating expression of *brlA*, *flbB*, *flbD* and *fluG* (28). The *hbxA* gene also influenced
78 germination rate and virulence in a neutropenic mouse model. Interestingly, as in the case of *A.*
79 *flavus*, *A. fumigatus hbxA* affected production of various secondary metabolites, including
80 fumigaclavines, fumiquinazolines, compounds that accumulate in asexual structures, whose
81 production is linked to *brlA* expression (29–32), and chaetominine, an alkaloid compound that is
82 being tested to combat leukemia cells(20). Both *A. flavus* and *A. fumigatus* studies indicate that
83 HbxA/Hbx1 is a global regulator of SM in these fungi, in addition to its role in morphogenesis.
84 HbxA also affects *A. nidulans* conidiation (33,34) in a similar manner as that in *A. flavus* and *A.*
85 *fumigatus*(17,27,28). To gain further inside into the regulatory scope of *hbxA* in the genus
86 *Aspergillus*, in the present study, we characterized its role in the model fungus *A. nidulans* by
87 transcriptome and metabolomics approaches. Our findings indicate that more than one thousand
88 genes were differentially expressed in the absence of this regulator or when it was over-

89 expressed, as compared to the wild type. These include several transcription factor genes,
90 including those involved in development and SM production. Our study revealed that numerous
91 secondary metabolites gene clusters are *hbxA*-dependent in *A. nidulans*. Furthermore, our
92 analyses also indicated that *A. nidulans* metabolome is affected by *hbxA*, including production of
93 some unknown novel compounds.

94 **MATERIALS and METHODS**

95 *Phylogenetic Analysis*

96 Deduced amino acid sequences of HbxA homologs were obtained from FUNGIDB
97 (<https://fungidb.org/fungidb/>) website. BLASTp was performed against the protein sequence
98 database (pdb). Percentage (%) similarity was found using Pairwise sequence alignment using
99 EMBOSS Needle (ebi.ac.uk/Tools/psa/emboss_needle/). The phylogenetic tree was constructed using
100 MEGA v6.0 and the Maximum Likelihood model with bootstrap value of 1000.

101 *Strains used and culture conditions*

102 The *A. nidulans* strains used in this study are listed in Table 1. Strains were grown on glucose
103 minimal medium (GMM) (35) with appropriate supplements for their respective auxotrophic
104 markers (35). For solid medium, agar (15 g/L) was added. Strains were stored as 30% glycerol
105 stocks at -80°C.

106 **Table 1: Strains used in this study**

Strain Name	Pertinent Genotype	Source
RMJP1.49	<i>pyrG89; argB2; Δnku::argB; pyroA4</i>	(36)

TSSP38.1	<i>pyrG89; argB2; Δnku::argB; pyroA4; ΔhbxA::pyrG; pyroA</i>	This Study
TSSP40.1	<i>pyrG89; argB2; ΔnkuA::argB; pyroA4; ΔhbxA::pyrG, hbxA::pyroA</i>	This Study
TSSP34.1	<i>pyrG89; argB2; ΔnkuA::argB; pyroA4; gpdA(p)::ΔhbxA::trpC(t)::pyrG; pyroA</i>	This Study
TRV50.2	Wild type	(37)

107

108 ***Generation of the hbxA deletion strain (ΔhbxA)***

109 The DNA cassette employed to obtain the deletion *hbxA* strain (TSSP38.1) was generated by
110 fusion polymerase chain reaction (PCR) through a previously described method (38). All
111 primers used in this study are listed in Table 2. The 1.5 kb 5` UTR region of the *hbxA* locus was
112 PCR amplified using P#2154/SD3 and P#2155 primers from genomic DNA of the *A. nidulans*
113 FGSC4 wild-type strain. Similarly, the 1.1 kb 3` UTR of *hbxA* was amplified using P#2156 and
114 P#2157 primers also from genomic DNA. The 1.9 kb *A. fumigatus pyrG* selectable marker was
115 amplified from plasmid p1439 (39) using P#2158 and P#2159 primers. The 5` and 3` UTR
116 fragments were fused to the selectable *pyrG* marker using P#2160/SD9 and P#2161 primers. The
117 resultant fusion product was transformed into RMJP1.49 strain using a polyethylene glycol
118 mediated protocol as described previously (38). Transformants were confirmed by diagnostic
119 PCR using P#2154 and P#963 primers. The selected deletion *hbxA* strain was then transformed
120 with a DNA fragment containing the *A. nidulans pyroA* gene, PCR amplified with primers
121 P#1042 and P#1045 from genomic DNA, resulting in strain TSSP38.1.

122

123 **Table 2: Primers used in this study**

Name	Sequence (5' → 3')
P#2154/SD3	CCCGCTGATGTATGGTGAGGC
P#2155	TGGTGTAGGATGCGATGCGG
P#2156	CATCTCCTCCTTCAACACCAGGG
P#2157	GGTCTGAGGTCTTGCCGTTTCC
P#2158	CCGCATCGCATCCTACACCAACCGGTCGCCTCAAACAATGCTCT
P#2159	CCCTGGTGTGAAGGAGGAGATGGTCTGAGAGGAGGCACTGATGCG
P#2160/SD9	CGCTCCCTTGAAACTCCGAGAG
P#2161	CACAGTAGGCACGAATGGCGTT
SD1	ACCGGTCGCCTCAAACAATGCTCT
SD2	GTCTGAGAGGAGGCACTGATGCG
SD4	GCGTTTTATTCTTGTTGACATGGGGTCCCTTAGCCGAAATTGGTGGG
SD5	CCCATGTCAACAAGAATAAAACGC
SD6	CCGAGTGGAGATGTGGAGT
SD7	ACTCCACATCTCCACTCGGGCCCATATCTTCCGTAGCAGTC
SD8	AGAGCATTGTTTGAGGCGACCGGTGACGGAGAGCTGAGAGTCCTAG
SD10	CAGAGCACCGCCGTGGTATTG
P#2962	GTCTCGTAGGTCTCTTGACGACCG
P#2238	AAAAAAGGCGCGCCATGAATTATATCCATCATCCATACCCTTTCGCTG

P#2239 AAAAAAAAAAGCGGCCGCTTAGCCGAAATTGGTGGGGGTC
P#1042 GCCGAAAAGGACCACGAATACCCGC
P#1045 CACCGCCAACGGAGACAATCAAGCC
P#963 GAGCAGCGTAGATGCCTCGAC
P#2093 GACCTAATACAGCCCCTACAACGACC
P#2218 GCGGCCGCTTAGCCGAAATTGGTGGGGGTC

124

125

126 ***Generation of the *hbxA* complementation strain (*hbxA-com*)***

127 The complementation strain (TSSP40.1) was generated by re-introducing the wild-
128 type *hbxA* allele into the $\Delta hbxA$ strain at the same locus. The complementation cassette was
129 generated as follows: first, a DNA fragment containing the *hbxA* coding region and a 3.7 kb
130 5' UTR was PCR amplified using P#2154/SD3 and SD4, and the *trpC* terminator fragment was
131 amplified with primers SD5 and SD6 using *A. nidulans* genomic DNA as a template. The *A.*
132 *fumigatus pyroA* gene (Afub_055620) was amplified from genomic DNA using primers SD7 and
133 SD8. *A. fumigatus pyrG* was amplified from plasmid p1439 (38) using primers SD1 and SD2.
134 All four PCR fragments were fused together using primers P#2160/SD9 and SD10 in a single
135 reaction using Prime Star DNA polymerase (Clontech, USA). The resulting fusion product was
136 then transformed into the *hbxA* deletion strain (TSSP38.1) using methods previously described
137 (38). Fungal transformants were confirmed using diagnostic PCR with primers P#2154 and
138 P#2962.

139 ***Generation of the *hbxA* overexpression strain (OE*hbxA*)***

140 To generate the over-expression *hbxA* strain (TSSP34.1), the coding region of *hbxA* was first
141 amplified from *A. nidulans* genomic DNA using P#2238 and P#2239 primers. The resulting PCR
142 product was digested with *AscI* and *NotI* and ligated to pTRS2 plasmid, previously digested with
143 the same enzymes. pTRS2 contains the *gpdA* promoter, *gpdA*_(p), and *trpC* terminator, *trpC*_(t).
144 The resulting plasmid, pSSP34.1, was transformed into the *A. nidulans* RJMP1.49 strain, and
145 transformants were screened by PCR using P#2093 and P#2218 primers. The selected
146 overexpression *hbxA* strain was then transformed with a DNA fragment containing the *A.*
147 *nidulans pyroA* gene, PCR amplified with primers P#1042 and P#1045 from genomic DNA,
148 resulting in strain TSSP34.1.

149 ***Transcriptome analysis***

150 *RNA purification and sequencing*

151 Plates containing 25 mL of solid GMM with the appropriate supplements were top-agar
152 inoculated with 5 mL of medium containing $\sim 5 \times 10^6$ spores/mL of wild-type (WT) control,
153 $\Delta hbxA$, *hbxA*-com or OE*hbxA* (Table 1). Cultures were incubated in the dark at 37°C. After 72 h
154 of incubation, mycelia were collected, frozen in liquid nitrogen, and lyophilized. Total RNA was
155 extracted from mycelia using an RNeasy Plant Mini Kit (Qiagen, Germantown, Maryland, USA)
156 following the manufacturer's protocol. RNA was further purified using Dynabeads mRNA
157 Purification Kit (Thermo Fisher Scientific Inc., Massachusetts, USA). RNA quality was assessed
158 using an Agilent Bioanalyzer. Sequencing was performed as a HiSeq 2000 single read 1x100bp
159 lane. The experiment was carried out with 3 biological replicates.

160 *Read mapping, decontamination and Read count*

161 The RNA reads were trimmed by trim_galore (40) with the default parameter. Kraken2 (41) was
162 run on trimmed reads to check the contamination. Then, reads were mapped to reference genome
163 downloaded from FungiDB (*Aspergillus nidulans* FGSC4)(42) . Unmapped reads were removed
164 to get clean reads. The clean reads were then repaired to pair-end reads with BBTools (43).
165 These final clean pair-end reads were remapped to reference genome again using hisat2 ((44).
166 Mapped reads in SAM format were sorted by coordinates with samtools (45) to obtain the BAM
167 format mapped reads. Then read count and TPM (Transcripts Per Kilobase Million) were
168 calculated by running StringTie (46) and python script. The parameters were set not to infer new
169 transcripts with the reference gene annotation file (also downloaded from FungiDB).

170 *Differentially expressed coding genes (DEGs)*

171 The read counts table was used as input for DEseq2 (47). This package was used to determine
172 DEGs by comparing read counts between two strains. Significant up regulated genes were
173 determined with $-\log_{10}$ q-value ≤ 2 and \log_2 fold change ≥ 2 , while significant down
174 regulated genes were defined with $-\log_{10}$ q-value ≤ 2 and \log_2 fold change ≤ -2 . Control vs.
175 OE*hbxA* and Control vs. Δ *hbxA*. python script was developed to convert gene id between
176 FungiDB and FungiFun2 so that the webserver of FungiFun2 can be used to perform FunCat
177 term annotation and enrichment of DEGs for Control vs. OE*hbxA* and Control vs. Δ *hbxA*(48).
178 Heat maps of TPM (transcript per million) values of DEGs of secondary metabolism clusters
179 were calculated by averaging all TPM values of all replicates.

180 Evaluation of differentially expressed ortholog genes in *A. nidulans* and *A. flavus* was carried out
181 by using the MCL algorithm in combination with all-versus-all protein BLAST search, similar to
182 a method previously described (49). Proteins with BLAST hits were filtered with the following

183 parameters: 1, query and subject coverage is greater than 60%. 2, e-value is less than 1^{-5} . 3, the
184 percent of identity is greater than 60%. And then, the filtered hits were fed into OrthoMCL with
185 an inflation parameter of 2 to generate orthogroups between these two species.

186 To analyze changes in the expression of genes in secondary metabolite biosynthetic gene clusters
187 (SMGs), 67 SMGs were extracted (50). SMGs expression related figures were plotted with
188 python seaborn package. In addition, expression of 521 transcript factors (TFs) was also
189 analyzed. The list of TFs and their function annotations were derived from a previous report
190 (51).

191 *Metabolomics*

192 *Thin- Layer Chromatography*

193 Wild-type control, $\Delta hbxA$, $hbxA$ -com, and OE $hbxA$ were top-agar inoculated with 5 mL of
194 medium containing $\sim 5 \times 10^6$ spores/mL on solid GMM and grown at 37°C for 3 days. Three 16-
195 mm diameter cores per plate were collected and extracted with chloroform. Overnight dried
196 extracts were resuspended in 200 μ L chloroform. Sample were separated using thin-layer
197 chromatography (TLC) as previously described (28,52) on silica gel plates using benzene and
198 glacial acetic acid [95:5(v/v)] as solvent system. Aluminum chloride (15% in ethanol) was then
199 sprayed, and plates were baked for 10 min at 80 °C. Bands were visualized under UV light (375
200 nm). Sterigmatocystin (ST) standard was purchased from Sigma-Aldrich (St. Louis, MO, USA).

201 *Analysis of secondary metabolites by liquid chromatography combined with mass spectrometry*
202 *(LC-MS)*

203 Chloroform extracted samples were also analyzed by LC-MS. Samples were resuspended in 500
204 μL of acetonitrile/water (50:50, v/v), shaken vigorously for 30 s and then treated with a sonicator
205 (Bransonic 221 Ultrasonic bath, Roucaire, Les Ulis, France) for 2 h. A volume of 250 μL of pure
206 ACN was added to each sample, followed by vigorous shaking (30s) and centrifugation (pulse).
207 Secondary metabolites analysis was performed using Acquity ArcSystem HPLC (Waters, Saint-
208 Quentin-en-Yvelines, France) combined with an LTQ Orbitrap XL high-resolution mass
209 spectrometer (Thermo Fisher Scientific, Les Ulis, France). A volume of 10 μL of the suspension
210 was injected into a reversed-phase 150 mm \times 2.0 mm, Luna[®] 5 μm C18 column (Phenomenex,
211 Torrance, CA, U.S.A.). Water acidified with 0.1% formic acid was used as phase A and 100%
212 acetonitrile was used as phase B with the following elution gradient: 0 min 20% B, 30 min 50%
213 B, from 35 to 45 min 90% B, from 50 to 60 min 20% B at 30 °C at a flow rate of 0.2 mL min⁻¹.
214 HRMS acquisitions were achieved with electrospray ionization (ESI) in positive and negative
215 modes, as previously reported (28). MS/MS spectra were obtained with CID mode at low
216 resolution and collision energy of 35%.

217 *Statistical analysis*

218 Statistical analysis was applied to analyze all quantitative data in this study utilizing analysis of
219 variance (ANOVA) in conjunction with a Tukey multiple-comparison test using a *p* value of
220 <0.05 for samples that are determined to be significantly different.

221

222 **RESULTS**

223 *HbxA is conserved in numerous fungal species*

224 Our phylogenetic analysis confirmed that the *hbxA* deduced amino acid sequence corresponds to
225 a transcription factor containing a homeodomain. HbxA homologs are present in other
226 *Aspergillus* species, including *A. flavus* (17,27), *A. fumigatus* (28), *Aspergillus niger* and
227 *Aspergillus terreus* (Fig 1, Table 3), as well as in species of other fungal genera, such as
228 *Alternaria alternata*, *Arthrotrrys flagrans*, *Ascospaera apis*, *Blastomyces dermatitidis*,
229 *Histoplasma capsulatum*, *Microsporium canis*, *Penicillioopsis zonata*, *Penicillium rubens*,
230 *Talaromyces marneffeii* and *Trichophyton tonsurans* (Fig 1, Table 3). Of the sequences analyzed,
231 *A. niger* HbxA was the closest homolog to *A. nidulans* HbxA, with 56.40% identity and 68.4%
232 sequence similarity.

233 **Table 3: Phylogenetic analysis of *A. nidulans* HbxA and homologs in other fungal species. HbxA**
234 **homologs were retrieved from FUNGIDB website and BLASTp was performed against protein**
235 **sequence database. % similarity was found utilizing Pairwise sequence alignment using *A. nidulans***
236 **HbxA as search query against each protein of interest using EMBOSS Needle.**

Species (sorted)	Identity% (Needle)	Similarity% global Pairwise alignment
<i>Aspergillus niger</i>	56.4	68.4
<i>Aspergillus flavus</i>	52.9	65.2
<i>Penicillioopsis zonata</i>	44.5	54.3
<i>Penicillium rubens</i>	43.7	56.9
<i>Talaromyces marneffeii</i>	37.1	49.3
<i>Blastomyces dermatitidis</i>	36.9	49.9
<i>Histoplasma capsulatum</i>	36.9	49.5
<i>Aspergillus terreus</i>	35.5	46.4
<i>Microsporium canis</i>	34.0	43.7

<i>Trichophyton tonsurans</i>	32.3	44.9
<i>Ascosphaera apis</i>	30.3	41.8
<i>Arthrotrrys flagrans</i>	21.5	34.3
<i>Alternaria alternata</i>	20	31.5

237

238 **Fig 1: Phylogenetic analysis of *Aspergillus nidulans* HbxA.** The phylogenetic tree was
239 constructed using MEGA v6.0 and the Maximum Likelihood model with bootstrap value
240 of 1000 (<http://megasoftware.net/>).

241

242

243 **Fig 2: Multiple sequence alignment of *A. nidulans* HbxA with other fungal**
244 **homologs.**

245 The HbxA deduced amino acid sequences were aligned using
246 clustalOmega(<https://www.ebi.ac.uk/Tools/msa/clustalo/>). Data was visualized with
247 boxshade using ENDScript server (<https://escript.ibcp.fr/ESPrpt/cgi-bin/ESPrpt.cgi>
248)(53)<https://doi.org/10.1093/nar/gku316>

249

250 ***hbxA* is required for normal development in *A. nidulans***

251 To determine the regulatory scope of *hbxA* in *A. nidulans*, three strains were generated, a
252 deletion strain, $\Delta hbxA$, a complementation strain, *hbxA-com*, and an over-expression strain,
253 OE*hbxA* (Fig 3). Deletion, complementation and overexpression strains were confirmed by
254 diagnostic PCR, yielding the expected 3.01 kb PCR product for $\Delta hbxA$, a 3.96 kb DNA fragment

255 for *hbxA-com* and a 3.16 kb DNA fragment for OE*hbxA*. Our results confirmed that absence of
256 *hbxA* results in a drastic reduction of conidiation (Fig 4), as previously shown(33,34).

257 **Fig 3: Generation of *A. nidulans hbxA* deletion, complementation and**
258 **overexpression strains.** Confirmation of the deletion ($\Delta hbxA$), complementation (*hbxA-*
259 *com*) and overexpression (OE*hbxA*) by diagnostic PCR. (A) The diagram shows
260 replacement of *hbxA* with the marker gene *pyrG* by a double cross-over event. Primers
261 P#2154/SD3 and P#963 were used for the diagnostic PCR, obtaining the predicted 3.01
262 kb product. (B) Schematic representation showing reintroduction of the wild-type *hbxA*
263 allele at the *hbxA* locus in the deletion strain TSSP38.1. PCR with primers P#2154/SD3
264 and P#2962 confirmed the reintroduction of *hbxA* in the selected deletion strain; the
265 expected 3.96 kb product was obtained. (C) Linear diagram of *hbxA* overexpression
266 plasmid pSSP34.1. The overexpression transformant was confirmed by PCR with primers
267 2093 and 2218, which yielded the predicted 3.16 kb product.

268

269 **Fig 4. *hbxA* is required for normal conidiation in *A. nidulans*.** Cultures of wild type,
270 deletion, complementation and overexpression *hbxA* strains, top-agar inoculated on
271 GMM and incubated for 7 days in the dark at 37°C.

272

273 *hbxA* regulates secondary metabolism

274 Our TLC analysis indicated that deletion of *hbxA* reduces sterigmatocystin (ST) production in *A.*
275 *nidulans* by approximately 50 % when compared with levels in the wild-type strain (Fig 5).

276 Importantly, overexpression of *hbxA* completely blocked ST production. Additionally, synthesis

277 of other metabolites was also affected by deletion or forced overexpression of *hbxA* compared to
278 the control strain. The absence of metabolites was particularly notable in the OE*hbxA* strain
279 extracts. These results suggested that the regulatory role of *hbxA* is broader than originally
280 expected, controlling not only developmental processes but also acting as a global regulator of
281 secondary metabolism.

282 **Fig 5. Effect of *hbxA* on the production of ST and other secondary metabolites in *A.***
283 ***nidulans*.** Wild type, deletion, complementation and overexpression *hbxA* strains were
284 top-agar inoculated on glucose minimum medium (GMM) and incubated for 3 days in the
285 dark. (A) Extracts were analyzed by TLC. Black arrows indicate ST standard. The
286 experiment was carried out with three replicates. (B) Densitometry of TLC analysis of ST
287 levels. The densitometry was performed using the
288 <http://biochemlabsolutions.com/GelQuantNET.html> website. Error bars represent the standard
289 error. Columns of different letters represent values that are statistically different *p* value
290 of <0.05

291

292 ***hbxA*-dependent transcriptome in *A. nidulans***

293 *More than one thousand genes are regulated by hbxA in A. nidulans*

294 RNA-sequencing analysis revealed that of the predicted 11286 genes present in *A. nidulans*
295 genome (54), 552 were downregulated, and 195 were upregulated in the Δ *hbxA* strain compared
296 with the wild-type control strain (Table 4, Fig 6). Over-expression of *hbxA* resulted in an even
297 more pronounced effect on the *A. nidulans* transcriptome, where 1044 genes were
298 downregulated, and 424 genes were upregulated in the OE*hbxA* strain in comparison to the wild

299 type. In strong contrast, the comparison of the complementation strain and wild type showed that
300 the two strains present very similar expression patterns. Expression of 618 genes in the *A.*
301 *nidulans* genome was altered by either deletion or overexpression of *hbxA*, many of them
302 presenting the same expression pattern of upregulation or downregulation when *hbxA* was either
303 deleted or overexpressed (Fig 6).

304 **Fig 6. Number of DEGs in *A. nidulans* when expression of *hbxA* is altered by *hbxA***
305 **deletion or overexpression. (A)** Number of significantly upregulated (purple) and
306 significantly downregulated (orange) DEGs estimated by DeSeq2. **(B)** Volcano plot of
307 log₂ fold change vs. -log₁₀ q-value of all the genes in $\Delta hbxA$, and OE*hbxA* vs. control.
308 Significantly upregulated genes are shown as red dots, significant down regulated genes
309 are shown as blue dot and other genes are shown as black. The x-axis represents the log₂
310 of the fold change determined by DeSeq2. The y-axis is the log₁₀ of the adjusted p-value
311 from DeSeq2. The cut off log₁₀ fold change value to determine the upregulated
312 expression is greater than 2 while -2 is for down regulated expression. The -log₁₀ q-value
313 cutoff was set to 2 to determine the significant expression or not. **(C-D)** Venn Diagrams
314 showing the overlap of DEGs in $\Delta hbxA$ and OE*hbxA* **(C)**, and the overlap of upregulated
315 **(D)** and downregulated DEGs **(E)** in $\Delta hbxA$ and OE*hbxA*. Venn Diagrams were
316 constructed using [https://bioinformatics.psb.ugent.be/cgi-](https://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.html)
317 [bin/liste/Venn/calculate_venn.html](https://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.html) website.

318 *Comparison of hbxA/hbx1 DEGs in A. nidulans and A. flavus*

319 The comparison of the current *A. nidulans hbxA*-dependent transcriptome study with the
320 previous *A. flavus hbx1* results (27) is shown in Fig 7. Only a small percentage of homologs were
321 differentially expressed in the absence of *hbxA* and *hbx1* in *A. nidulans* and *A. flavus*,

322 respectively, with respect to the corresponding wild types. Most of the DEGs in *A. nidulans* are
323 not DEGs in the *A. flavus* study.

324 **Fig 7: Comparison of orthologous genes affected by deletion of *hbxA* in *A. nidulans***
325 **and *A. flavus*.** Both upregulated orthologous genes were colored in red. Both
326 downregulated orthologous genes were colored in blue. No expression changed
327 orthologous genes are colored in grey. Two orthologous genes having different regulation
328 status are colored in purple. The significantly regulated genes were defined as $|\log_2 \text{fold}$
329 $\text{change}| \leq 2$ and $q\text{-value} \leq 0.05$.

330

331 *Expression of numerous TF genes is hbxA-dependent in A. nidulans*

332 Based on our analysis, 74 out of 521 TFs genes in *A. nidulans* (51) were regulated by *hbxA* under
333 the culture conditions assayed (Table 5). Some of these differentially expressed TF genes also
334 presented the same expression pattern of upregulation or downregulation when *hbxA* was either
335 deleted or overexpressed (Fig 8).

336 **Fig 8. Number of transcription factor (TF) genes controlled by *hbxA* in *A. nidulans*.**

337 **(A)** Venn Diagram showing the overlap of differentially expressed TF genes in $\Delta hbxA$
338 and OE*hbxA*. **(B-C)** Venn Diagrams showing the overlap of upregulated **(B)** or
339 downregulated **(C)** TF genes in $\Delta hbxA$ and OE*hbxA*. Venn Diagrams were constructed
340 using https://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.html website.

341

342 Our results indicated that overexpression of *hbxA* caused upregulation of developmental
343 regulators, including genes of the central developmental pathway, *brlA*, *abaA* (55–58), *fluffy*
344 genes *flbC* and *flbD* (59), and another HD-TF gene, *hbxB*, that regulates asexual and sexual
345 development in *A. nidulans*(34). In addition, the developmental regulatory gene *zcfA* (60) was
346 also upregulated by *hbxA* overexpression. Some of the upregulated TFs genes in OE*hbxA* are
347 involved in both governing development as well as SM, such as the master transcription factor
348 *mtfA* (37,61,62), *urda*, (63), *sclB* (64), *osaA* (65), and *velB* (66). Other upregulated *hbxA*-
349 dependent TF-DEGs annotated to be putatively involved in SM regulation include AN8391 and
350 AN6788. Other upregulated TF genes have an important role in primary metabolism, such as
351 *glcD*, which has a putative role in protein dimerization and activation of *areB*, (67), *galR*, which
352 is known to regulate the D-galactose catabolic pathway (68) and *creA* repressor of carbon
353 catabolite (69). Other upregulated TF genes were *rfeC*, whose ortholog in *Saccharomyces*
354 *cerevisiae* promotes *FLO11* expression (70), the *mcnB* fork-head like transcription factor (71), as
355 well as expression of some other uncharacterized putative transcription factors genes (Table 5).

356 Overexpression of *hbxA* in *A. nidulans* caused downregulation of other developmental genes
357 such as *flpA*, with a role in sexual development (72), *mat1*, involved in activation of the alpha-
358 domain mating-type protein (73). Overexpression of *hbxA* also caused downregulation of *metZ*,
359 a transcription factor involved in the regulation of sulfur metabolism (74). TF genes AN8377,
360 AN8645, AN3385 and AN8918 predicted to be involved in SM, are also downregulated in this
361 strain (Table S2).

362 Interestingly, deletion of *hbxA* also resulted in an increase in the expression of *brlA*, *abaA*, and
363 *urda*, as in the case of OE*hbxA*. It also increased the expression of *tah-3*, which is involved in
364 conidiophore development and tolerance for harsh plasma environment (75) (Table 5). Deletion

365 of *hbxA* also upregulated *veA* (Table 4). The *veA* gene product, VeA, which contains a NF- κ -B
366 like DNA-binding domain (76), is well known as a global regulator that interacts with at least nine
367 other proteins, LlmF, VapA, VipA, VipC, VelB, MpkB, FphA, LreB and LaeA (77), governing
368 several signaling pathways and consequently multiple cellular processes, including development
369 and SM (25).

370 Absence of *hbxA* in *A. nidulans* downregulated the expression of various transcription factors,
371 including the gene encoding the alpha-domain mating-type protein, *mat1*(73), as in
372 overexpression of *hbxA*. Deletion of *hbxA* also showed downregulation of *metZ*, involved in
373 methionine biosynthesis (78) the nitrogen-dependent *mdpE*, which regulates production of a
374 secondary metabolite called monodictyphenone (79). The putative SM TF gene AN4933 is
375 downregulated, and AN3385, AN8645 and AN8918 are also downregulated in deletion *hbxA*, as
376 in OE*hbxA*.

377

378 *hbxA* affects the expression of genes in SM gene clusters and biosynthesis of natural products
379 in *A. nidulans*

380 Our TLC analysis revealed that both deletion and overexpression of *hbxA* negatively affect ST
381 production (Fig 5) as well as the production of other secondary metabolites. Furthermore,
382 FunCat enrichment analysis revealed that differentially regulated genes in the Δ *hbxA* versus wild
383 type and OE*veA* versus wild type comparisons have significant functional overlap (Fig 9). DEGs
384 genes are dramatically enriched for secondary metabolism-related processes for both; most of
385 those genes are downregulated when *hbxA* is either deleted or overexpressed, particularly in the

386 latter. Other categories showing enrichment include disease, virulence, and defense; virulence
387 disease factors; C-compound and carbohydrate metabolism; and detoxification.

388 **Fig 9. FunCat enrichment of significant DEGs found in (A) $\Delta hbxA$ and (B) OE*hbxA***
389 **vs. control.** The $-\log_{10}$ of the q-value of DEGs in each term is proportional to the length
390 of the bars. FunCat annotations and q-value is determined by FungiFun2 webserver.
391 Downregulated genes are to the left of the origin and up regulated genes to the right.

392

393 To gain further understanding of the effect of *hbxA* on SM in *A. nidulans*, as part of our
394 transcriptome analysis, we identified DEGs in SM gene clusters and analyzed concomitant
395 production of secondary metabolites by a metabolomics approach. Our study revealed that
396 production of nidulaninA, nidulanin B and nidulanin D are *hbxA*- dependent (Fig 10A-C). Both,
397 deletion and overexpression of *hbxA*, completely inhibited the production of these compounds. In
398 addition, the Heatmap shown in Fig 10D indicates downregulation of some of the genes in the
399 nidulanin cluster (80), including the NRPS coding gene, *nlsA*, in both $\Delta hbxA$ and OE*hbxA*. This
400 reduction in *nlsA* expression was particularly notable in the latter.

401 **Fig 10: *hbxA* regulates the production of nidulanins in *A. nidulans*.** Wild-type (WT),
402 deletion ($\Delta hbxA$), complementation (*hbxA-com*) and overexpression (OE*hbxA*) strains
403 were top-agar inoculated on solid glucose minimum medium (GMM) at 37°C for 72 h,
404 when samples were collected, extracted with chloroform and analyzed by LC-HRMS in
405 positive mode (A-C) Quantification of nidulanin A (m/z 604.34943), B (m/z 620.34404),
406 D (m/z 536.28659) respectively. (D) Heat map of TPM values of nidulanin cluster
407 (DEGs) expression in *A. nidulans* $\Delta hbxA$ and OE*hbxA* with respect to wild type strain on

408 a log scale found in Inglis et al.(50). The TPM value of each gene was calculated by
409 averaging all the TPM values of all replicates.

410

411 LC-MS analysis of ST confirmed the TLC results, indicating that production of this mycotoxin
412 was reduced in $\Delta hbxA$ and absent in the overexpression strains (Fig 11). Unexpectedly, the
413 Heatmap in Fig 11B shows that most of the ST genes were not downregulated in the deletion
414 strain with respect to the wild type. However, most of the genes in this cluster were
415 downregulated in the overexpression strain, excluding the structural genes *stcK*, *stcJ*, *stcF* and
416 *stcC*, and the regulator, *aflR*.

417 **Fig 11. *hbxA* regulates the production of ST in *A. nidulans*.** Wild type (WT), deletion
418 ($\Delta hbxA$), complementation (*hbxA-com*) and overexpression (OE*hbxA*) strains were top-
419 agar inoculated on solid glucose minimum medium (GMM) at 37°C for 72 h, when
420 samples were collected, extracted with chloroform and analyzed by LC-HRMS in
421 positive mode. (A) Quantification of ST (*m/z* 325.07014). (B) Heat map of TPM values
422 of ST cluster (DEGs) expression in *A. nidulans* $\Delta hbxA$ and OE*hbxA* with respect to wild
423 type strain on a log scale found in Inglis et al. (50). The TPM value of each gene was
424 calculated by averaging all the TPM values of all replicates.

425

426 In addition, both $\Delta hbxA$ and OE*hbxA* strains were unable to synthesize the meroterpenoids
427 austinol and dehydroaustinol under conditions conducive to their production in the wild type (Fig
428 12). The genes involved in the synthesis of these two compounds are grouped in two clusters, A
429 and B (81). Our transcriptome analysis revealed that most of the genes in these two clusters are

430 downregulated in the *hbxA* deletion and also in the overexpression strains compared to the
431 control (Fig 12C and D). For example, genes *ausA-D* are down regulated in both $\Delta hbxA$ and
432 OE*hbxA* in gene cluster A. In cluster B, genes *ausE-G* and *ausM* are also downregulated in
433 $\Delta hbxA$ and OE*hbxA*. Additionally, expression of *ausH*, *ausL* and *ausN* is reduced in OE*hbxA*
434 with respect to the wild type.

435 **Fig 12. *hbxA* regulates the production of austinol and dehydroaustinol in *A.***
436 ***nidulans*.** Wild type (WT), deletion ($\Delta hbxA$), complementation (*hbxA-com*), and
437 overexpression (OE*hbxA*) strains were top-agar inoculated on solid glucose minimum
438 medium (GMM) at 37°C for 72 h, when samples were collected, extracted with
439 chloroform and analyzed by LC-HRMS in positive mode. Quantification of (A) austinol
440 (*m/z* 459.20059) and (B) dehydroaustinol (*m/z* 457.18524) compounds by full MS spectra
441 resolution of 60,000 with a range of mass-to-charge ratio (*m/z*) set to 50 to 800. (C &
442 D) Heatmap of TPM values of austinol cluster (DEGs) expression in *A. nidulans* $\Delta hbxA$
443 and OE*hbxA* with respect to wild type strain on a log scale found in Inglis et al. (50). The
444 TPM value of each gene was calculated by averaging all the TPM values of all replicates.

445

446 Our metabolomics study also indicated that the production of three novel, unknown secondary
447 metabolites was altered when *hbxA* was not expressed at wild-type levels. Two of these
448 compounds (*m/z* 423 and *m/z* 518 observed in negative mode) were absent in the *hbxA* deletion
449 strain and also in the overexpression strain (Fig 13). The third novel compound (*m/z* 489 in
450 negative mode) was produced at remarkably high levels in the *hbxA* deletion strain compared to
451 those in the wild type (Fig 13B).

452 **Fig 13: *hbxA* regulates the production of novel uncharacterized metabolites in *A.***
453 ***nidulans*.** Wild type (WT), deletion ($\Delta hbxA$), complementation (*hbxA-com*), and
454 overexpression (*OEhbxA*) strains were top-agar inoculated on solid glucose minimum
455 medium (GMM) at 37 °C for 72 h, when samples were collected, extracted with
456 chloroform and analyzed by LC-HRMS in negative mode. (A-C) Quantification of novel
457 uncharacterized metabolites with *m/z* of 423.18012, 489.18082, and 518.16482,
458 respectively.

459

460 DISCUSSION

461

462 HD-TFs have been shown to govern development in eukaryotes (13–15), including fungi
463 (13,14,16–21). Previous reports, together with the present study, indicate that these regulators are
464 conserved across different fungal genera. (Zheng et al., 2012; Ghosh et al., 2015). In *A. flavus*,
465 *hbxA*, an ortholog of *hbxA*, is also required for developmental processes, regulating genes in the
466 conidiation central pathway, such as *brlA* and *wetA* (17,27) as well as *flbA*, *flbC*, *flbD*, *flbE*, *fluG*
467 and *mat1-1*(27). In *A. fumigatus*, *hbxA* promotes *brlA*, *abaA* and *wetA*, as well as *flbB*, *flbD* and
468 *fluG* expression(28). Similarly, *hbxA* regulates conidiation in *A. nidulans*(33,34); our
469 transcriptome study showed that *hbxA* not only regulates *brlA*, as shown in (34), but also *abaA*,
470 *flbC* and *flbD*. These studies support that the *hbxA*-dependent regulatory mechanism of
471 conidiation is at least in part conserved in these three *Aspergillus* species and possibly in other
472 species of this genus.

473 Interestingly, our results revealed a broader regulatory scope for *hbxA* in *A. nidulans*, with more
474 than one thousand DEGs when *hbxA* was deleted or overexpressed in this model organism,
475 including numerous transcription factor genes. This was also the case for *A. flavus hbx1*(27).
476 However, most of the DEGs in *A. nidulans* are not DEGs in *A. flavus*; only a small percentage of
477 homologs were DEGs in the *hbxA* and *hbx1* mutants with respect to the controls. This suggests
478 that although the conservation of some of the regulatory mechanisms controlling conidiation
479 appears conserved, a great part of its regulatory input is specialized in different fungal species.

480 Some of the TF genes involved in governing development that were found *hbxA*-dependent also
481 control secondary metabolism in *A. nidulans*, for example, *mtfA* (37,61,62), *urdA* (63), *sclB* (64),
482 *osaA* (65) and *velB* (66). Furthermore, FunCat functional enrichment analysis showed that the
483 category of secondary metabolism-related processes was, by far, the most enriched in *A.*
484 *nidulans*. Our study showed that in *A. nidulans*, numerous genes in SM gene clusters were
485 regulated by *hbxA*. The secondary metabolism category was also enriched in *A. flavus*
486 (27)However, the wide variation of biosynthetic gene clusters across fungal species, even in
487 those phylogenetically close (82) could explain that although the major functional category is the
488 same in both species, namely SM, the percentage of differentially expressed homologs is low.
489 For example, *A. flavus hbx1* regulates genes in the aflatoxin, cyclopiazonic acid, aflatrem,
490 asparasone, piperazine, and aflavarin gene clusters(27), while in *A. nidulans*, our study shows
491 that *hbxA* controls genes in other gene clusters such as those responsible for the synthesis of
492 nidulanins A, B and D, austinol and dehydroaustinol. *A. nidulans* HbxA also control genes in the
493 ST gene cluster, which is partially conserved with that of aflatoxin in *A. flavus*. The regulatory
494 pattern was similar; absence of both *hbxA* and *hbx1* resulted in a reduction of toxin production
495 (17,27). In *A. flavus* deletion of *hbx1* downregulated *aflR* and other genes in the aflatoxin gene

496 cluster. However, this was not the case in *A. nidulans*, suggesting that the lower levels of ST in
497 the deletion strain, verified by both TLC and LC-MS, could be due to other factor(s). Our study
498 showed *veA* expression is *hbxA*-dependent. VeA is a global regulator that orchestrates numerous
499 biological processes in fungi (25,26), such as development and SM. VeA has been shown to
500 regulate the production of aflatoxins in *A. parasiticus* (83). It is possible that *hbxA*, in a *veA*-
501 dependent manner, could also influence compartmentalization of ST production in *A. nidulans*.
502 This reduction in ST in the deletion strain, contrast with a previous report (34) where an increase
503 in ST was described. It is possible that different experimental conditions in both studies could
504 have resulted in different outcomes. Nevertheless, the most striking result is the effect of *hbxA*
505 overexpression on ST biosynthesis as well as on the production of other metabolites. The
506 complete elimination of ST production by *hbxA* overexpression was, in this case, accompanied
507 by the downregulation of genes in the ST gene cluster. However, this downregulation of ST
508 genes was, as in the case of the deletion strain, not mediated by changes in *aflR* expression.

509 Our study revealed that *hbxA* regulates key genes in the nidulanin gene cluster and,
510 consequently, affects the production of the cyclic tetrapeptides nidulinins A, B and D. These
511 compound are found in *Aspergillus* and *Penicillium* species. The function of nidulanins is not yet
512 known. As in the case of ST, both deletion or overexpression of *hbxA* resulted in reduction or
513 elimination of nidulinins A, B and D production, suggesting that, as in the case of VeA, certain
514 balanced stoichiometry with respect to other regulatory factors could be needed for proper
515 function, perhaps also interacting with other regulatory proteins. One of the genes downregulated
516 in both deletion *hbxA* and overexpression *hbxA* strains is *nlsA*, encoding a non-ribosomal peptide
517 synthase necessary for the synthesis of nidulanin. This enzyme has been shown to also be

518 involved in the synthesis of fungisporin (84), which presents antibacterial activity (85), however
519 fungisporin was not detected in our study under the conditions tested.

520 LC-MS indicated that *hbxA* also controls austinol and dehydroaustinol production. These are two
521 meroterpenoids produced from polyketide and terpenoid precursors. Both austinol and
522 dehydroaustinol have been shown to inhibit the neuraminidase enzyme, suggesting a potential
523 for the development of new antiviral drugs (86). Austinol also showed antibacterial activity (87).
524 Alteration of wild-type *hbxA* transcription by deletion or forced overexpression also resulted in a
525 lack of production of these compounds, further supporting the possibility of a necessary
526 stoichiometry with other regulatory partners. Two separate gene clusters, A and B (81,88), are
527 required for the synthesis of these compounds. Both deletion and overexpression of *hbxA* showed
528 profound changes in the expression profile of both gene clusters, with numerous downregulated
529 structural genes, including the polyketide synthase gene *ausA*. The prenyltransferase gene *ausN*
530 was also downregulated in the overexpression strain.

531 In addition, our metabolomics analysis indicated that *A. nidulans hbxA* also controls the
532 production of three unknown novel compounds. Synthesis of two of these metabolites (m/z &423
533 and m/z 528) did not occur in the absence of *hbxA* or when this gene was overexpressed, while
534 the third novel compound (m/z 489) was produced at strikingly high levels in the *hbxA* deletion
535 strain. The identity, the association with MS gene clusters, or bioactive properties of these
536 compounds are still known and will be the subject of future studies.

537 Regarding additional roles of *hbxA* in *A. nidulans*, besides those in development and SM, our
538 FunCat functional enrichment analysis also indicated a possible role in primary metabolism, with
539 enrichment in the carbon-compound and carbohydrate metabolism category, particularly in the

540 *hbxA* overexpression strain. Upregulation of the carbon catabolite repressor TF gene *creA* (69)
541 was observed in this strain. *creA* is also under *hbx1* regulation in *A. flavus*(27). Other *A. nidulans*
542 *hbxA*-dependent regulatory genes involved in primary metabolism were, for example, *galR*,
543 which regulates the D-galactose catabolic pathway (68), and *glcD*, which has a putative role in
544 protein dimerization with and activation of *areB*, involved in nitrogen metabolism (67). Other
545 enriched categories were detoxification, virulence and disease factors and defense, suggesting its
546 possible involvement in pathogenesis. This agrees with the fact that the *hbxA* homolog in *A.*
547 *fumigatus* was shown to affect virulence in *A. fumigatus* (28)

548 In conclusion, we have shown that the regulatory TF gene *hbxA* governs the expression of
549 hundreds of genes in *A. nidulans*, modulating not only developmental genes, but also multiple
550 regulatory pathways. Consequently, *hbxA* governs different important aspects of this fungus'
551 biology, including a remarkable role in SM, regulating expression of several SM gene clusters
552 and natural product biosynthesis, including some novel compounds. Additionally, genes
553 associated with other cellular processes such as primary metabolisms, as well as defense and
554 virulence, are also influenced by *hbxA*. Interestingly, a functional conservation exists between
555 *hbxA* homologs in other *Aspergillus* species and possibly in other fungi.

556

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559

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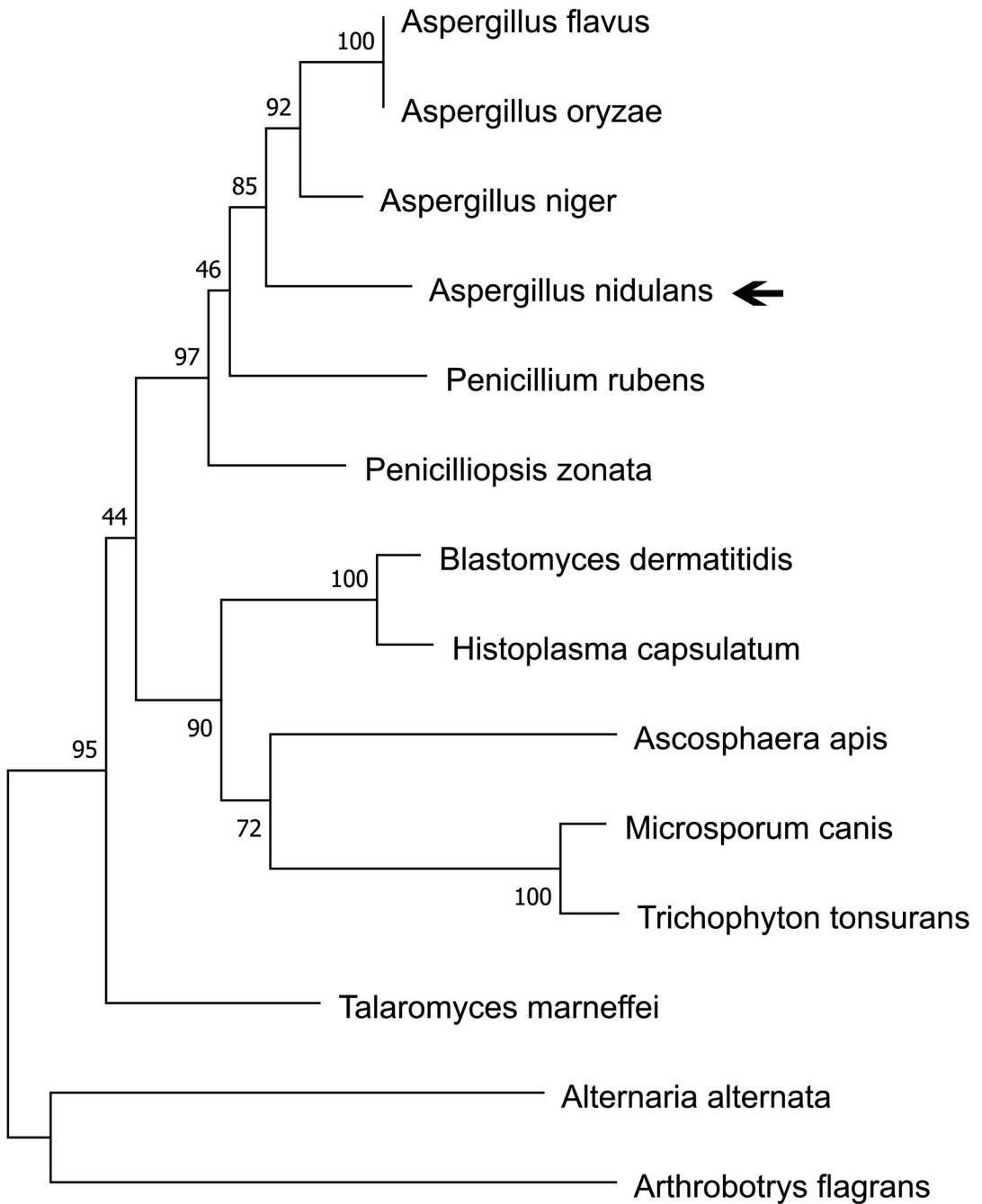
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1           10           20           30           40
Aspergillus_nidulans  MNYIHHP YPFAGHPS VPMEQH LAYD..... T MVHP TM MHH..... PMDGY Y
Aspergillus_niger    MNYLHHP YAF TGHAA VPMEQP VAFD..... PTMAHP SM H..... PMDGY L
Aspergillus_flavus   MNYLHHP YAYAGHAA VPMEQP IAYD..... PTMAHP SM H..... PMEGY I
Aspergillus_oryzae   MNYLHHP YAYAGHAA VPMEQP IAYD..... PTMAHP SM H..... PMEGY I
Penicillium_zonata   MSYLHHS YPYSSHPA VPLDQP LVFD..... PAMAHSSM I PQ..... PMDGY L
Penicillium_rubens   MSYIHP S WNYQGHQG IPMDQH MAYD..... PSMVPP PM H..... PIDGY M
Talaromyces_marneffei MNYLHHP APYGVHAG IHLDQP GLVH..... P..... AIA N..... NIDGY V
Blastomyces_dermatitidis MSYLHHP FPFGGHA IPVDQP VGYG..... VP I RHP QL G H..... SVDTY L
Histoplasma_capsulatum MSYLHHP FPFSGHHA IPVDQP FDYR..... VP I RHP QL S H..... PVDTY L
Microsporium_canis   MNYLQTP FQY... S VPMDDQP MGYG..... MP I SHHP Y D Q..... PMDQCY Y
Trichophyton_tonsurans MNYLQTP FQY... A MPMDQP MGYG..... MP V SHHP Y E Q..... PMDQCY Y
Ascosphaera_apis     MNYLHNP YGYGAPTGLPIDQQ MGYG..... VP I HPHP H P H P D Y H H V P G M P F F Y
Arthrobotrys_flagrans MEV... LSYDNKGS MP SQNP NV P V R S D V H V T Q G P S P Q H L N V P R H P A A N S G P H I E T F I
Alternaria_alternata .....
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50           60           70           80           90           100
Aspergillus_nidulans  YAQP P F DMVD YYHQ... PIMDYDEEY AENLSRPRLTKE QVETLEA QFQAHPKPSSNV KRQLA
Aspergillus_niger    YPHP P F DMVD FYHQ... PIMDYDEEY AENLSRPRLTKE QVETLEA QFQAHPKPSSNV KRQLA
Aspergillus_flavus   YPHP P F DMID FYHQ... PIMDYDEEY AENLSRPRLTKE QVETLEA QFQAHPKPSSNV KRQLA
Aspergillus_oryzae   YPHP P F DMID FYHQ... PIMDYDEEY AENLSRPRLTKE QVETLEA QFQAHPKPSSNV KRQLA
Penicillium_zonata   YPHP P F EMVD FYPP... PIMDYDEY AENLSRPRLTKE QVETLEA QFQAHPKPSSNV KRQLA
Penicillium_rubens   YPHP P M EMD FYHQ... PIMDYDEY AENLSRPRLTKE QVETLEA QFQAHPKPSSNV KRQLA
Talaromyces_marneffei LTRPAY ELADYYTHMP LME DYE EY AENLSRPRLTKE QVDTLEA QFQAHPKPSSNV KRQLA
Blastomyces_dermatitidis LPNAP I DLAEYYHQAAAL DFE EY AENLSRPRLTKQ QVDTLEA QFQAHPKPSSNV KRQLA
Histoplasma_capsulatum LPNAP I DLAEYYHQAAAL DFE EY AENLSRPRLTKD QVDTLEA QFQAHPKPSSNV KRQLA
Microsporium_canis   VPYHQ NDIHG FYTT... GAIE EY E EY V ENLSRPRLTKE QVEI L E S QFQAHPKPSSNV KRQLA
Trichophyton_tonsurans IPYHQ NDIHG FYTT... GAIE EY E EY V ENLSRPRLTKE QVEI L E S QFQAHPKPSSNV KRQLA
Ascosphaera_apis     GHHV P V E L A N S F Y Q A V T L... E D T E S L S R P R L T K E Q V D T L E A Q F Q A H P K P S S V K R Q L A
Arthrobotrys_flagrans GNHF P V... I P R F T P... P V S G P N E E S V K E P R R F K C S R E Q T L A L E R L Y Q C C K P N Q A T K R D L A
Alternaria_alternata ..... M D S S G G S T T... A P S A P K P Q G S D V K P R L T K D Q H D I L E Q H F L A Q H K P S T N V K K E F A
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110           120           130           140           150
Aspergillus_nidulans  QQTHLSLPRVANWFQNRRAKAKQKKRQEE... YERMQ KAKAEAE AAKRKS E S S V P E . S
Aspergillus_niger    AQTNLSLPRVANWFQNRRAKAKQKKRQEE... FEKMQ KAKAEAE AARGK S E S T E S S . D
Aspergillus_flavus   AQTNLSLPRVANWFQNRRAKAKQKKRQEE... FERMQ KAKTEAE AARIKIENA EKS . E
Aspergillus_oryzae   AQTNLSLPRVANWFQNRRAKAKQKKRQEE... FERMQ KAKTEAE AARIKIENA EKS . E
Penicillium_zonata   AQTNLSLPRVANWFQNRRAKAKQKKRQEE... FERMQ KAKAQAE AARGK S D T A . D Q P . E
Penicillium_rubens   AQTNLSLPRVANWFQNRRAKAKQKKRQEE... FEKMT KAKAEAE AAKRKS E T L D Q L . S
Talaromyces_marneffei VQTNLSLPRVANWFQNRRAKAKQKKRQEE... FERMQ REAK E K E D . Q S K S I K D E E Q D Y G L P
Blastomyces_dermatitidis AQTNLTLPVANWFQNRRAKAKQKKRQEE... FERMQ ASEK D E Q W . Q N N D A P Q K . . . . . E
Histoplasma_capsulatum AQTNLTLPVANWFQNRRAKAKQKKRQEE... FERMQ ASG N G E Q W . K N N D T K Q K . . . . . E
Microsporium_canis   LQTSLTLPVANWFQNRRAKAKQKKRQEE... FEKMQ AKEKAMA A E G S E N Q Q Q S . . . . . E
Trichophyton_tonsurans LQTSLTLPVANWFQNRRAKAKQKKRQEE... FEKMQ AKEKAMA A E E A E S K Q . S A . . . . . E
Ascosphaera_apis     MQTNLTLPVANWFQNRRAKAKQKKRQAE... YEKKLAE E K A E K E Q N G K . . . . . E
Arthrobotrys_flagrans HA I N L S P T R V N I W F Q N R R A R A K K H K E I Q E . . . . . A K M A Q I L E T A G R E R M A K I A L G T P M A L . . . S
Alternaria_alternata TRLGVPLDKINNWFQNRRAKVKQDRKKL M N Q Y N M T M S L P F G H S H V P A M . . . . . S
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160           170           180           190
Aspergillus_nidulans  SDSQRSAAEKDEKKQD... DSKA P T P K P . S . . . . . K P A S D D Q . . . . . K Q S E A P A E S N H
Aspergillus_niger    SK... ED AK D S K D E T . . D K D T P K Q S V . E . . . . . N T A E R T K . . . . . T P A . P S S S R P K H
Aspergillus_flavus   SN... P... DVKEET . DKE T P K Q S S . D . . . . . Q T M S D D R . . . . . T K T P A S N S R S K H
Aspergillus_oryzae   SN... P... DVKEET . DKE T P K Q S S . D . . . . . Q T M S D D R . . . . . T K T P A S N S R S K H
Penicillium_zonata   SGSTVKAETPDESSNA... S . L A P Q K K S A E . . . . . T T S S T A S T R . . . . . S H V P A T S A P R S R H
Penicillium_rubens   GSRKGS I... ANE E S E . K S A T P K Q T P . T . . . . . S T S . S G H . . . . . A K T D S T S S R S K H
Talaromyces_marneffei GCDQKSP I H K D D N S H G T T K S P T P T Q A S . . . . . N Y T K D R P . . . . . Q T S D S S S L S R P K H
Blastomyces_dermatitidis GES... K Q S E L P E S S T T P T O R P A S . . . . . I S S C . . . . . S S L P S P A K Q E E Q
Histoplasma_capsulatum GAS... K E Q S E R L E S S A T P T O Q P D P . . . . . S S S S . . . . . L N P S E . V E K E K Q
Microsporium_canis   SSDEQQKSEQDQKNSI... L T N . . T R G A . . . . . S S S C S E Q E D H G
Trichophyton_tonsurans SSDEQQRSEQDQKSSM... S T P T D T R G A . . . . . S S S C S E Q E D H G
Ascosphaera_apis     GESKEKQESQPQESQ T N E . A E A T Q A A . . . . . S S S L P L L T T E . . . . . F N P A . . . . . S S T S
Arthrobotrys_flagrans ES... S . M . S D L E T P T S A T S A . . . . . Q S S L P L L T T E . . . . . F N P A . . . . . S S T S
Alternaria_alternata NHY... A... H P Q E Q Q H P H M L M Q P D F Y P N A D I S P A S L P V Q I G E G P S A L D L G P Q L S L Q Q H H
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200           210           220
Aspergillus_nidulans  QQTRS..... E S N R V A S L A S... L Q R A M D A A A... Q Y Q
Aspergillus_niger    QKTRS..... E S A R E A T F A S... L Q R A L N A A V... A A R
Aspergillus_flavus   HKT K S... E S A R E A T F A S... L Q R A L N A A V... A A R
Aspergillus_oryzae   HKT K S... E S A R E A T F A S... L Q R A L N A A V... A A R
Penicillium_zonata   QKT P S... E S A R E A T F A S... L Q R A L N A A V... A A R
Penicillium_rubens   HKT K S... E S A R E A T F A S... L Q R A L N A A C... A A R
Talaromyces_marneffei QKT G S... D L A Q E K T Y A S... L Q R A I S A A V... A A R
Blastomyces_dermatitidis QQASD..... F T T K T G C P E S... P Q K A M N V S M... P S S
Histoplasma_capsulatum QQASN..... S I S Q P G L P E P... P Q K A M K A T M... P V S
Microsporium_canis   LQTPA..... E E K P E P R F D A... A G H Q S K V Q A... P V S
Trichophyton_tonsurans LQTPA..... D E E P E P K F E V... V G H T T E A Q V... P V S
Ascosphaera_apis     ... D K A K A A V P P Q... P Q Q T V N A Q S... S S E
Arthrobotrys_flagrans T S P P Y... P S S K E A A A A S... L A R S I T I A S... A Y A
Alternaria_alternata QQHQHQQQQQQFDMQHGLHSVP E P D R S A S Y R S N D L M H S I M A A T N G A Y M H N S G M S L N A Q E P
```



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Aspergillus_nidulans      . . . . . KKPARSLSLPAETDR.GLPRVGTSRSTSMLS.TS..TMS
Aspergillus_niger        . . . . . QLVN...EGD...RSSWKEPSKELDLAARRKRPRPAAIGTSRSSSMLT.GSS..TMS
Aspergillus_flavus       . . . . . GLPI...RTD...RSSWKEAGKELDLAARRKRPRPAAIGTSRSSSMLA.GSAA.SMS
Aspergillus_oryzae       . . . . . GLPI...RTD...RSSWKEAGKELDLAARRKRPRPAAIGTSRSSSMLA.GSAA.SMS
Penicillium_zonata        . . . . . HLNP...QRVDPAAWKEPGKELDLAARRKRPRPAAIGTSASGRSSLAAGTVSMS
Penicillium_rubens       . . . . . GLSQ...PVDREVATWKEPGKELDLAARRKRPRPAAIGTSGRPLANSTMSLS
Talaromyces_marneffei    . . . . . G...SMDSSMWRPEKELDLAARRKRPRPAAIGTAHHRLS...TNPMSV
Blastomyces_dermatitidis QNTPHLNLAQLHHQAEPSTNWRYPEKELVDIARRKRPRPAAIGTSMSRS...YGPSMS
Histoplasma_capsulatum  RNTPHLNMAQLRHQVDPSTNWRYPEKELVDIARRKRPRPAAIGTPAMRS...YGPSMS
Microsporium_canis       SE..G...RQLPRLHISTSDNAIGLAARRKRPRPAAIGTSLRSAL..GGPMS
Trichophyton_tonsurans  PE..G...RQPPRLHISTSDNAIGLAARRKRPRPAAIGTSGFGRTV..GGPMS
Ascosphaera_apis        MAP...PTVSDMDTWRQFKKELVDIARRKRPRPAAIGTATLGRSF..TGPSMS
Arthrobotrys_flagrans   .AAPGM...ERSQYTERPSLQEIALLRRLRMPSPSLGNARTQRIFHG.KI.NGS
Alternaria_alternata     . . . . . IRNSTPDGFPDPPDQSSIAARRK.RPVNLLSSAMRSASYSAA..PMS
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Aspergillus_nidulans     PTTRG..QNY..GT..VKQSKSAQNLG...SRYAGVRKPS.AQRSPNLSTFAEAGVL
Aspergillus_niger       PSTRL..PSY..GNHAVRQSKSAQGLN...SRYAGVRKASAAQRSPNLSTFAEAGAL
Aspergillus_flavus      PTTRL..PSY..GSAPGVRQSKSAQCLN...SRYAGVRKASAAQRSPNLSTFAEAGAL
Aspergillus_oryzae      PTTRL..PSY..GSAPGVRQSKSAQCLN...SRYAGVRKASAAQRSPNLSTFAEAGAL
Penicillium_rubens      PTARM..PSS..GAGNSMRQSKSTQSLN...SRYAGVRKASAAQRSPNLFSTFAESGI
Talaromyces_marneffei   PNARM..ATF..GAPHTTRHAKSSHTLG...SRYAGVRKLSATQRSPNLFSTFAEATA
Blastomyces_dermatitidis PTTTRI..HGM..GAGHVLRHAKSTONLSPSHTSRYPGIRKASAPQRSPLGITSTFAESLF
Histoplasma_capsulatum PTTTRI..QGM..GAGHVLRHAKSTONLSP...NRYPGIRKASVAQRSPLGITSTFAESRF
Microsporium_canis      PTRRV..SSA..AWGGVRKSSQLAELS...PRYASVRKLSGSPRSPFPYSLEGRHAL
Trichophyton_tonsurans PTRRV..SSA..AWSGVRKSSQLAELS...PRFGMRKISGSPRSPFPYSLEGRHAL
Ascosphaera_apis       PTLGVTRPGYGPGHCHTLRQTKSTQSLGHSARSLSGIRKTSYNSRSPNLSSTFENSLL
Arthrobotrys_flagrans  HS...VHGTPLETPPSDADF...NATVQHKLKRKPSDLKSEHS...
Alternaria_alternata    PG...GNGDKVLRIRISSGIPNA..GGRV.QKSQPGSAQRSPMVSSTFSDAAS
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Aspergillus_nidulans     S.SAKT...ELSTMLOPV.TTNSLAPPTPLTPEDLHHLLPTTPSTDG.YCLS.AQPTAHLF
Aspergillus_niger       G.S.KA...DMSSMLQPAVTTGG.LAPPTPLTPEDLHHLLPTTPSDG.YCLS.AQPTSQLF
Aspergillus_flavus      G.TSKP...EMSSMLSPAVTTGG.LAPPTPLTPDDLHHFIPNTPSDGG.YCLS.AQPTSQLF
Aspergillus_oryzae      G.TSKP...EMSSMLSPAVTTGG.LAPPTPLTPDDLHHFIPNTPSDGG.YCLS.AQPTSQLF
Penicillium_rubens      S.AAKA...EMLOP.SVSANTLAPPTPLTPEDFQHLLEPASPAGG.YCLSAHPASQQL
Talaromyces_marneffei   A.AANASSESRQKHLRHTSASVGNLAPPTPLTPEDFQHMLLPTTSTDMQN..FSTPHLT
Blastomyces_dermatitidis N.CANAT...DMMSVPLVTTT.LAPPTPLTPEDLRTLPLTPND.SQYCVSPTDDM...
Histoplasma_capsulatum N.CANTA...DLMSLPLGLVTTT.LAPPTPLTPEDLQTLPLTPND.SQYCVSPTDDM...
Microsporium_canis      S.NT...DLAVP.SSTSSLP.PATPLTPDEM.QYLLEP.TPIDNQ.YCLS.PQEM...
Trichophyton_tonsurans S.NA...DLAVP.SSTSSLP.PATPLTPDEM.QYLLEP.TPIDNQ.YCLS.PQEM...
Ascosphaera_apis       G.SALP...TISPLATPMTPDGARSLMP.TPND.AYAS.PMEPCP...
Arthrobotrys_flagrans  PKFART...FSTSSATTIIGHGGS.LAPPTPLTPQDFGNYWGGAA...L.VIRPHSAMPDH
Alternaria_alternata
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Aspergillus_nidulans     . . . . . PTTOPMQINIAS. . . . . PPAATPLGMDTMS...SYPVHSVAP.PMSAPA
Aspergillus_niger       . . . . . PTTOPMQINIAS. . . . . PPAATPLAVDVL.S...SYPYQGVAP.PMSAPA
Aspergillus_flavus      . . . . . PTTOPMQINIAS. . . . . PPAATPMAMDMLS...TYQVHSVAP.PMSAPA
Aspergillus_oryzae      . . . . . PTTOPMQINIAS. . . . . PPAATPMAMDMLS...TYQVHSVAP.PMSAPA
Penicillium_zonata      FQSTTATTOPMQIHIAS. . . . . PPSSTPLTMEVLS...PFAYITLAP.PMSAPA
Penicillium_rubens      . . . . . PTTOPMQVNMAS. . . . . PPAATPL..DIYS...PFVQNVAP.PMSAPA
Talaromyces_marneffei   DTQGNFVPTQSMQINVAS. . . . . PPEATPLTLDFVS...AMQYQNMGP.PLSATP
Blastomyces_dermatitidis GCARFFPTISOPMQVHIES. . . . . PPTPLHLGLVPS...HLOQYQSMGVP.PMSASP
Histoplasma_capsulatum GCARLFPTMSOPVQVHIES. . . . . PPTPLHLGLVQS...HLOQYQSMGVP.PMSASP
Microsporium_canis      GYAHSPPTSQSMNFDENQ. . . . . ESKRQPPFVVMVGMPH...AQSYQSFTE.PMSAPP
Trichophyton_tonsurans GYSHSPPTSQSMTFDECO. . . . . ESKRQPPFVVMVGMPH...PQSYQFTE.PMSAPP
Ascosphaera_apis       SYA..APPPTGMSLEPES. . . . . PPGTFFNFVGYGMPRPSLHHTSFVP.PRSAPP
Arthrobotrys_flagrans  . . . . . TNEDAPIKQNLAS. . . . . PPTPETAGLAAVNGSFKEL...E.SSDQ
Alternaria_alternata    . . . . . NSESMHTNWS.DQAGNVIAKTTS.PSSSLDLQSRFVND..ALYRDT.PMSAPA
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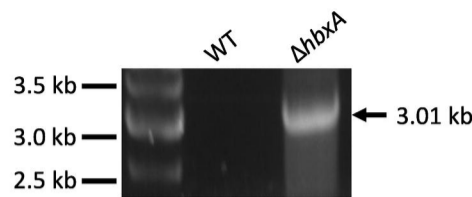
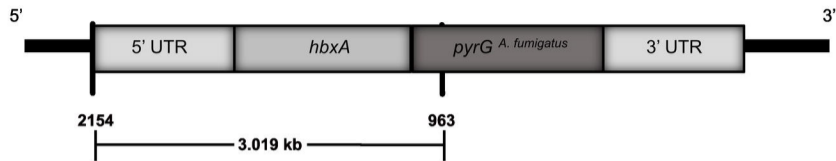
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Aspergillus_nidulans     NFTSFP.DYS...CD.GSFQGRNWE.ATSMPSPEVVPFQSQCH...Q..M
Aspergillus_niger       HYSFA.DYAS...CE.APLTGRS.WTDATSMPSPEASFQSPCQ...LPQA
Aspergillus_flavus      HYSFP.DYVT...CEGAPLGRS.WTGANSMP.SPEAAFNVRV...ITQA
Aspergillus_oryzae      HYSFP.DYVT...CEGAPLGRS.WTGANSMP.SPEAAFNVRV...ITQA
Penicillium_zonata      QYTFPEYAG...SCEVPLTARS.WAEAVPMP.SPEMNFVGMF...PSIA
Penicillium_rubens      QVSSFP.EYLT...CDSVPIPARS.WADTGSIS.SPEYPAGLQVP...HS.S
Talaromyces_marneffei   QYASFT.DYSP...ITSEPLTGV.SWAVSTPDSL...FP.SLQ...SQR
Blastomyces_dermatitidis QRTTFQEYALS...VPTNPMNGGL.WSDVSSMPSPETSHLQPT..IHMPPQ
Histoplasma_capsulatum QHTTFQEYALS...IPTNPMNGGL.WSDVSSMPSPETSHLQPT..IHMPPQ
Microsporium_canis     NFTTFNELIPD...CGQQQQOQQALTSSEADPS...LNV..IHMPPR
Trichophyton_tonsurans NFTTFNELIPG...CGQQQQOQQALTSSEADPS...LNV..IHMPPR
Ascosphaera_apis       NQHGFPDSSNF.MPIPGSAPGTAAGPHAMS.DYAWSTGSS.LSPDTPHLQNSH..FSPHPT
Arthrobotrys_flagrans  QYTNFTKMLEQSSPIVSTAAFDGHSDFQPNVEHWISGHQPYPYWTPTHQSDNHVNDISQQ
Alternaria_alternata    TQQHFPRTSYMQ...QPQMAAA...FHS.TTDLTIQPKP
```

		540		550
<i>Aspergillus nidulans</i>	N	F	..
<i>Aspergillus niger</i>	D	L	..
<i>Aspergillus flavus</i>	D	V	..
<i>Aspergillus oryzae</i>	D	V	..
<i>Penicillium zonata</i>	Q	H	S
<i>Penicillium rubens</i>	T	V	..
<i>Talaromyces marneffei</i>	Q	Q	P
<i>Blastomyces dermatitidis</i>	T	H
<i>Histoplasma capsulatum</i>	T	H
<i>Microsporium canis</i>	T	H
<i>Trichophyton tonsurans</i>	T	H
<i>Ascosphaera apis</i>	H	Q	M	L
<i>Arthrotrrys flagrans</i>	S	Q	A	Y
<i>Alternaria alternata</i>	E	H

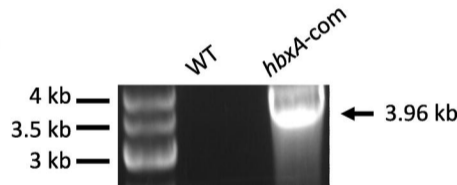
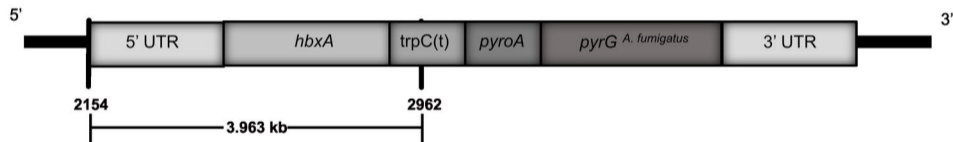
	560	570	580	590
<i>Aspergillus nidulans</i>	S	Q	S	P
<i>Aspergillus niger</i>	F	S	L	V
<i>Aspergillus flavus</i>	P	P	L	M
<i>Aspergillus oryzae</i>	P	P	L	M
<i>Penicillium zonata</i>
<i>Penicillium rubens</i>	P	S	L	I
<i>Talaromyces marneffei</i>	P	P	A
<i>Blastomyces dermatitidis</i>	P	Q	C	T
<i>Histoplasma capsulatum</i>	P	H	C	A
<i>Microsporium canis</i>	P	N	S	P
<i>Trichophyton tonsurans</i>	P	N	S	P
<i>Ascosphaera apis</i>	P	Q	M
<i>Arthrotrrys flagrans</i>
<i>Alternaria alternata</i>	H

	600	610
<i>Aspergillus nidulans</i>	Q	L
<i>Aspergillus niger</i>	Q	L
<i>Aspergillus flavus</i>	Q	L
<i>Aspergillus oryzae</i>	Q	L
<i>Penicillium zonata</i>	A	Q
<i>Penicillium rubens</i>	H	L
<i>Talaromyces marneffei</i>	Q	L
<i>Blastomyces dermatitidis</i>	E	Q
<i>Histoplasma capsulatum</i>	E	Q
<i>Microsporium canis</i>	Q	L
<i>Trichophyton tonsurans</i>	Q	L
<i>Ascosphaera apis</i>	A	M
<i>Arthrotrrys flagrans</i>
<i>Alternaria alternata</i>	R	R

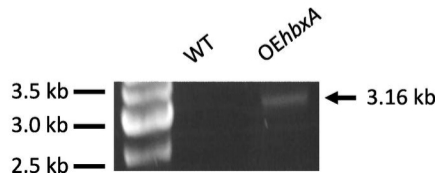
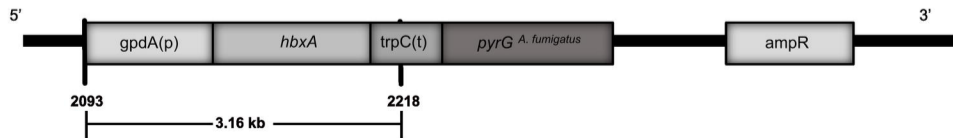
A



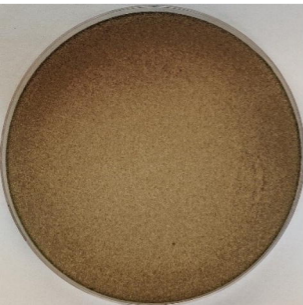
B



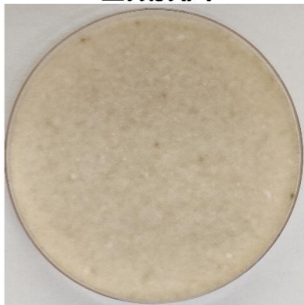
C



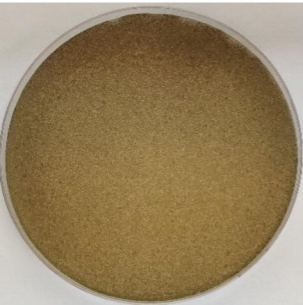
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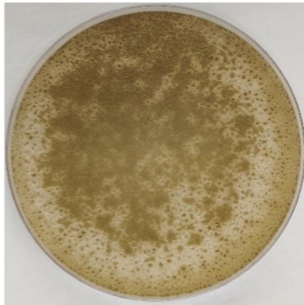
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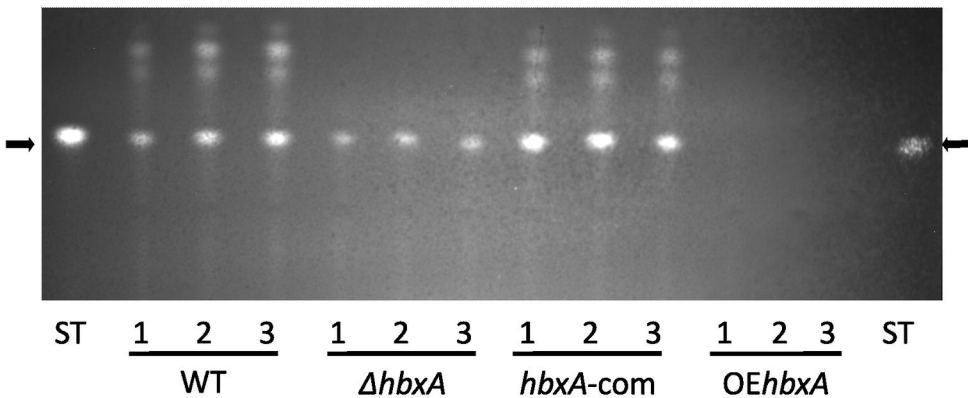
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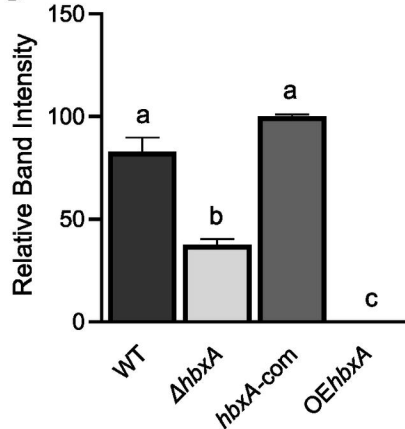
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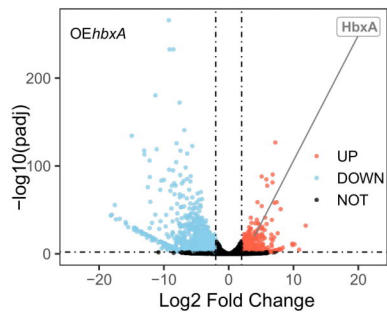
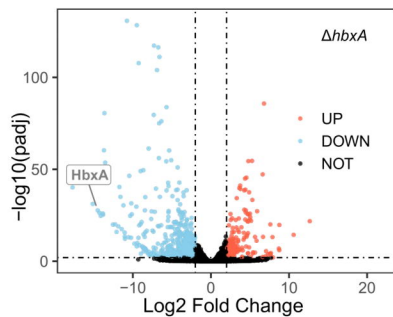
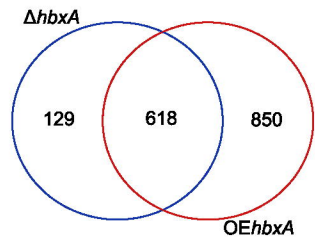
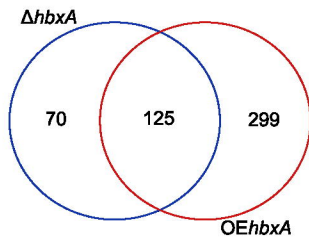
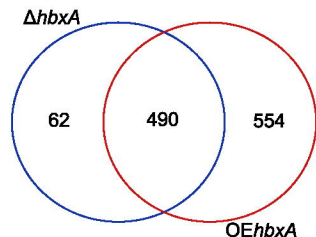


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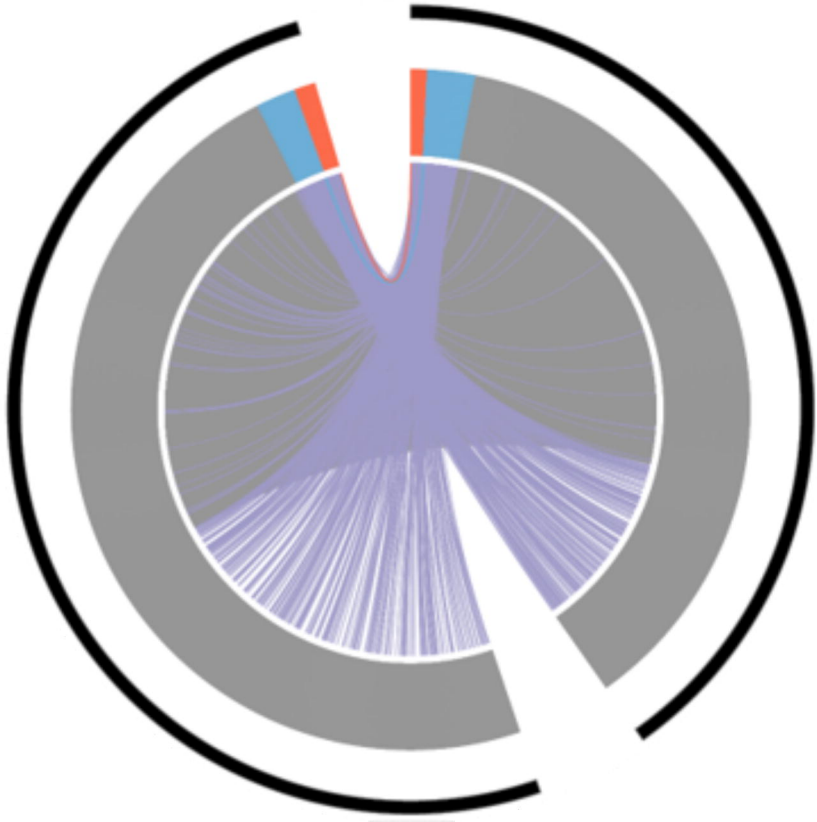


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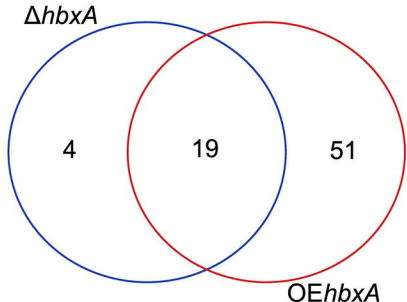
A**B****C****D****E**

A. flavus

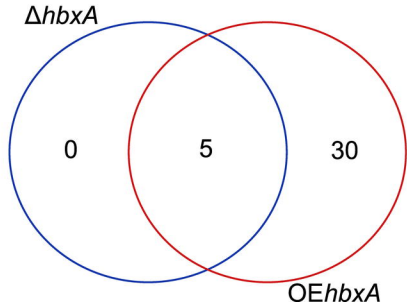


A. nidulans

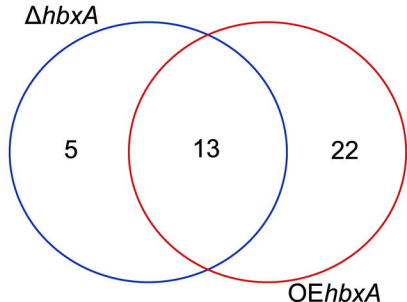
A



B

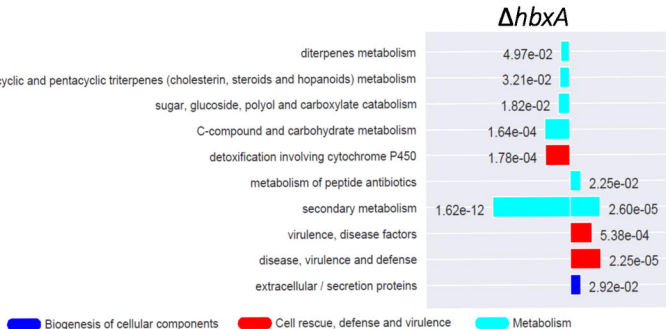


C



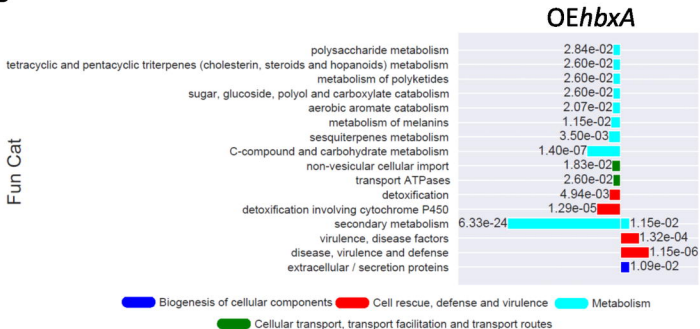
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Fun Cat



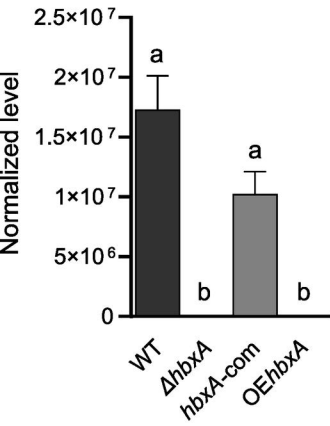
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Fun Cat



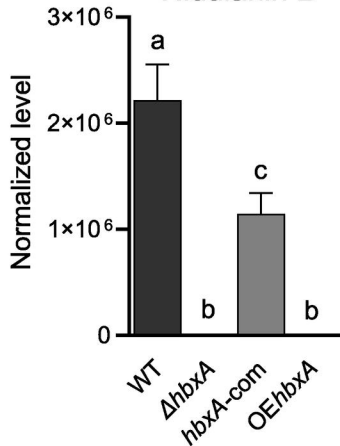
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Nidulanin A



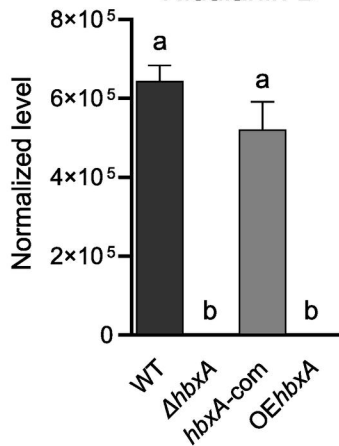
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Nidulanin B

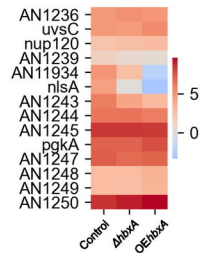


C

Nidulanin D

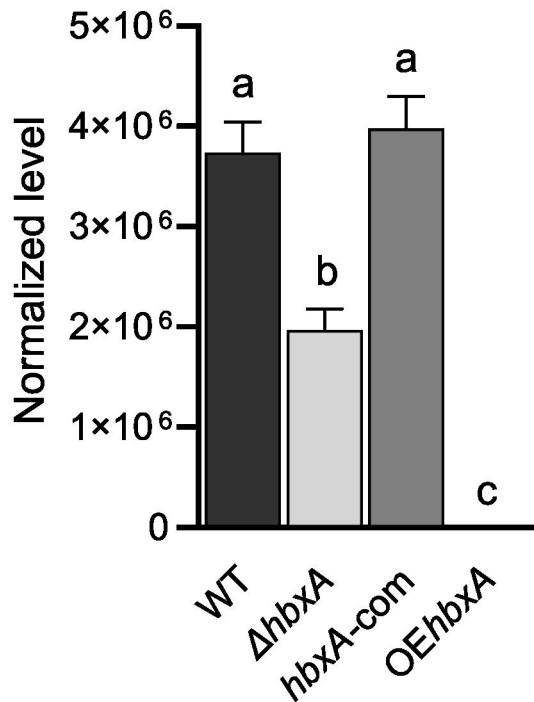


D

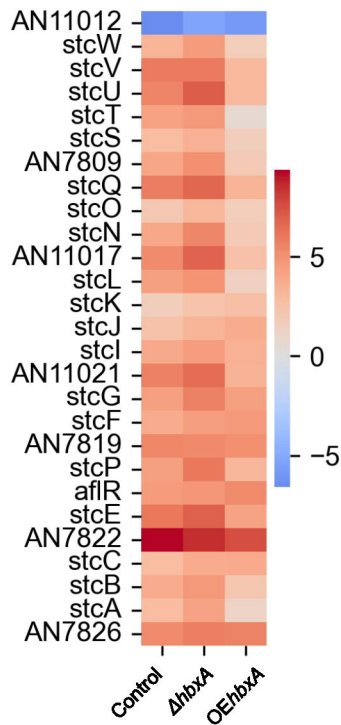


A

Sterigmatocystin

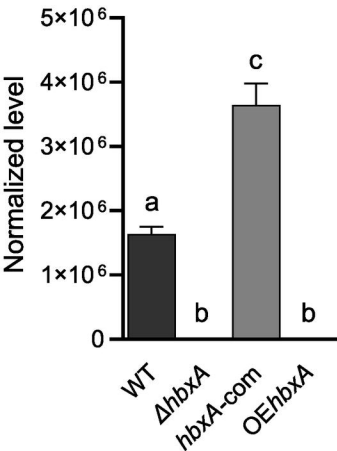


B



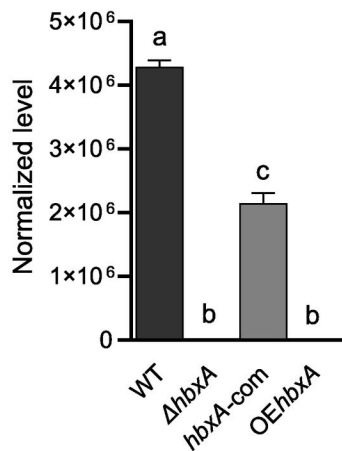
A

Austinol

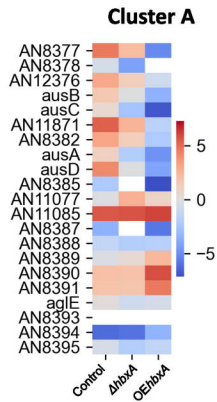


B

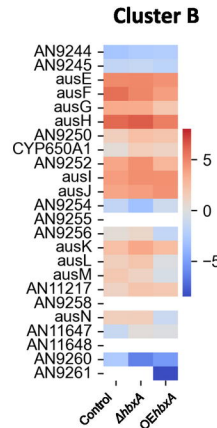
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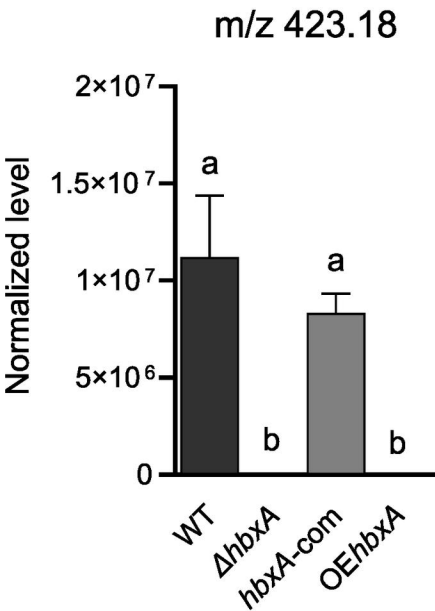
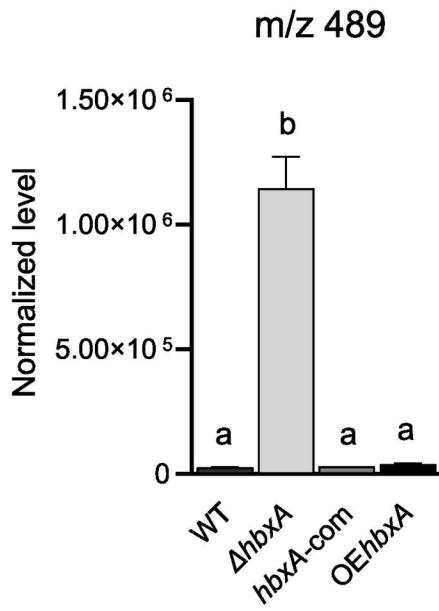


C



D



A**B****C**