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1 # Conservation and Divergence in the Asexual Sporulation Gene
2 Regulatory Network Across a Genus of Filamentous Fungi

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15 Running Head: The WetA regulatory network in *Aspergillus* conidiation

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23 # Abstract

24 Asexual sporulation is fundamental to the ecology and lifestyle of filamentous fungi and can
25 facilitate both plant and human infection. In *Aspergillus*, the production of asexual spores is
26 primarily governed by the BrlA→AbaA→WetA regulatory cascade. The final step in this
27 cascade is controlled by the WetA protein and not only governs the morphological
28 differentiation of spores but also the production and deposition of diverse metabolites into
29 spores. While WetA is conserved across the genus *Aspergillus*, the structure and degree of
30 conservation of the *wetA* gene regulatory network (GRN) remains largely unknown. We carried
31 out comparative transcriptome analyses between *wetA* null mutant and wild type asexual spores
32 in three representative species spanning the diversity of the genus *Aspergillus*: *A. nidulans*, *A.*
33 *flavus*, and *A. fumigatus*. We discovered that WetA regulates asexual sporulation in all three
34 species via a negative feedback loop that represses BrlA, the cascade's first step. Furthermore,
35 ChIP-seq experiments in *A. nidulans* asexual spores suggest that WetA is a DNA-binding
36 protein that interacts with a novel regulatory motif. Several global regulators known to bridge
37 spore production and the production of secondary metabolites show species-specific regulatory
38 patterns in our data. These results suggest that the BrlA→AbaA→WetA cascade's regulatory
39 role in cellular and chemical asexual spore development is functionally conserved, but that the
40 *wetA*-associated GRN has diverged during *Aspergillus* evolution.

41 # Importance

42 The formation of resilient spores is a key factor contributing to the survival and fitness of many
43 microorganisms, including fungi. In the fungal genus *Aspergillus*, spore formation is controlled
44 by a complex gene regulatory network that also impacts a variety of other processes, including
45 secondary metabolism. To gain mechanistic insights into how fungal spore formation is
46 controlled across *Aspergillus*, we dissected the gene regulatory network downstream of a major

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47 regulator of spore maturation (WetA) in three species that span the diversity of the genus: the
48 genetic model *A. nidulans*, the human pathogen *A. fumigatus*, and the aflatoxin producer *A.*
49 *flavus*. Our data shows that WetA regulates asexual sporulation in all three species via a
50 negative feedback loop and likely binds a novel regulatory element we term the WetA Response
51 Element (WRE). These results shed light on how gene regulatory networks in microorganisms
52 control important biological processes and evolve across diverse species.

53 # Introduction

54 The ability to produce numerous asexual spores is one of the key factors contributing to the
55 fecundity and fitness of filamentous fungi. Fungal asexual spores are highly efficient for
56 genome protection, survival, and propagation. Spores are also the primary means of infecting
57 host organisms for many pathogenic fungi (1). Importantly, in some filamentous fungi,
58 morphological development is coordinated with the production of secondary metabolites with
59 toxic and antibiotic properties (2–4).

60 Asexual development (conidiation) in the fungal class Eurotiomycetes results in the formation
61 of mitotically derived asexual spores known as conidiospores or conidia. As asexual
62 sporulation is widespread among fungi, it represents a simple, highly tractable system for
63 understanding how gene regulatory networks (GRNs) evolve in microbial eukaryotes and how
64 this evolution has influenced developmental and metabolic phenotypes.

65 Members of the genus *Aspergillus* are ubiquitous in most environments, and include various
66 beneficial, pathogenic, and/or toxigenic species (5) All aspergilli produce conidia as the main
67 means of dispersion and infection. Importantly, the asexual development and the production of
68 certain secondary metabolites including mycotoxins are intimately associated (2).

69 The three distantly related species *Aspergillus nidulans*, *Aspergillus flavus*, and *Aspergillus*
70 *fumigatus*, whose pairwise levels of genome similarity are similar to the genomic similarity
71 between the human and fish genomes (6), form distinct conidiophores with varying sizes of

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72 conidia. The regulatory mechanisms of conidiation have been extensively studied in *A.*
73 *nidulans* (7–23). The regulatory genes can be divided into central regulators, upstream
74 activators, negative regulators, light-dependent regulators, and the *velvet* regulators (24, 25).
75 The central genetic regulatory cascade BrlA→AbaA→WetA is present in *Aspergillus* and
76 governs both conidiation-specific GRNs and the resulting morphological pathway of
77 conidiation (Fig 1A) (22, 24, 26). BrlA is a C_2H_2-zinc-finger type transcription factor (TF),
78 which recognizes and interacts with BrlA Response Elements (BRE) (Fig 1B) (27, 28). The
79 *brlA* gene is expressed in the early phase of conidiation and mediates vesicle formation and
80 budding-like cell growth (11). The *abaA* gene is activated by BrlA and regulates the formation
81 of metulae and phialides. Similar to BrlA, AbaA is a TF, containing an TEA/ATTS DNA
82 binding motif and a potential leucine zipper that recognizes the AbaA Response Elements
83 (AREs) (Fig 1B) (29). The *wetA* (wet-white A) gene, activated by AbaA, functions in the late
84 phase of conidiation that completes sporogenesis. The BrlA→AbaA→WetA central regulatory
85 cascade acts in concert with other genes to control conidiation-specific gene expression and
86 determine the order of gene activation during the cellular and chemical development of spores.
87 The WetA protein plays a pivotal role in the coordinated control of *Aspergillus* conidiogenesis;
88 however, the precise molecular mechanisms of WetA function have been unknown. WetA is
89 highly and broadly conserved in *Ascomycetes* (8, 10–13, 15–23, 26, 30), plays an essential role
90 in the synthesis of crucial conidial wall components, and makes the conidia both impermeable
91 and mature (20, 21, 30). The *Aspergillus* WetA proteins have a conserved ESC1/WetA-related
92 domain (PTHR22934: SF23) with putative DNA-binding functions, a predicted transcription
93 activation domain (TAD) (31), and a nuclear localization signal (NLS) (32, 33) near the C
94 terminus (16, 34), suggesting that WetA is likely a DNA-binding TF (30) (Fig 1B). As
95 summarized in Table 1, the deletion of *wetA* results in a plethora of conidial defects, including
96 the formation of colorless conidia that undergo autolysis in *A. nidulans* (10–13, 20–22, 26), *A.*

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97 *fumigatus* (8, 15), *A. oryzae* (18), and *A. flavus* (30). The metabolism and expression of several
98 conidial components are perturbed in the Δ*wetA* conidia, leading to reduced stress tolerance
99 and spore viability (8, 30).

100 In this report, we have investigated the structure and degree of conservation of the
101 BrlA→AbaA→WetA central regulatory cascade of *Aspergillus* conidiation and the broader
102 *wetA* GRNs in three representative *Aspergillus* species: the genetic model *A. nidulans*, the
103 mycotoxin producer *A. flavus*, and the human pathogen *A. fumigatus*. Specifically, we carried
104 out comparative transcriptome analyses between *wetA* null mutant and wild type (WT) asexual
105 spores in the three species. We also investigated the WetA-chromatin interaction in asexual
106 spores via ChIP-seq in *A. nidulans* spores, which enabled us to identify the consensus WetA-
107 DNA binding sequence. Further comparative genome-wide analyses revealed that the WetA-
108 associated GRN has diverged during *Aspergillus* evolution, uncovering important yet
109 unexplored regulatory networks of asexual sporulation and metabolic remodeling in
110 *Aspergillus*. Our findings provide the first clear and systematic dissection of the evolutionarily
111 conserved WetA developmental regulator governing the diverged processes of cellular
112 differentiation, chemical development, and cell survival across a genus of filamentous fungi.

113 Results

114 Conserved and diverged roles for WetA in the control of gene
115 expression in *Aspergilli*

116 To investigate the conserved and divergent regulatory roles WetA plays in the three *Aspergillus*
117 species, we carried out comprehensive analyses of gene expression differences between the
118 WT and *wetA* null mutant conidia. We found that WetA plays a broad regulatory role in conidia
119 in all three *Aspergillus* species; approximately 52%, 57%, and 43% of all genes showed
120 differential accumulation of mRNAs in the Δ*wetA* conidia in comparison to WT conidia in *A.*

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121 *nidulans*, *A. fumigatus*, and *A. flavus*, respectively (Table 2). Among the differentially
122 expressed genes (DEGs), 46%, 48%, and 50% were underexpressed and 54%, 52%, and 50%
123 were overexpressed in the Δ*wetA* conidia compared to the WT conidia in *A. nidulans*, *A.*
124 *fumigatus*, and *A. flavus*, respectively (Table 2).
125 Functional category analysis was carried out by determining Gene Ontology (GO) terms that
126 were enriched in DEGs. Specifically, the biological process GO categories that were enriched
127 in the Δ*wetA* conidia included "asexual sporulation", "secondary metabolic process", and
128 "toxin biosynthetic process". Moreover, over 70% of all genes in the cellular component GO
129 category, "fungal-type cell wall", were also regulated in each species. These top enriched GO
130 categories are consistent with the phenotypes of the Δ*wetA* mutants, suggesting that WetA plays
131 key a role in carbohydrate metabolism, secondary metabolism, and conidial wall integrity (30).
132 To explore the conserved and diverged regulatory roles of WetA, we examined the mRNA
133 expression profiles of orthologous groups of genes (orthogroups) in the three *Aspergillus*
134 genomes. In total, 8,978 orthogroups were identified and 6,466 of these contained orthologs in all
135 three species. Of the 8,978 total orthogroups, 7,301 (81%) had at least one gene that showed
136 differential expression in the Δ*wetA* conidia, but only 1,294 orthogroups show consistent
137 WetA-regulation (i.e., all orthologs in the group were either overexpressed or underexpressed).
138 The enriched GO categories of the 1,294 orthogroups whose genes showed the same
139 differential expression pattern suggest that WetA is functionally conserved in controlling stress
140 response, pigmentation, spore trehalose formation, cell wall organization, and cellular
141 development, which are also consistent with the phenotypes observed in Δ*wetA* strains. In
142 contrast, the remaining 6,007 WetA-regulated orthogroups show divergent differential
143 expression patterns, implying that a substantial portion of the WetA-controlled GRNs has
144 functionally diverged among the three species. Furthermore, of the 6,466 *Aspergillus*
145 orthogroups that contain orthologs in all three species, only 788 exhibited a conserved pattern

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146 of differential expression (i.e., all genes were either overexpressed or underexpressed in the
147 Δ*wetA* conidia in all three species) (Fig 2, Table S2).

148 WetA-regulated genes involved in asexual development, signal 149 transduction, and conidial integrity are divergently regulated 150 among *Aspergilli*

151 To explore the conserved and diverged molecular roles of WetA in conidiation in the three
152 species, we examined mRNA levels of genes related to asexual development, signal
153 transduction, and conidial integrity (Fig 3 , Table S3), phenotypes previously implicated to be
154 controlled by WetA (8, 10–13, 20–22, 26, 30, 37).
155 Our data show that WetA negatively regulates asexual development in conidia produced by
156 species across the genus *Aspergillus* via a negative feedback loop that represses the pathway's
157 upstream regulator, *brlA*. Specifically, both *brlA* and *abaA* expression are increased in the
158 Δ*wetA* conidia relative to WT in all three species (Figure 4). However, to achieve the conserved
159 repression of *brlA* and *abaA* mRNA accumulation, WetA shows species-specific regulatory
160 effects on *brlA* upstream regulatory networks. For example, in the *velvet* protein family and
161 complex, *vosA* was consistently underexpressed in the three Δ*wetA* conidia, but the WetA
162 effects on *veA*, *velB*, *velC*, and *laeA* expression are not conserved in each species. Similarly,
163 the light-dependent regulators were differentially regulated by WetA. The blue-light-dependent
164 regulators *lreA* and *lreB* were unaffected in the Δ*AflwetA* conidia but repressed in both the
165 Δ*AniwetA* and Δ*AfuwetA* conidia. Taken together, the WetA-mediated feedback repression of
166 asexual development is functionally conserved across the genus *Aspergillus* but the specific
167 GRNs appear to have diverged during the evolution of the genus *Aspergillus*.
168 Our previous study showed that *Afl*WetA is involved in regulating G-protein regulatory
169 pathways (30). Expanding this analysis in the three *Aspergillus* species showed that *gprC*, *gprF*,

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170 *gprG*, *nopA*, *flbA*, and *pkaA* were consistently differentially regulated in the Δ*wetA* conidia,
171 while other members in the G-protein regulatory pathways were either not affected by WetA or
172 showed species-specific regulatory patterns in the Δ*wetA* conidia (Table S4).
173 WetA is involved in other signal transduction pathways. Total 110, 126, and 92 kinase-encoded
174 genes were differentially expressed in the Δ*AniwetA*, Δ*AfuwetA*, and Δ*AflwetA* conidia
175 respectively; however, only 21 of them were consistently over- or underexpressed in the Δ*wetA*
176 conidia of all three species (Table S5). Similarly, 132, 153, and 142 putative TF-encoded genes
177 in each species were differentially expressed in Δ*AniwetA*, Δ*AfuwetA*, and Δ*AflwetA* conidia
178 respectively; however, only 32 were consistently over- or underexpressed in all three of the
179 Δ*wetA* conidia (Table S5).
180 We further investigated the mRNA levels of the genes in the secondary metabolite gene (SMG)
181 clusters in each species (30, 38, 39) (Table S7). In total, 96% (64/67), 100% (33/33), and 92%
182 (68/74) of SMG clusters in the Δ*AniwetA*, Δ*AfuwetA*, and Δ*AflwetA* conidia, respectively, had
183 at least one gene that showed altered mRNA expression levels (Table 3). One of the SMG
184 backbone genes, *wA*, is conserved in all three species and it encodes a polyketide synthase
185 (PKS) necessary for the formation of a key conidial pigment (40). Previous studies showed that
186 *wA* is activated by WetA (20), consistent with the colorless conidia phenotype of the Δ*wetA*
187 mutants. Although *wA* was underexpressed in the Δ*AniwetA* and Δ*AflwetA* conidia as expected,
188 it was overexpressed in Δ*AfuwetA*, suggesting that the regulation of the conidial pigmentation
189 pathway in *A. fumigatus* differs from that in the other two species.
190 Finally, we examined the expression levels of genes involved in conidia content and conidial
191 wall integrity. Most of the DEGs associated with trehalose biosynthesis were underexpressed
192 in the Δ*wetA* conidia in all three species, while *treA*, involved in trehalose degradation, was
193 overexpressed in the Δ*AniwetA* and Δ*AfuwetA* conidia but underexpressed in the Δ*AflwetA*

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194 conidia (Fig 4). Loss of *wetA* resulted in overexpression of almost all genes involved in the
195 biosynthesis of chitin and β-(1,3)-glucan, but genes involved in the biosynthesis and
196 degradation of α-(1,3)-glucan were both overexpressed and underexpressed relative to WT.
197 (Fig 4).

198 Moreover, our results show that WetA is a key regulator of hydrophobins, DHN-melanin
199 biosynthesis, and pyomelanin biosynthesis. Somewhat unexpectedly, although we observed the
200 conserved "wet" and "white" phenotypes of the Δ*wetA* conidia in all three species, all of the
201 genes proposed to be related to the "wet" (hydrophobin) and "white" (DHN-melanin and
202 pyomelanin) phenotypes were overexpressed in the Δ*AfuwetA* conidia (Fig 5).

203 Identification of WetA response elements (WREs)

204 To better understand WetA regulatory mechanisms in conidia, we carried out chromatin
205 immunoprecipitation (ChIP) experiments, followed by high-throughput sequencing of the
206 enriched DNA fragments (ChIP-seq) in the *A. nidulans* conidia. We identified 157 peaks from
207 two independent ChIP-seq experiments, using a False Discovery Rate (FDR) cutoff of less than
208 or equal to 0.001 and a Fold Change (FC, sample tag counts divided by input tag counts) cutoff
209 greater than or equal to 2. Of the 157 peaks, 135 were located in at least one of the following:
210 a protein coding region, an intron, an upstream region, or a downstream region (Table S8).
211 Upstream and downstream regions were defined as locations within 1.5 kb of the translation
212 start or stop site, respectively. Many peaks were located within multiple features due to the
213 condensed nature of the *A. nidulans* genome; therefore, 212 genes were considered "peak-
214 associated". Only a few peaks were located within protein coding regions (18) or introns (5);
215 however, 105 peaks were in upstream regions and 59 peaks were in downstream regions. Of
216 the 212 peak-associated genes, 139 showed differential expression in the *A. nidulans* RNA-seq
217 dataset. Multiple previously described genes are in the list of peak-associated genes, including
218 *flbA*, *mtfA*, *nopA, velB*, *sfaD*, *wetA*, *vosA*, *hsp70*, *srbA*, and *tpsA* (Table S8).

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219 A putative WRE was predicted by MEME-ChIP (41). The 100 bp surrounding the summits of
220 all peaks was used as input for the MEME-ChIP analysis. The only statistically significant
221 motif identified (E-value = 8.8e-8) was 5'-CCGYTTGCGGC-3' and it exists in the upstream
222 region of *AniwetA*. Potential *Ani*WetA-recognized regions were identified by searching for the
223 predicted motif in the upstream regions of ORFs in the *A. nidulans* genome with FIMO (42).
224 In total, 2,217 genes were predicted to contain the WRE within their upstream regions in *A.*
225 *nidulans* (Table S9). Functional analysis shows that many biological processes were enriched
226 in these potential *Ani*WetA targeted genes, including "trehalose metabolic process" and "cell
227 wall mannoprotein biosynthetic process", consistent with what is known about WetA function
228 in conidiation.

229 To investigate the expression profile of potential *Ani*WetA target genes in conidia, data from
230 the transcriptomic analysis was utilized. In total, 1,176 WRE-containing genes were
231 differentially expressed in *A. nidulans*, including 2 G-protein signaling pathway-associated
232 genes, 22 conidial integrity-associated genes, 14 putative kinase-encoding genes, 22 putative
233 transcription factor-encoding genes, 5 SMG backbone genes, and 11 asexual development-
234 associated genes (Table 4).

235 Since the *Aspergillus* WetA proteins have a highly conserved putative DNA-binding domain
236 and have conserved functions in the overall conidiation process, we hypothesized that *Afu*WetA
237 and *Afl*WetA recognize a similar *Ani*WetA WRE. To test our hypothesis, we searched the
238 *Ani*WetA WRE in the *A. fumigatus* and *A. flavus* genomes and summarized the results in Fig 6.
239 Only 15 genes, including *wetA*, that contain a WRE in their upstream 1.5 kb regions in all three
240 species also exhibit consistent differential expression (Table 5). We further searched for WRE
241 occurrences in the 1.5 kb sequence upstream of other *wetA* orthologs in different *Aspergillus*
242 and other fungal species and found that the WRE in the upstream region of *wetA* genes is
243 completely conserved throughout the family Aspergillaceae (Fig 7).

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244 Taken together, we conclude that WetA regulates the *Aspergillus* conidial transcriptomes
245 through both direct and indirect methods and controls species-specific GRNs to achieve
246 conserved and diverged functions.

247 Discussion

248 While WetA is well known as the key regulator of multiple cellular and chemical
249 developmental processes in Ascomycetes (7, 8, 17–23, 30, 9–16), the regulatory mechanisms
250 behind it employs are not known. In this study, we investigated the roles of WetA-mediated
251 GRNs in the model organism *A. nidulans*, the human pathogen *A. fumigatus*, and the aflatoxin
252 producer *A. flavus* and further identified a potential WetA binding motif in *A. nidulans*.
253 Previous studies suggested that *Ani*WetA is required for activating a set of genes whose
254 products comprise, or direct the assembly of, the conidial wall layers and also ensure proper
255 cytoplasmic metabolic remodeling including massive trehalose biogenesis (20, 22, 43). We also
256 reported that *Afl*WetA is involved in the regulation of conidial secondary metabolism and
257 hypothesized that this was done by WetA controlling a group of TFs and signaling pathways
258 (30). Our RNA-seq results here show that 52%, 57%, and 43% of *A. nidulans*, *A. fumigatus,*
259 and *A. flavus* transcriptomes were differentially regulated in the Δ*wetA* conidia respectively,
260 suggesting a broad regulatory role for WetA in aspergilli (Table 2, Fig 6). While *Ani*WetA,
261 *Afu*WetA, and *Afu*WetA are functionally conserved in many aspects of developmental
262 processes in conidia, the specific genes regulated by WetA are divergent in each species.
263 Although WetA regulates a large number of common orthogroups in aspergilli, only 9% of
264 *Aspergillus* orthogroups were consistently all over- or underexpressed in Δ*wetA* conidia from
265 the three species, suggesting that while the WetA-mediated regulation is functionally conserved,
266 the WetA-mediated GRNs have been highly rewired (Fig 8).

267 An example of a divergent WetA-mediated GRN whose output is the conserved regulation of

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268 a biological process is *Aspergillus* asexual development (Fig 3). In all three species, loss of
269 *wetA* leads to increased levels of the central regulator *brlA* in conidia and shuts down asexual
270 development. However, the set of regulatory events that result in WetA-mediated repression of
271 *brlA* are unique to each species (Fig 3).

272 Although WetA shows broad regulatory effects in *Aspergillus* species, only 15 genes with the
273 WRE in their upstream regions were consistently under- or overexpressed in the Δ*wetA* conidia.
274 The list of peak-associated genes in *A. nidulans* includes *wetA* and the important developmental
275 regulators *vosA* and *velB*, suggesting that these genes may play crucial roles in conidiation and
276 thus be conserved during evolution. VosA and VelB are both members of the *velvet* family of
277 proteins (44–46). Moreover, the VosA-VelB complex is a crucial functional unit controlling
278 conidia maturation (45–47). Loss of *vosA* causes some phenotypes similar to those by the loss
279 of *wetA*, like a reduction in trehalose amount (48), suggesting that part of the WetA-mediated
280 GRN may be controlled by regulating VosA. Previous studies show that *Ani*WetA contains an
281 *Ani*VosA binding motif in its upstream 2 kb region (44), implicating the cross feedback
282 regulation of WetA by VosA. Taken together, the WetA-mediated regulatory pathway may
283 cross-talk with the *velvet* regulatory pathways via the cooperative activity of WetA/VosA/VelB.

284 We further examined the WetA-mediated GRNs controlling other pathways based on
285 previously characterized, conserved WetA functions. First, we analyzed genes involved in
286 conidial integrity for their WetA-regulation. The genes associated with trehalose biosynthesis
287 are almost all underexpressed in all three of the Δ*wetA* conidia (Fig 4). Similarly, almost all the
288 genes associated with β-(1,3)-glucan biosynthesis were overexpressed in all three of the Δ*wetA*
289 conidia (Fig 4). These results explain the dramatically reduced amount of trehalose and
290 increased content of β-(1,3)-glucan in the Δ*wetA* conidia (8, 30) and suggest a conserved WetA-
291 mediated GRN for activation of trehalose biogenesis and repression of β-(1,3)-glucan

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292 biosynthesis. WetA's function is likely diverged in α-(1,3)-glucan metabolism. *Ani*WetA
293 upregulates the α-(1,3)-glucan synthase *Aniags2* but downregulates all the genes associated
294 with α-(1,3)-glucan degradation except AN1604 (Fig 4). In contrast, *Afu*WetA downregulates
295 all the α-(1,3)-glucan synthases (*Afuags1*, *Afuags2*, and *Afuags3*), but has mixed effects on the
296 genes associated with α-(1,3)-glucan degradation in conidia (Fig 4). In conidia, *Afl*WetA shows
297 mixed effects on both the genes associated with α-(1,3)-glucan biosynthesis and degradation
298 (Fig 4).

299 WetA is involved in the regulation of hydrophobin expression. Only one of the five
300 hydrophobin-encoding genes in *A. nidulans* was not differentially expressed in the Δ*wetA*
301 conidia, and only *AnidewA* was underexpressed (Fig 5). In *A. fumigatus*, all six hydrophobin-
302 encoding genes were overexpressed in the Δ*wetA* conidia (Fig 5). In *A. flavus*, three of five
303 hydrophobin-encoding genes were underexpressed in the Δ*wetA* conidia, one of them was not
304 regulated, and only *AflrodA* was up-regulated (Fig 5). Since the loss of *wetA* causes lower
305 hydrophobicity of conidia, there might be other unidentified hydrophobins controlled by
306 *Afu*WetA.

307 *Afu*WetA is diverged relative to *Ani*WetA and *Afl*WetA in its regulation of melanin biosynthesis.
308 A previous study showed that *wA*, the first regulator in the DHN-melanin synthesis pathway, is
309 activated by WetA in *A. nidulans* conidia (20, 49). Our RNA-seq analyses have revealed that
310 both *AniwA* and *AflwA* were underexpressed in the Δ*AniwetA* and Δ*AflwetA* conidia (Fig 5).
311 Moreover, *Aflayg1*, the second gene in the DHN-melanin pathway (50) was underexpressed in
312 the Δ*AflwetA* conidia (Fig 5). Surprisingly, although the Δ*AfuwetA* conidia are colorless, all the
313 DEGs associated with both DHN-melanin and pyomelanin biosynthesis were overexpressed in
314 the Δ*AflwetA* conidia (Fig 5), suggesting the melanin biosynthesis pathway in *A. fumigatus* may
315 have uniquely evolved.

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316 We identified a potential WRE (5'-CCGYTTGCGGC-3'), which is highly similar to the
317 *Saccharomyces cerevisiae* Ixr1, Dal81, and Leu3 motifs (51–53). Although 53% of genes in
318 the *A. nidulans* genome were differentially regulated in the Δ*AniwetA* conidia, only 21% of
319 them contain a WRE in their upstream 1.5 kb regions (Fig 6), suggesting that *Ani*WetA might
320 serve as a conserved regulatory hub which controls a group of regulators of various biological
321 processes. Our data support a model where the WetA-mediated regulation is carried out via
322 both direct and indirect interactions to control a downstream cascade of genes (Figure 9). We
323 also scanned the *A. fumigatus* and *A. flavus* genomes for instances of the WRE and found that,
324 while similar numbers of genes contained the WRE compared to *A. nidulans*, the makeup of
325 that list of genes was different.

326 In conclusion, our studies provide the first clear and systematic dissection of WetA, an
327 evolutionarily and functionally conserved regulator of morphological and chemical
328 development of filamentous fungal conidiation. Moreover, we have revealed the molecular
329 mechanisms of WetA as a DNA-binding, multi-functional regulator governing the diverged
330 processes of cellular differentiation, chemical development, and cell survival across a genus of
331 filamentous fungi, which advances our knowledge of spore formation in pathogenic and
332 toxigenic fungi.

333 Materials and methods

334 Strains, media, and culture conditions

335 All strains used in this study are listed in Table S1. The fungal strains were grown on minimal
336 medium (MM) with appropriate supplements as described (48, 54), and incubated at 37°C (*A.*
337 *nidulans* and *A. fumigatus*), or 30°C (*A. flavus*). For liquid cultures, conidia were inoculated in
338 liquid MM and incubated at 37°C or 30°C, 220 rpm. Conidiation induction was performed as
339 described (55).

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340 ## Generation of *wetA* deletion and complemented strains

341 We generated the deletion (Δ) and complement (C') strains of *wetA* in *A. nidulans* (*AniwetA*).
342 The oligonucleotides used in this study are listed in Table S1. Briefly, the deletion construct
343 containing *A. fumigatus pyrG* marker with 5' and 3' flanking regions of *AniwetA* were
344 introduced into the recipient strain RJMP1.59 (56). To generate complemented strains, a WT
345 *AniwetA* gene region, including its 2 kb upstream region, was cloned to pHS13 (45). The
346 resulting plasmid pMY1 was then introduced into the recipient Δ*AniwetA* strain TMY3,
347 resulting in isolation of TMY4. Multiple Δ*AniwetA* strains were generated and all behaved the
348 same in every assay. Multiple C' *AniwetA* strains were generated and all behaved identically to
349 one another as well. The Δ*AfuwetA* (TSGw4), Δ*AflwetA* (TMY1), and C'*AflwetA* (TMY2)
350 strains were generated in previous studies (8, 30).

351 ## Nucleic acid manipulation

352 The genomic DNA and total RNA isolation for Northern blot analyses was performed as
353 described (55, 57, 58). For RNA-seq and ChIP-seq, fresh conidia from 2-day-old solid cultures
354 grown at 37°C or 30°C of WT and Δ*wetA* strains were harvested.

355 ## RNA sequencing

356 Total RNA from 4 *A. nidulans* biological replicates, 3 *A. flavus* biological replicates, and 3 *A.*
357 *fumigatus* biological replicates was extracted and submitted to ProteinCT Biotechnologies
358 (Madison, WI) and the University of Wisconsin Gene Expression Center (Madison, WI) for
359 library preparation and sequencing. For each replicate, a strand-specific library was prepared
360 from total RNA using the Illumina TruSeq Strand-specific RNA sample preparation system.
361 All replicates' libraries were sequenced (PE100bp for *A. nidulans* and SE100bp for *A.*
362 *fumigatus* and *A. flavus*) using the Illumina HiSeq2500.

363 The *A. flavus* expression data were analyzed as previously reported (30). The following

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364 analyses were carried out for the *A. fumigatus* and *A. nidulans* data. The overall quality of the
365 raw sequence reads was verified using version 0.11.5 of FastQC (59). The genomes and
366 annotation were downloaded from FungiDB and used for mapping (60). Mapping of the raw
367 sequence reads to the genome was carried out with version 2.1.1 of Tophat2 (61), and the
368 default settings were used except that the max intron length was set to 4,000 bases. The
369 alignment files were compared against the gene annotation file, and raw counts for the number
370 of reads mapping to each gene were generated using version 0.6.1p1 of HTSeq-count (62).
371 Differential expression analysis of the raw counts was carried out using version 1.14.1 of
372 DESeq2 (63). Genes were considered differentially expressed between the WT and Δ*wetA*
373 conidia if their adjusted p-value was less than 0.05 and their $\log_2$ fold-change was smaller than
374 -1 or greater than 1. All RNA-seq data files are available from the NCBI Gene Expression
375 Omnibus database (*A. nidulans* and *A. fumigatus*: GSE114143; *A. flavus*: GSE95711).

376 Functional Enrichment and Orthogroup identification

377 Gene Ontology enrichment analyses were carried out using the tool available at FungiDB (60).
378 Unless otherwise stated, default settings were used in FungiDB, and redundant terms were
379 collapsed with the REVIGO tool (64) using the "Tiny" setting for allowed similarity.
380 Orthologs were identified using OrthoMCL with the settings: p-value cutoff of 6e-6, percent
381 identity cut-off of 30%, percent match cut-off of 70%, MCL inflation value of 2, and Maximum
382 weight allowed of 180.

383 Chromatin immunoprecipitation sequencing (ChIP-seq)

384 ChIP assays were performed using MAGnify ChIP assays (Invitrogen) according to the
385 manufacturer's instructions. Briefly, 10^9 of *A. nidulans* WT conidia were cross-linked with 1%
386 formaldehyde, lysed and broken as described (65). Cell lysates were sonicated to shear DNA
387 to 300-500 bp and were immunoprecipitated with the rabbit anti-WetA polyclonal antibodies
388 (GenScript, NJ). Two experiments were performed, each with biological triplicates. In the first

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389 experiment, 10% of the supernatants was kept as an input control (input represents PCR
390 amplification of the total sample) and compared to the ChIP sample. In the second experiment,
391 the ChIP sample from the WT strain was compared to the ChIP sample from the Δ*wetA* strain.
392 ChIP DNA samples were sent for ChIP-Seq service (ProteinCT, WI). Libraries were prepared
393 using the TruSeq ChIP Library Preparation Kit (Illumina, CA) and sequenced on a HiSeq2500
394 with single reads of 50 bp. Approximately 8-30 M reads were achieved per replicate.
395 ChIP-seq reads were first trimmed using version 0.36 of the Trimmomatic software (66) and
396 then version 0.7.15 of the BWA-MEM software (67) was used to map reads to the *A. nidulans*
397 (FGSC A4) genome. Reads with any of the following flags were removed: unmapped,
398 secondary alignment, or supplementary mapped read. Reads with a mapping quality (MAPQ)
399 score of 0 were also removed. Duplicate reads were removed and samples were pooled using
400 version 1.3 of the SAMtools software (68). Version 2.1.1.20160309 of the MACS2 software
401 (69) with the settings -g 2.93e7 –s 101 --nomodel --extsize was used to call peaks. Extension
402 sizes were calculated using SPP (70, 71). Peaks that exhibited a fold-change greater than 2 and
403 a q-value less than 0.001 were used in further analyses. Peak lists were combined from each of
404 the ChIP experiments. The ChIP-seq data is available from the NCBI Gene Expression
405 Omnibus database (GSE114141).

406 Motif discovery analyses

407 To discover the WetA-Response Element (WRE), 100 bp of sequence surrounding the summits
408 of the 157 combined peaks were pulled from the *A. nidulans* genome using the bedtools
409 software, version 2.26.0 (72) and submitted to the MEME-ChIP software, version 4.12.0 (41).
410 MEME was instructed to search for 10 motifs, 5-21 bp in length; all other settings were left at
411 default. Instances of the WRE were identified in the upstream regions (1.5 kb upstream of the
412 translation start) of all genes in the three *Aspergillus* genomes using the FIMO software (42)
413 with a p-value cutoff of 5e-5.

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628
Figure Legends

629 **Fig 1 The central regulatory pathway of *Aspergillus* conidiation**

630 (A) A cartoon depiction of genetic interactions of the central regulators in *A. nidulans*
631 conidiogenesis. The central regulators cooperatively activate the conidiation-specific genes
632 responsible for the morphogenesis of conidiophores. (B) The predicted protein architectures
633 for the three conserved central regulators of conidiation in *A. nidulans*, *A. fumigatus*, and *A.*
634 *flavus*. The blue box and the red hexagon represents the C2H2 zinc finger domain and
635 TEA/ATTS domain in BrlA and AbaA, respectively, and were identified in a blastP (version
636 2.6.0) search (35). The red circle represents a putative transcription activation domain (TAD),
637 which was predicted by 9aaTAD using the "Less stringent Pattern" setting (31). The blue
638 diamond represents the nuclear localization signal (NLS) predicted by NLStradamus using the
639 4 state HMM static model (32). The orange rectangle represents the ESC1/WetA-related
640 domain (PTHR22934) predicted by the PANTHER classification system (36).

641 **Fig 2 Overview of the WetA-regulated-orthologs in *A. nidulans*, *A. fumigatus*, and *A.***

642 ***flavus***

643 The 6,466 genes belonging to an orthogroup that possessed at least one member from *A.*
644 *nidulans*, *A. fumigatus*, and *A. flavus* are represented by the black arcs next to their respective
645 species labels. Gray, orthologs whose expression did not change between Δ*wetA* and WT
646 conidia. Green, orthologs that were differentially expressed in only one species. Blue, genes
647 that showed the same differential expression pattern in two out of the three species. Red, genes
648 that showed the same differential expression pattern in all three species. Orange, genes that
649 showed a divergent differential pattern in two or more species. Lines connect expressed genes
650 from the same orthogroup.

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651 **Fig 3 WetA-mediated regulation of asexual development in the three *Aspergillus* species**

652 Schematic diagram of the WetA-mediated regulatory model of conidiation. Genes with
653 increased, decreased, and unaffected mRNA levels in the Δ*wetA* conidia are labeled with red
654 (WetA-inhibited), blue (WetA-activated), and grey (not affected by WetA) circles and the
655 WetA-regulatory effects in the Δ*AniwetA*, Δ*AfuwetA*, and Δ*AflwetA* conidia are listed under the
656 gene name from left to right, respectively. There are two orthologs of *fphA* in *A. fumigatus*, one
657 of which is WetA-inhibited and the other is not regulated by WetA.

658 **Fig 4 WetA-regulatory effects on trehalose, chitin, β-(1,3)-glucan, and α-(1,3)-glucan**
659 **metabolism in *Aspergillus* species**

660 **Fig 5 WetA-regulatory effects on DHN-melanin, pyomelanin, and hydrophobin**
661 **biosynthesis in *Aspergillus* species**

662 In summary, our RNA-seq analyses suggest that WetA exerts broad regulatory effects in
663 conidiation by controlling about half of the transcriptome in each of the three *Aspergillus*
664 species we tested. Even though WetA-mediated regulation results in similar phenotypes and
665 pathways being regulated across the three species, the nature of that regulation is different when
666 comparing individually regulated genes among the species. These results suggest that, although
667 the WetA-mediated GRNs have diverged during the evolution of *Aspergillus*, their regulatory
668 logic appears to have remained conserved.

669 **Fig 6 Overlap between DEGs and WRE-containing genes in three *Aspergillus* species.**

670 The percentages of genes differentially expressed in the Δ*wetA* conidia (DEG), the percentage
671 of genes that contain predicted WRE sequences in their upstream 1.5 kb regions (WRE), and
672 the DEGs with a WRE in their upstream 1.5 kb regions (DEG w/ WRE) are shown. The *A.*

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673 *nidulans*, *A. fumigatus*, and *A. flavus* genes are shown in light green, light blue, and light orange,
674 respectively.

675 **Fig 7 WRE occurrences in the upstream regions of *wetA* orthologs in representative**
676 **fungi**

677 WRE occurrences were identified in a series of regions located upstream of *wetA* orthologs.
678 Numbers to the left of the sequence indicate at what position relative to the translation start site
679 the sequence shown begins. The sequences shown are from 15 bp upstream of the WRE
680 occurrence that was identified by FIMO (42) with the lowest p-value, to 14 bp downstream of
681 the WRE occurrence. Bases are colored black if they are conserved in at least 60% of the
682 species. Green – Aspergillaceae. Orange – Trichocomaceae. Blue – Onygenales. Purple –
683 Sodariomycete. (RC) – Reverse Complement.

684 **Fig 8 Proposed model of the rewired WetA-mediated GRNs in *Aspergilli***

685 We propose that WetA-mediated GRNs have been rewired during the evolution of *Aspergillus*
686 species but that their regulatory logic (i.e., their regulation of chemical development, cellular
687 development, and other biological processes) remains conserved. Red circle: WetA. Blue circle:
688 Targets that are consistently up-/down-regulated by WetA. Yellow circle: Targets that are
689 divergently regulated by WetA. Grey circle: Targets that are not regulated by WetA.

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691 **Table 1 The roles of WetA in three *Aspergillus* species**

	Δ*AniwetA*	Δ*AfuwetA*	Δ*AflwetA*
Conidia			
Colorless and autolyzed conidia (8, 10–13, 20–22, 26, 30)	+	+	+
Reduced conidia size (8)	+	+	+
Disrupted conidial wall structure (8, 20, 21, 30, 37)	+	+	+
Disrupted C2 layer thickness (8, 30)	Thicker	Thicker	Thinner
Reduced viability & stress tolerance (8, 30)	+	+	+
Reduced trehalose amount M-Y Wu and J-H Yu unpublished data, (8, 30)	+	+	+
Increased β-(1,3)-glucan amount (30)	+	NA	+
Reduced light-dependent conidiation (30)	NA	NA	+
Disrupted conidiation time M-Y Wu and J-H Yu unpublished data, (8, 30)	-	Postponed	Advanced
Hyphae			
Reduced hyphal growth rate M-Y Wu and J-H Yu unpublished data, (8, 30)	+	+	+
Higher branching rate M-Y Wu and J-H Yu unpublished data, (8, 30)	+	+	+
Reduced aflatoxin production (30)	NA	NA	+

692 NA: not applicable

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694 **Table 2 Summary of DEGs in the three *Aspergillus* Δ*wetA* conidia**

	A. nidulans	*A. fumigatus*	*A. flavus*
Unaffected genes	5,246 (48%)	4,374 (43%)	7,730 (57%)
DEG	**5,742 (52%)**	**5,756 (57%)**	**5,755 (43%)**
Overexpressed in Δ*wetA*	3,107 (28%)	2,996 (30%)	2,899 (21%)
Underexpressed in Δ*wetA*	2,635 (24%)	2,758 (27%)	2,856 (21%)
Total	**10,988**	**10,130**	**13,485**

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697 **Table 3 WetA-mediated SMG regulation**

	A. nidulans	*A. fumigatus*	*A. flavus*
Total cluster number	67	33	74
Clusters with at least one WetA-regulated gene	64 (96%)	33 (100%)	68 (92%)
Clusters not regulated by WetA	3 (4%) Cluster 41 Cluster 56 Cluster 63	0 (0%)	6 (8%) Cluster 2 Cluster 5 Cluster 14 Cluster 19 Cluster 38 Cluster 68
Clusters where every gene was regulated by WetA	5 (7%) Emericellamide Terriquinone Cluster 26 Cluster 37 Cluster 60	8 (24%) Ferricrocin DHN Melanin Fumigaclavine Endocrocin Helvolic Acid Fumisoquin Fumiquinazolines Cluster 31	8 (11%) Cluster 23 Cluster 35 Cluster 41 Cluster 46 Cluster 48 Cluster 52 Cluster 54 Cluster 71
Whole cluster is upregulated in Δ*wetA* conidia	2 (3%) Emericellamide Terriquinone Cluster 26	6 (18%) Ferricrocin DHN Melanin Endocrocin Helvolic Acid Fumisoquin Fumiquinazolines	1 (1%) Cluster 71
Whole cluster is downregulated in Δ*wetA* conidia	0 (0%)	1 (3%) Cluster 31	2 (3%) Cluster 23 Cluster 52

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700 **Table 4 WetA targeted DEGs in Δ*AniwetA* conidia**

G-protein pathway	*gprC, nopA*
Conidial integrity	*tppC*, *ccg-9*, *tpsC*, *treA*, *chsA*, *chiA*, *btgD*, *eglC*, *engA*, *btgC*, *eng7*, *bglA*, *ags2*, *agnD*, *agnC*, *hmgX*, *hppD*, *maiA*, *dewA,* AN0499, AN1069, AN1837
Kinase	*rio2*, *aromA*, *nimO*, *isr1*, *teaR*, *pho80*, *ffkA*, *panK*, *nimX*, AN3619, AN8213, AN8843, AN10188, AN10551
TF	*fcr1*, *aflR*, *dbaA*, *cpcA*, *vosA*, *mdpE*, *wetA*, *zapA*, AN6295, AN1217, AN0817, AN3502, AN3769, AN0094, AN4773, AN6790, AN8111, AN8355, AN8949, AN11169, AN0388, AN10550
SMG backbones	*apdA, inpB*, AN0016, AN1242, AN9129
Asexual development	*ams1, chsB, cnaB, cpcB, dewA, gprC, odeA*, *tpsC, velB, wetA, wsc1*

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703 **Table 5 Conserved DEGs with a WRE in *Aspergilli***

	A. nidulans ID	***A. flavus ID***	***A. fumigatus ID***
Up-regulated in Δ*wetA* conidia	AN1156	AFLA_068310	Afu1g11450
	AN10598	AFLA_101220	Afu3g07020
	AN6088	AFLA_045760	Afu2g09282
	AN4836	AFLA_102180	Afu3g07290
	AN3752	AFLA_073850	Afu7g04580
Down-regulated in Δ*wetA* conidia	AN4464	AFLA_112150	Afu4g07690
	AN1524	AFLA_078640	Afu8g05330
	AN5715	AFLA_127800	Afu1g06770
	AN10265	AFLA_014960	Afu2g15910
	AN10551	AFLA_112710	Afu4g07140
	AN5215	AFLA_087840	Afu6g07490
	AN8763	AFLA_131750	Afu6g02960
	AN9037	AFLA_023560	Afu3g15190
	AN4716	AFLA_091920	Afu5g08580
	AN1937	AFLA_052030	Afu4g13230

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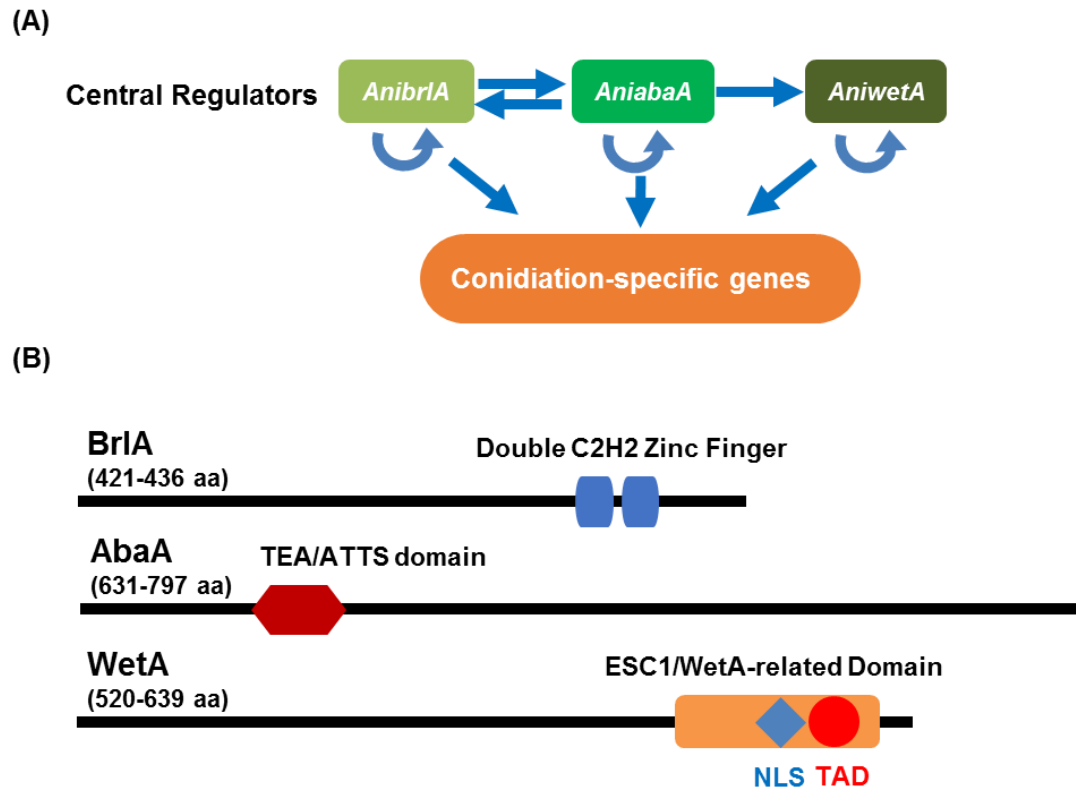

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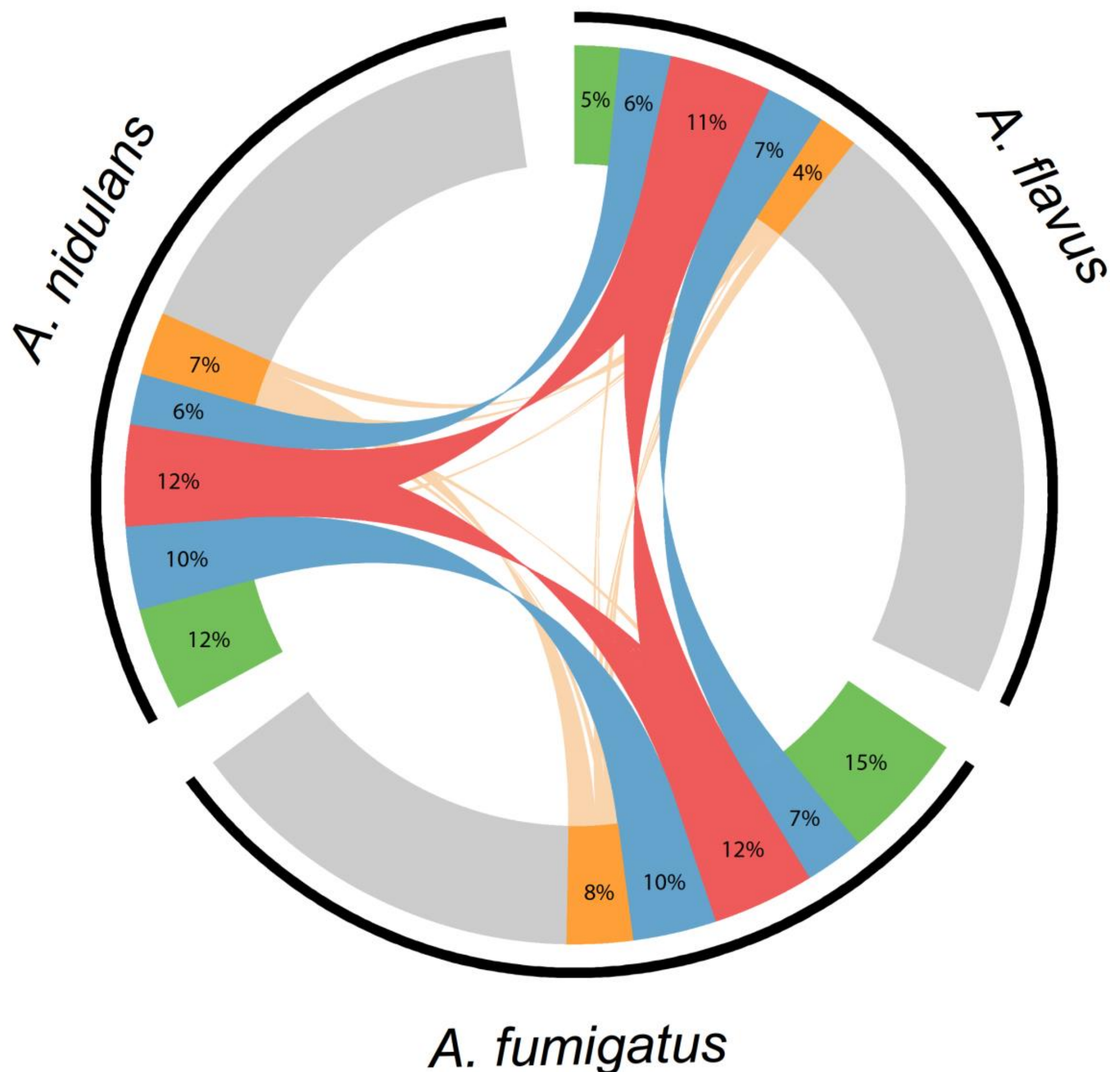

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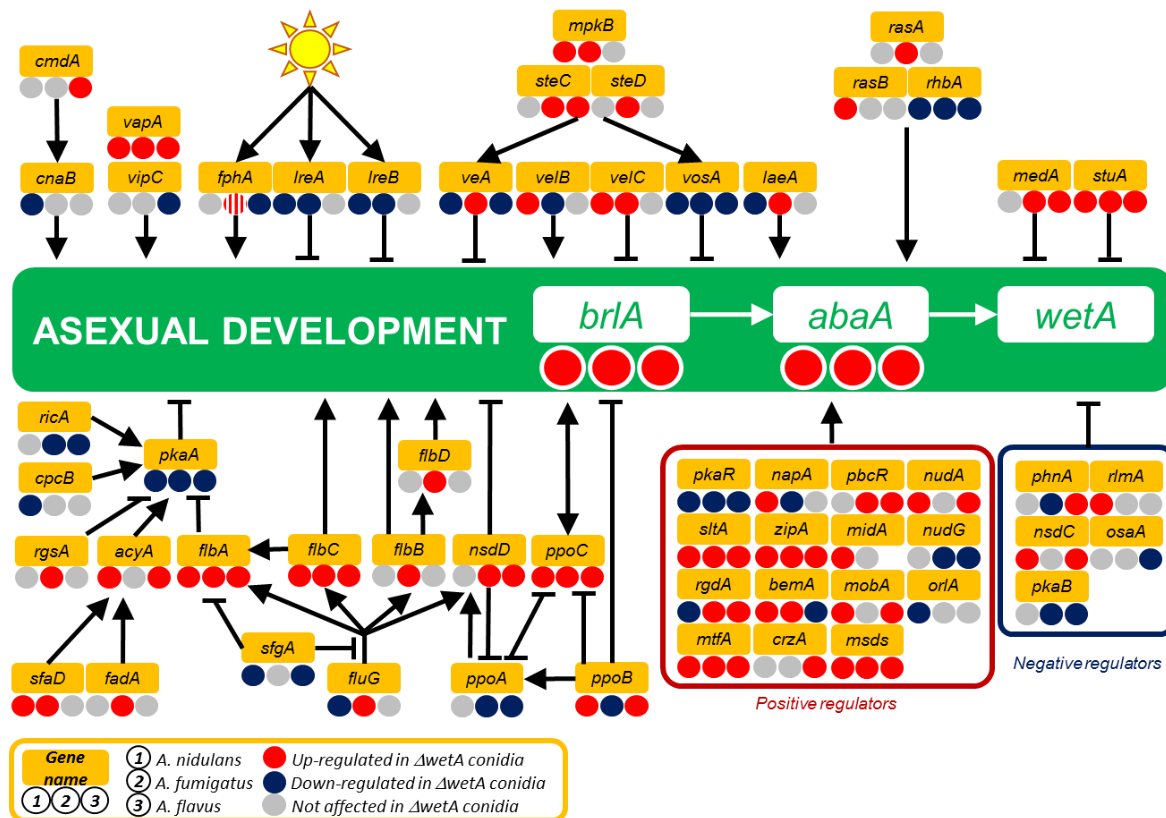

Fig 3 WetA-mediated regulation of asexual development in the three *Aspergillus* species

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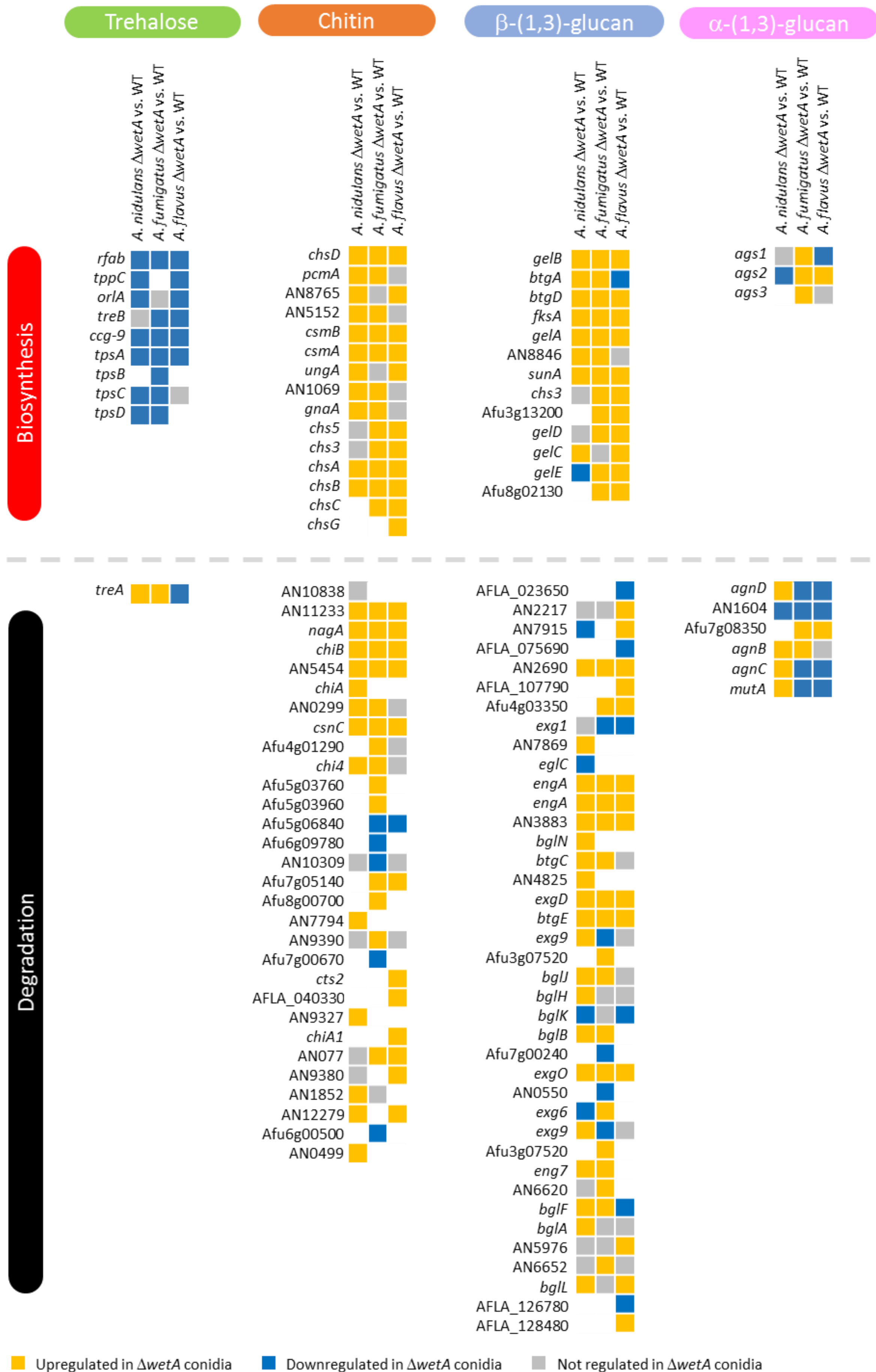

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metabolism in *Aspergillus* species

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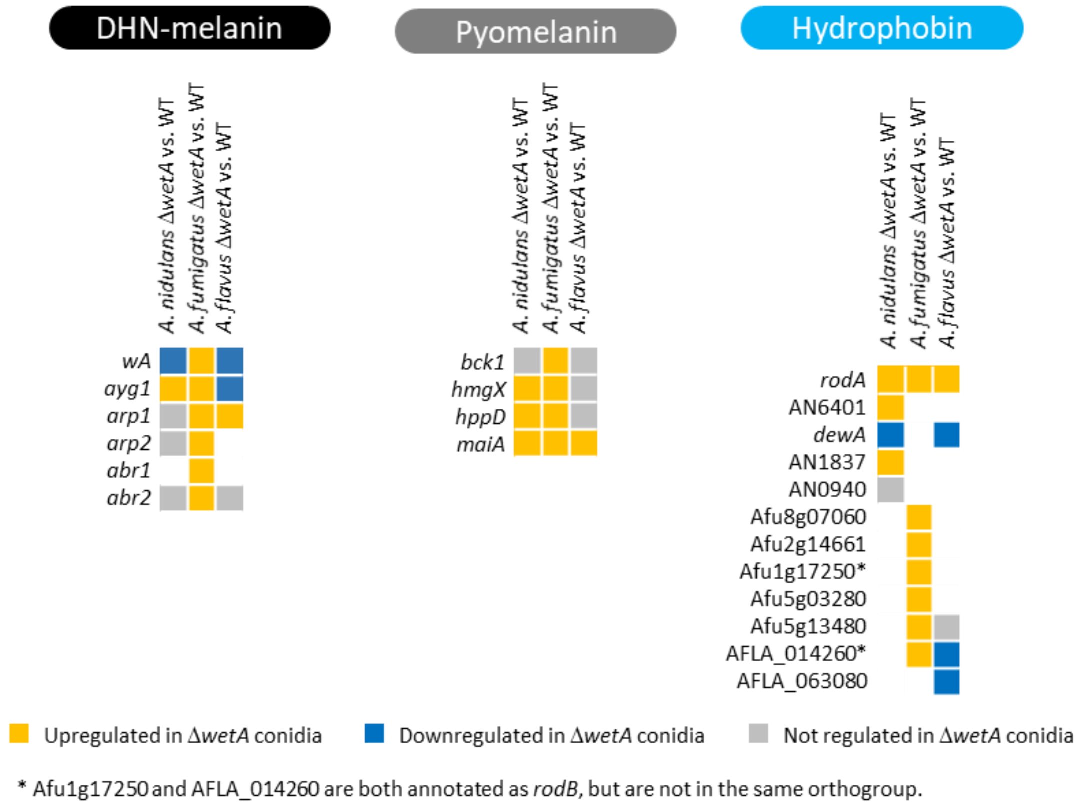

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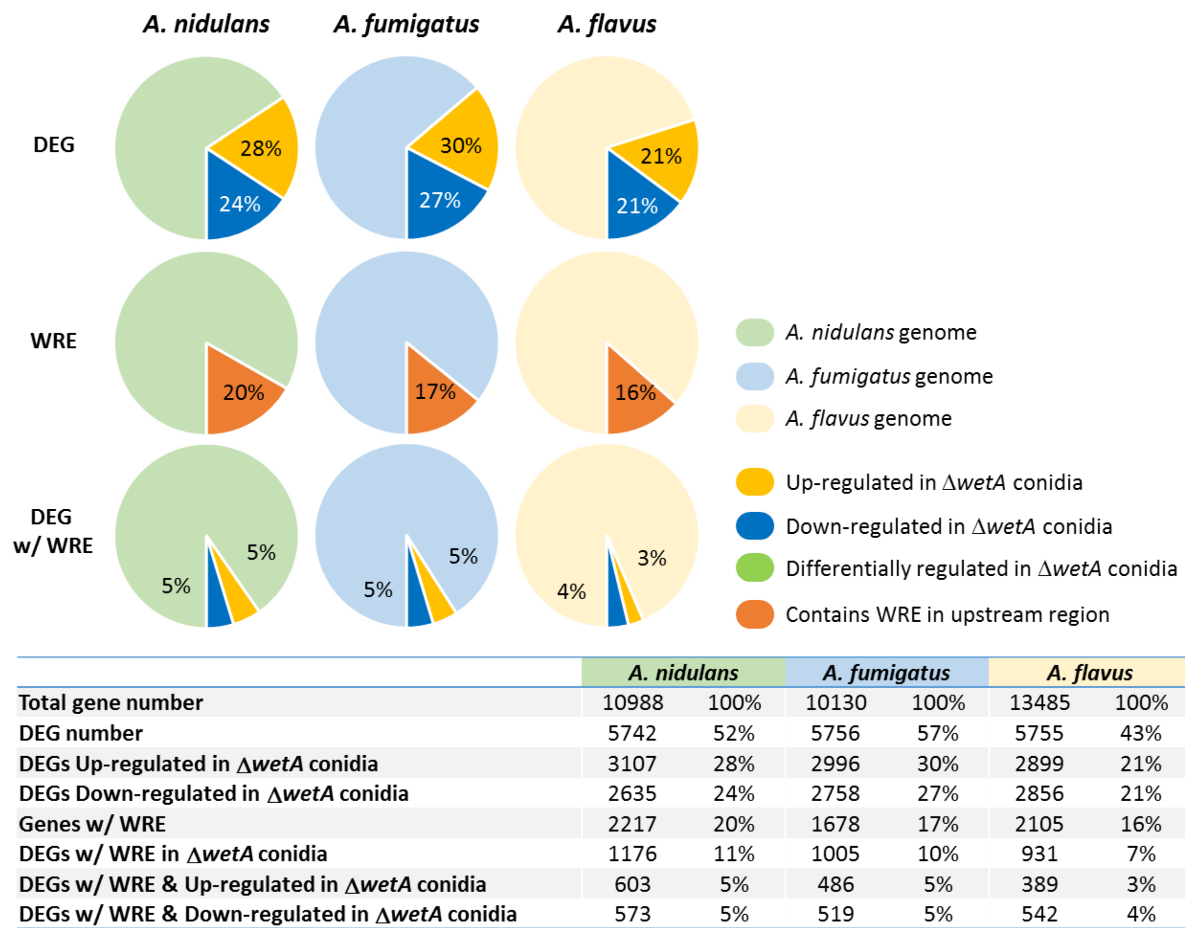

	A. nidulans		*A. fumigatus*		*A. flavus*	
Total gene number	10988	100%	10130	100%	13485	100%
DEG number	5742	52%	5756	57%	5755	43%
DEGs Up-regulated in Δ*wetA* conidia	3107	28%	2996	30%	2899	21%
DEGs Down-regulated in Δ*wetA* conidia	2635	24%	2758	27%	2856	21%
Genes w/ WRE	2217	20%	1678	17%	2105	16%
DEGs w/ WRE in Δ*wetA* conidia	1176	11%	1005	10%	931	7%
DEGs w/ WRE & Up-regulated in Δ*wetA* conidia	603	5%	486	5%	389	3%
DEGs w/ WRE & Down-regulated in Δ*wetA* conidia	573	5%	519	5%	542	4%

Fig 6 Overlap between DEGs and WRE-containing genes in three *Aspergillus* species.

The percentages of genes differentially expressed in the Δ*wetA* conidia (DEG), the percentage of genes that contain predicted WRE sequences in their upstream 1.5 kb regions (WRE), and the DEGs with a WRE in their upstream 1.5 kb regions (DEG w/ WRE) are shown. The *A. nidulans*, *A. fumigatus*, and *A. flavus* genes are shown in light green, light blue, and light orange, respectively.

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Species	Position	Sequence
A. niger	-48	CTACTCTGGTCGTCGCCGCAAACGGAAAGCGGCACCCCGT
A. nidulans	-56	CCTCTTCCATCATCGCCGCAAACGGAAAGGTGATCACCCG
A. flavus	-44	TTGGAACCACCATCGCCGCAAACGGAAATAGAATATCCGA
A. oryzae	-44	TTGGAACCACCATCGCCGCAAACGGAAATAGAATATCCGA
A. terreus (RC)	-41	TCGCTCTCTTTGTCTCCGCAAACGGAAAGCATCTGGCAAC
A. fumigatus	-44	TTCTCAGCTCTTTTGCCGCAAACGGAAAGACAGAGCAGCC
A. fischeri	-101	CTTCTCAGCTCTTTGCCGCAAACGGAAAGACAGAGCAGCC
A. clavatus	-44	GCTCTCACATCATTGCCGCAAACGGAAAGAGGACGGCAGC
P. chrysogenum	-33	ACTTCTATATTGTCGCCGCAAACGGAAAACAAGATGTTCG
A. zonatus	-42	ATTAGCCGCGGACGGCCGCAAACGGAAAGGAAAGCCATCA
T. marneffei	-51	GACATTTTCCTCTAACCAAAAGCGGAAAACACTGCCCACC
T. stipitatus	-1,521	ATCCGAGGGGCTCCCCTGCAAGCATTTCATGACCCAATTG
C. immitis (RC)	-324	AGAAGGAAACAGCCTCGAAACCAGCAGCGGACTCTTAACA
P. brasiliensis	-1,224	TAAAACGCCAGATGACCCAAACAGTCTCCACAGAGCAGAC
H. capsulatum (RC)	-210	ACAGGGTTTTTCCCCCGCAAATGGCACCTACAGGCAATGA
F. graminearum (RC)	-1,380	TTAGGGACGGGAGCTTGCATACAGCGCATGCAACATCCCT

Fig 7 WRE occurrences in the upstream regions of *wetA* orthologs in representative fungi

WRE occurrences were identified in a series of regions located upstream of *wetA* orthologs. Numbers to the left of the sequence indicate at what position relative to the translation start site the sequence shown begins. The sequences shown are from 15 bp upstream of the WRE occurrence that was identified by FIMO (42) with the lowest p-value, to 14 bp downstream of the WRE occurrence. Bases are colored black if they are conserved in at least 60% of the species. Green – Aspergillaceae. Orange – Trichocomaceae. Blue – Onygenales. Purple – Sodariomycete. (RC) – Reverse Complement.

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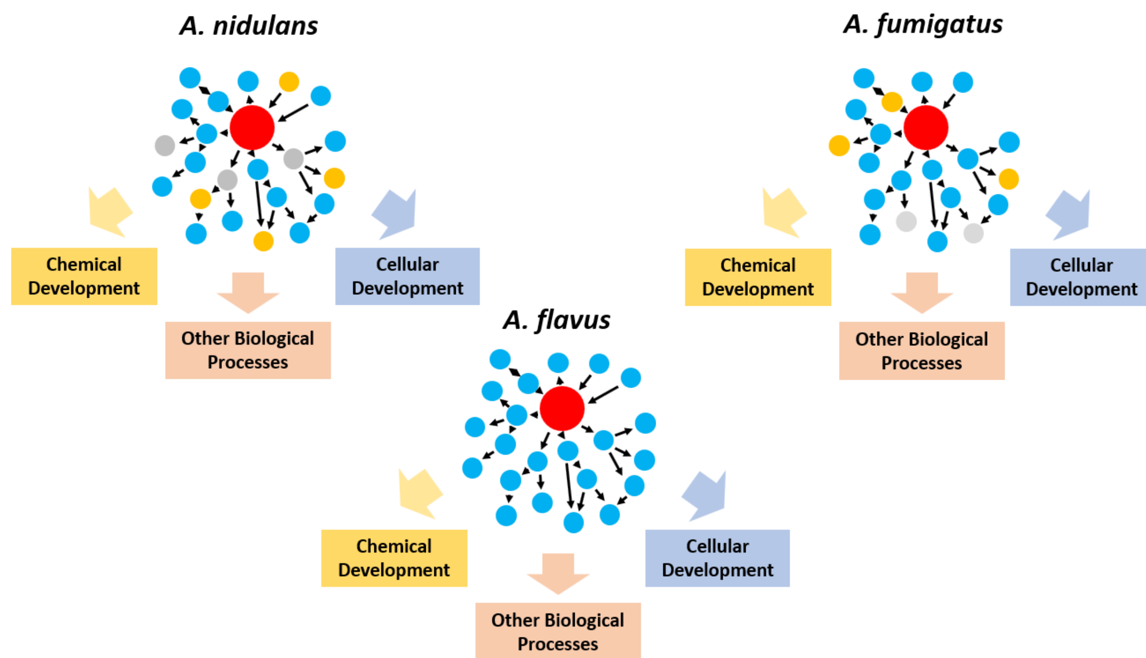

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