Distinct function of Mediator subunits in fungal development, stress response and secondary metabolism in fungal pathogen *Fusarium verticillioides*

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ABSTRACT

Mediator is a conserved, nucleus-localized, multi-subunit complex highly conserved across eukaryotes. It interacts with RNA polymerase II transcription machinery as well as various transcription factors to regulate gene expression. However, systematic characterization of Mediator complex has not be thoroughly performed in filamentous fungi. In current study, our aim was to investigate key biological functions of Mediator subunits in a mycotoxigentic plant pathogen *Fusarium verticillioides*. While we recognized some level of divergence in subunits constituent, overall structure remained conserved between *Saccharomyces cerevisiae* and *F. verticillioides*. We were able to generate 11 Mediator subunit deletion mutants and characterize serveal important phenotypes associated with fungal development, environmental stress, nutrient utilization, virulence as well as secondary metabolism. While each Mediator subunit deletion mutant showed deficiencies in at least three of the phenotypes tested, it is important to note that different modules or subunits showed unique regulatory role in different cellular processes. Significantly, the deletion of *FvMed1* led to increased FB_1 production, and we were able to confirm that FvMed1 is transported from the nucleus to the cytoplasm under fumonisin-producing conditions. Taken together, our study characterized the different biological functions of Mediator subunits in plant pathogen *F. verticillioides* and the possibility of select subunits unique cytoplasmic function independent of the core Mediator.

IMPORTANCE (150 words)

Mediator is a highly conserved component of RNA polymerase II pre-initiation complex (PIC) in eukaryotes. Studies in yeast have demonstrated the important roles of Mediator subunits, but we lack deeper understanding in filamentous fungi. Here, we selected *Fusarium verticillioides*,

a major fungal pathogen of maize worldwide, to study transcriptional profile followed by mutational analyses of all Mediator genes. Each Mediator subunit deletion mutant showed some shared deficiencies but it is also important that different modules or subunits showed unique regulatory role in different cellular processes. Significantly, *FvMed1* deletion mutant showed elevated levels of FB_1 production, and we were able to confirm that FvMed1 translocates from the nucleus to cytoplasmic organelles under fumonisin-producing conditions. This is a first study demonstrating that a Mediator subunit regulate cellular functions while not directly associating with PIC in filamentous fungi.

KEYWORDS *Fusarium verticillioides*, Mediator, fungal development, fumonisin B1, cytoplasmic translocation

58 **INTRODUCTION**

59

60 Cellular growth, differentiation and development is a complex and highly regulated
61 process that relies on differentially and methodically coordinated expression of numerous
62 genes in fungi. The coordinated expression, including protein-coding genes and various non-
63 coding RNAs, is accomplished by the RNA polymerase II (Pol II) complex. Notably, Pol II
64 holoenzyme does not carry out transcription alone but performs function as a macromolecular
65 structure known as the pre-initiation complex (PIC), which consist of Pol II, Mediator, TFIIA,
66 TFIIB, TFIID, TFIIE, TFIIF and TFIIH (1, 2). Among these components, Mediator serves as
67 the central scaffold of PIC and is known as the main regulator of Pol II activity. However,
68 owing to its structural complexity and expansive size, our understanding of the biochemical
69 and genetic functions of Mediator in filamentous fungi is rudimentary.

70 Mediator complex was first discovered in *Saccharomyces cerevisiae* (3), and their
71 organization and structure seem to be evolutionarily conserved among eukaryotes. Mediator
72 complex contains 30 (human) or 25 (yeast) subunits which can be classified into four functional
73 modules: Head, Middle, Tail and Kinase. Significantly, the subunit 14 (Med14) serves as a
74 scaffold and holds the Head, Middle and Tail modules together as a complex, which is defined
75 as the 'core Mediator' (4-6). Published studies indicated that the 'core Mediator' serves as a
76 bridge between DNA-binding transcription factors (TFs), Pol II as well as other components
77 in the transcription machinery, which collectively plays a crucial role in transcriptional
78 activation (7-10). The Kinase module, which consists of four subunits Med12, Med13, Cdk8
79 (cyclin-dependent kinase 8) and Ssn (cyclin C), could associate reversibly with the 'core
80 Mediator', thereby regulating Pol II activity and transcription (11-13).

81 While the functions of Mediator appear highly conserved among eukaryotes, analyses of
82 Mediator mutants in various cells and tissues have shown that each subunit of the Mediator

83 complex regulates distinct subsets of genes during various developmental processes. For
84 example, Ssn3 and Ssn8 are required for glucose metabolism and biofilm formation in *C.*
85 *albicans* (14). In *S. cerevisiae*, Med5 is involved in expression of genes associated with nuclear
86 and mitochondrial oxidative phosphorylation (OXPHOS) (15). It is interesting to note that
87 while Med7 is essential for yeast viability, its ortholog in *C. albicans* is not essential and is
88 involved crucial developmental control (16). Med31 is a maternal-associated gene in
89 *Drosophila* (17), whereas no defects have been detected during early embryonic growth in
90 mouse mutants (18). These reports support gene- and species-specific functions within
91 Mediator even though it has many conserved roles across eukaryotes. Although the biological
92 and physiological functions have been extensively studied in a select number of model
93 organisms, little is known about Mediator functions filamentous fungi.

94 *Fusarium verticillioides* is the fungal pathogen responsible for devastating stalk rot, ear
95 rot and seedling blight diseases in maize, which can lead to severe yield losses worldwide (19,
96 20). Importantly, *F. verticillioides* produces a variety of mycotoxins during maize infestation
97 in the field or in storage. Among these mycotoxins, fumonisins B_1 (FB_1) poses a particular
98 concern, with links to birth defects and cancer in humans as well as acute and chronic toxicosis
99 in animals (21-24). Therefore, the concentration of fumonisins in maize and food product is
100 regulated by governmental agencies worldwide (25). One of the key challenges in developing
101 effective mitigation strategies for fumonisins in food and feeds is our limited understanding of
102 how fumonisin biosynthesis is regulated in *F. verticillioides*. Published studies showed that
103 Kinase module subunits, namely cyclin C and cyclin C-dependent kinase, play a crucial role in
104 mycotoxin biosynthesis in *Fusarium* species (26-29), but the role of other Mediator subunits is
105 uncharacterized to date.

106 In order to investigate the functions of Mediator subunits, we implemented a systematic
107 study of all Mediator subunit genes in *F. verticillioides* by transcriptional analyses and gene

knockout strategy. In our study, 23 Mediator subunit genes were found in *F. verticillioides*, and the functional roles of 11 Mediator subunits were investigated in this study. Our results provide further evidence that different modules and subunits play a unique role in transcriptionl regulation of specific subsets of genes in *F. verticillioides*. Significantly, the translocation of a Mediator subunit from the nucleus to the cytoplasm was observed in fumonisin-producing conditions and non-producing conditions, suggesting that Mediator subunits may perform yet-to-be determined functions in the cytoplasm independent of Pol II.

RESULTS

Mediator subunit composition in *S. cerevisiae* and F. *verticillioides*. In all eukaryotes, including *S. cerevisiae*, Mediator subunits have been categorized into four functional modules: Head, Middle, Tail and Kinase (4, 15, 30, 31). We used *S. cerevisiae* subunits as queries and screened *F. verticillioides* predicted proteins for putative orthologs. Table 1 describes how Mediator subunits in two fungi can be comparatively organized. Although the Mediator subunit organization is conserved between *F. verticillioides* and *S. cerevisiae*, we did learn that *S. cerevisiae* subunits Med20, Med10, Med19 and Med2 were not identifiable in *F. verticillioides*. Also, we found that *F. verticillioides* harbors 15 copies of *FvMED15* gene while the gene encoding *MED15* resides as a single copy locus in *S. cerevisiae*. These genes are named as the telomere-associated genes (*TLOs*) and may suggest the evolutional divergence of Med15 between *F. verticillioides* and *S. cerevisiae*.

Transcription profile of Mediator genes. To determine whether certain Mediator subunits are transcriptionally associated with FB_1 biosynthesis regulation, we analyzed their expression levels in RNA-Seq libraries generated from *F. verticillioides* cultured in maize

133 inbred line B73 kernels (moderate FB_1 production) versus hybrid 33K44 kernels (high FB_1
134 production). We followed the relative transcription levels of Mediator subunits in 2, 4, 6, 8 days
135 post inoculation (dpi) samples (32). Overall, the expression of each Mediator gene increased
136 through the time course and highest level at 8 dpi, especially noticeable for *FvMed14*
137 (*FVEG_01411*), *FvMed12* (*FVEG_02030*), *FvMed31* (*FVEG_02237*), *FvMed7*
138 (*FVEG_04284*), *FvMed21* (*FVEG_06141*) and *FvMed3* (*FVEG_07603*) (Fig. S1). However, it
139 is important to note that the expression of all Mediator genes showed no statistical difference
140 between two RNA-Seq libraries as shown in Fig. 1 where we highlighted key Head
141 (*FVEG_03198*), Middle (*FVEG_02872*, *FVEG_02237*), Tail (*FVEG_07603*) and Kinase
142 (*FVEG_11159*, *FVEG_11306*) subunits. Notably, transcription profile of cyclin C *FvFcc1*
143 (*FVEG_11306*) and Cyclin-dependent kinase *FvFck1* (*FVEG_11159*) were in agreement with
144 earlier published studies (26, 27). These outcomes led us to predict Mediator subunits do not
145 regulate FB_1 biosynthesis transcriptionally but rather by forming complex with core Mediator
146 or PIC in *F. verticillioides*.
147
148 **Construction of Mediator complex deletion mutants.** In an effort to test the biological
149 functions of Mediator subunits in *F. verticillioides*, we generated knockout mutation of each
150 gene via split-marker approach (Fig. S2). All transformants were analyzed by PCR and qPCR
151 assays with corresponding primers to verify null mutation (Table S1). For 11 Mediator subunits,
152 at least two knockout mutants with similar phenotypes were obtained (Table S2). As for the
153 other 10 putative Mediator subunits (Table 1), we were not able to retrieve viable gene-deletion
154 mutants after screening more than 200 transformants from at least four independent
155 transformation experiments. This outcome led us to conclude that the null mutation of these
156 subunits is deemed lethal in *F. verticillioides*. Significantly, these subunits are also recognized
157 as essential genes for *S. cerevisiae* viability (33).

158

159 **Mediator complex play different roles in fungal development processes.** When we
160 tested vegetative growth in mutants, strains with Head module gene deletion (ΔFvMed18) and
161 Kinase module gene deletion (ΔFvMed12, ΔFvMed13, ΔFvFcc1 and ΔFvFck1) exhibited
162 significantly reduced growth rate compared to the wild-type progenitor on V8 agar (Fig. 2A
163 and Table 2). We also compared colony morphology and growth rate of 11 Mediator mutants
164 on 0.2×PDA and Myro agar plates (Table 2 and Fig. S3). Among these 11 mutants, 6 showed
165 reduced conidiation by > 30% compared to the wild-type progenitor. Surprisingly, ΔFvMed9
166 and ΔFvMed5 exhibited significantly increased conidiation (Fig. 2B and Table 2). We also
167 measured conidia germination rate in all Mediator subunit mutants and found that 9 mutants
168 exhibited dramatic defect in conidia germination (Fig. 2C and Table 2). Interestingly,
169 ΔFvMed18 conidia fail to germinate even after a 6.5-hour incubation on water agar (WA) plate.
170 In addition, we observed that mycelial surface of some Mediator mutants exhibited different
171 level of hydrophobicity compared with the wild-type progenitor. As showed in Fig. 2D, a 5-μL
172 water droplet could persist for more than 1 h on WT and the majority of Mediator mutants.
173 However, ΔFvMed18, ΔFvMed5, ΔFvMed16 and ΔFvMed12 exhibited different degree of
174 reduced hydrophobicity (Fig. 2D). Taken together, these results suggested that Mediator
175 complex is critical for fungal development processes in *F. verticillioides*, but each subunit
176 likely have different principal functions.

177

178 **Involvement of Mediator complex in plant virulence.** To test the role Mediator subunits
179 play in *F. verticillioides* virulence, we used maize seedling infection assay as previously
180 described (34). After a 10-day incubation at room temperature, all mutants exhibited reduced
181 virulence when compared to the wild-type progenitor. In particular, seedling rot symptoms
182 caused by ΔFvMed18, ΔFvMed12, ΔFvMed13, ΔFvFcc1 and ΔFvFck1 were restricted around

183 the inoculation sites (Fig. 3), suggesting that Head and Kinase modules in the Mediator
184 complex play more crucial roles compared to Middle and Tail modules in fungal virulence.
185
186 **Mediators show different levels of stress and fungicide sensitivity in *F. verticillioides*.**
187 We asked whether Mediator complex subunits play a role in regulating responses to
188 environmental stress agents and subsequently tested all mutants against cell wall-damaging
189 agent, oxidative and osmotic stresses, and fungicides. Our results showed that only 5 mutants
190 (ΔFvMed18, ΔFvMed12, ΔFvMed13, ΔFvFcc1 and ΔFvFck1) exhibited slightly decreased
191 sensitivity to cell wall-damaging agent Congo Red (Fig. 4 and Fig. S4). Overall, no sensitivity
192 differences were observed among 11 Mediator mutants to osmotic stress in comparison with
193 the wild-type progenitor in our experiments (data not shown).
194 In human pathogens, Mediator has been shown to play an important role in azole fungicide
195 resistance (35-39). Intriguingly, when we assayed the sensitivity of *F. verticillioides* Mediator
196 mutants, deletion of Head and Kinase module subunits (ΔFvMed18, ΔFvMed12, ΔFvMed13,
197 ΔFvFcc1 and ΔFvFck1) caused significant increase in ketoconazole and fluconazole resistance
198 compared to the wild-type progenitor. However, no sensitivity difference was detected among
199 other mutants (Table S3). These results demonstrated that Head and Kinase modules are
200 negatively associated with azole resistance in *F. verticillioides*.
201
202 **Mediators are involved in carbon and fatty acid utilization.** In earlier published studies,
203 two homologs of Kinase module, CycC and Cdk8, were suggested as a link between ambient
204 environment sensing and transcriptional regulation in *F. verticillioides* (26, 27). To further
205 understand the roles of the Mediators in carbon source utilization, each mutant was cultured on
206 minimal medium (MM) amended with glucose, fructose, lactose, starch or acetate as a sole
207 carbon nutrient. As results showed, the vegetative growth of wild-type strain and all mutants

208 were similar on MM amended with fructose, lactose and starch when compared to glucose (Fig.
209 S5A). Interestingly, most of the mutants in 'core Mediator' exhibited reduced growth rate with
210 acetate (except for ΔFvMed31 and ΔFvMed5). In contrast, mutants in Kinase module showed
211 increased relative growth rate on MM amended with acetate as compared with glucose (Fig. 5).
212 These results indicated that core Mediator plays contrasting role when compared to Kinase
213 module in acetate utilization in *F. verticillioides*.

214 We further tested carbon nutrient utilization using fatty acids. We observed that ΔFvMed18
215 exhibit reduced relative growth rate with butyrate sodium but increased relative growth rate
216 with palmitic acid and corn oil compared with glucose (Fig. S5B). However, mutants in Kinase
217 module showed increased relative growth rate with all fatty acids (excluding Med13 with
218 butyrate sodium) tested in our study (Fig. S5B). These results suggested that subunits in Head
219 and Kinase module are involved in fatty acids utilization, but their functions are discrepant.

220

221 **Mediator complexes play distinct roles in secondary metabolism.** Fumonisin B_1 (FB_1)
222 is the most harmful secondary metabolite produced by *F. verticillioides* (40). We assayed the
223 FB_1 production in Mediator mutants when they were incubated for 7 days on autoclaved corn
224 kernels (26). After normalization with fungal ergosterol level in each sample set, we were able
225 to confirm that FB_1 production was significantly reduced in 10 Mediator mutants in comparison
226 with the wild-type progenitor. One unexpected outcome was that ΔFvMed1 exhibited
227 drastically higher FB_1 biosynthesis than the wild type (Fig. 6A and Table S4). Consistently, the
228 expression level of *PKS11* (*FUM1*) was significantly up-regulated in ΔFvMed1 (Table S5).
229 These results led us to hypothesize that FvMed1 functions as a negative regulator of FB_1
230 biosynthesis in *F. verticillioides* while majority of other Mediator subunits serve as the positive
231 regulator.

232 To test the scope of Mediator complex subunit in regulating secondary metabolism in *F.*

233 *verticillioides*, all mutants were cultured in YEPD (to promote vegetative growth) and Myro
234 (to promote secondary metabolite production) liquid media for 3 days. As results showed,
235 ΔFvMed3, ΔFvMed12, ΔFvMed13, ΔFvFcc1 and ΔFvFck1 exhibited higher levels of dark
236 pigmentation in comparisons with the wild-type progenitor (Fig. 6B). Furthermore, qRT-PCR
237 assays showed that the expression of gene associated with fusarubins biosynthesis, *PKS3*, is
238 significant up-regulated in ΔFvMed18 and ΔFvFcc1, while deletion of ΔFvMed1 significant
239 decrease *PKS3* expression (Table S5). Taken together, our results indicated that different
240 Mediator modules and subunits regulate specific subset of genes involved in secondary
241 metabolism in *F. verticillioides*.
242
243 **Subcellular localization analysis indicates functional variety of FvMed1.** It is
244 recognized that Mediator subunits are localized in cell nucleus and regulates gene transcription
245 processes as an indispensable component of PIC (29, 41, 42). However, our FB_1 assay results
246 indicated that FvMed1 plays a unique role unlike other subunits under FB_1 producing
247 conditions. When we analyzed *in silico* putative Med1-interacting protein in *S. cerevisiae*,
248 majority of the published and predicted interacting proteins were nuclear proteins, *e.g.*,
249 subunits of Mediator complexes and histone proteins. However, we learned that certain non-
250 nuclear proteins, e.g. cytoplasmic proteins, mitochondrial proteins and endoplasmic reticulum
251 proteins, were identified as Med1-interacting proteins in *S. cerevisiae*. This raised a question
252 whether Med1 can translocate outside the nucleus in response to certain ambient signals or
253 conditions. In order to test this idea, we generated a FvMed1-GFP fusion construct with its
254 native promoter and transformed it into the wild-type protoplast. All transformants were
255 verified by PCR assay and observed under fluorescence microscope. Under FB_1 non-producing
256 condition, we observed weak GFP signals likely in the nucleus but clearly not in vacuoles in
257 each septate mycelial cell (Fig. 7A). Under FB_1-producing condition, although Med1-GFP

258 could be found in the nucleus, we also observed multiple punctate and rounded structures in
259 the cytoplasm (Fig. 7B - D). Significantly, we did not observe GFP signals localizing to
260 vacuoles as well as a large number of lipid bodies, which are commonly observed when F.
261 verticillioides is grown in secondary metabolite-inducing media. Also, that fact that the GFP
262 signal was not diffused throughout the cytoplasm suggests that FvMed1 translocates to a
263 defined cellular organelle when it moves out of the nucleus. When assayed for *FvMed1*
264 expression in YEPD and Myro cultures, the transcript level of *FvMed1* showed no difference
265 between two media (data not shown), suggesting that *FvMed1* may be constitutively expressed
266 in different growth conditions. These results showed that FvMed1 proteins were transported to
267 the cytoplasm in response to FB_1 promoting signals.
268

269 **DISCUSSION**

270 Mediator is a conserved protein complex that performs indispensable cellular functions as
271 a regulator of gene transcription. Here, we identified 21 genes encoding putative *F.*
272 *verticillioides* Mediator homologs by using 25 *S. cerevisiae* Mediator subunits as queries.
273 Although the modular organization of Mediator complexes are predicted to be similar between
274 *S. cerevisiae* and *F. verticillioides*, the biological functions of certain subunits show significant
275 differences in *F. verticillioides*. For instance, the gene encoding *Med15* resides as a single copy
276 locus in *S. cerevisiae*, but the homolog in *F. verticillioides* seems to have expanded to 11 copies
277 of telomere-associated genes (*TLO*s). We are making this prediction base on previous report
278 that showed *S. cerevisiae Med2* being replaced by a subset of *TLO*s in *C. albicans* (42).
279 Furthermore, when we studied 11 viable gene knockout mutants, we observed some distinct
280 phenotypic outcomes in different mutants.

281 It is perceived that Mediator is important for fungal development processes, secondary
282 metabolism and plant infection. Intriguingly, we observed phenotypic variations when

283 comparing between modules and subunits, indicating that certain modules or subunits of
284 Mediator complex preferentially control the expression of different subset of target genes, thus
285 regulating specific developmental processes. Disruption of a Kinase module exhibited defects
286 in growth and conidia production in *F. graminearum* (28, 29). Another study showed that
287 *CaMed7* is required for hyphal development (16). In our study, 5 Mediator mutants displayed
288 significant defect in vegetative growth, including mutants in Head and Kinase modules. In
289 addition, while the conidia production in ΔFvMed18 was similar to the wild-type strain, the
290 conidial germination was conspicuously suppressed. Furthermore, we observed that conidia
291 produced in ΔFvMed9 and ΔFvMed5 were significantly increased compared to the wild-type
292 progenitor. These results indicated that each module or subunit of Mediator have unique
293 functions during fungal development processes in *F. verticillioides*.

294 In *Cryptococcus neoformans*, *Cdk8* and *Ssn8* deletion mutants showed increased
295 sensitivity to cell wall and oxidative stress (44). The authors provided further evidence that this
296 outcome was due to mitochondria morphology defect and the resulting transcriptional
297 regulation defects were associated with oxidation-reduction mechanism. In our study, we
298 observed that mutants in Head and Kinase modules exhibited reduced tolerance to cell wall
299 damage agent. However, no significant difference was found in wild-type strain and all
300 Mediator mutants in regard to oxidative stress agents (data not shown). These results suggested
301 that Mediator is involved in regulating environment stress responses, but there may be species-
302 specific functions against various stress agents. Elucidating the mechanism in which these
303 processes are disrupted in *F. verticillioides* may require further study.

304 Glucose is the most suitable carbon source for eukaryotes, especially fungi. However,
305 fungi can utilize a wide array of carbon sources, even the most complex carbohydrates, to
306 survive and thrive in the most challenging environmental conditions. Previous research showed
307 that certain subunits of Mediator complex are involved in glucose metabolism and carbon

308 source utilization (14, 44-46). Our study showed how each Mediator mutant can differ in its
309 ability to utilize different carbon sources. While most of the mutants in the core Mediator
310 showed defects in using acetate as sole carbon source, mutants in Kinase module exhibited
311 increased growth rate in MM amended with acetate when compared to glucose. Surprisingly,
312 these results do not agree with those observed in *C. neoformans* ΔCdk8 and ΔSsn8 mutants
313 (44), indicating that Mediator has species-specific functions in regulating genes involved in
314 glucose metabolism.

315 A number of studies have demonstrated the importance role of Mediator in virulence. In
316 *Candida glabrata*, the Med2 subunit was shown to control the Epa adhesins biosynthesis, with
317 the mutant displaying elevated adherence to epithelial cells (37). In *C. albicans*, Med13 and
318 Med31 subunits can activate genes associated with adhesion thereby stimulating fungal
319 infection (47). Here, we observed that all Mediator mutants in *F. verticillioides* exhibited
320 attenuated disease symptoms on maize seedling, despite each Mediator mutant showing
321 varying reduced virulence levels. Additionally, our results are not consistent with those
322 observed in *C. albicans*, further supporting the divergence of function within Mediator despite
323 their conserved roles across eukaryotes.

324 Our effort to effectively control fungal diseases in crop production faces persistent
325 difficulty due to the limitation in antifungal agent availability. More seriously, this difficulty is
326 exacerbated due to the emergence of resistant pathogen population in the field. Many studies
327 showed that Mediator plays an important role in azoles resistance by regulating transcriptional
328 level of efflux pump genes. In *C. glabrata*, the expression of efflux pump genes *CgCDR1* and
329 *CgCDR2* were significantly decreased in *CgMed15A* mutant, which resulted in increased
330 sensibility to azole fungicides (36, 48). In addition, deletion of *CgMed2* also resulted in the
331 reduction of *CgCDR1* expression and sensitivity to azoles (37). However, *CgMed1* deletion
332 mutant did not exhibit any changes to azole sensitivity and *CDR2* expression (48). In our study,

333 we tested the sensitivity of wild-type and all Mediator mutants against two azole fungicides
334 ketoconazole and fluconazole. We observed that mutants in Head and Kinase module exhibited
335 increased drug resistance compared to the wild-type and other mutant strains. However, qPCR
336 assays showed that the expression of efflux pump genes in these mutants showed no statistical
337 difference in comparison to the wild-type progenitor and other mutants. Intriguingly, recent
338 studies showed that the deletion of *Ssn3* or *Ssn8* could increase azole resistance, which is
339 associated with chromatin remodeling (49, 50). Thus, we hypothesize that the increased drug
340 resistance in *F. verticillioides* Mediator mutants share similar mechanism to that in *C. albicans*.

341 In our study, FB_1 biosynthesis was significantly reduced in majority of the Mediator mutant
342 except ΔFvMed1 and ΔFvMed9. Similar results were observed in *F. graminearum* (28, 29).
343 Surprisingly, we found that FB_1 biosynthesis in ΔFvMed1 was about 2-fold higher than wild-
344 type progenitor. We questioned whether FvMed1 plays different roles in other secondary
345 metabolism compared to other Mediator subunits, and analyzed the expression of all 15 *PKS*
346 genes in ΔFvMed1 under toxin-inducing conditions. We learned that the expression of *PKS11*
347 (*FUM1*) was significantly higher in ΔFvMed1. Additionally, we observed that the expression
348 of other *PKS* genes showed significantly difference among 4 Mediator mutants tested (Table
349 S5). These results implied that Mediator complex is critical for secondary metabolism in
350 phytopathogen, but each subunit played unique roles and regulated specific subsets of genes.

351 Although many reports focused on the functions of Mediator subunits in the nucleus and
352 as a component to RNA Pol II, an earlier study revealed their possible roles in the cytoplasm
353 (51). In lung cancer cells, Med12 was found in both the nucleus and the cytoplasm. When
354 localized in the cytoplasm, Med12 participated in TGF-beta signaling regulation via binding
355 with TGF-beta receptor 2, thereby affecting its glycosylation and cell membrane expression
356 (51). We hypothesize that FvMed1 can translocate to the cytoplasm under FB_1-producing
357 conditions and perhaps perform its unique function as a cytoplasmic protein. Our result raised

358 a possibility that FvMed1 can control yet-to-be determined cytoplasmic signaling pathways
359 associated with FB_1 biosynthesis. Although the potential cytoplasmic FvMed1-interacting
360 proteins remains unknown, this outcome provides us with motivation to expand our efforts to
361 gain novel insight into the cytoplasmic function of Mediator subunits in filamentous fungi.
362

363 **MATERIALS AND METHODS**

364

365 **Strains and culture assays.** *F. verticillioides* wild-type strain M3125 was used for the
366 construction of the derived mutants used in this study (34). All strains used in this study were
367 grown and evaluated at 28˚C on V8, 0.2×PDA and Myro agar plates for mycelial growth (34).
368 For conidia gemination assay, conidia (10^6) grown on V8 plates for 6 days were harvested and
369 subsequently cultivated in water agar plates for 6.5 h at 28˚C in dark. For stress response assays,
370 5-mm diameter mycelial plugs taken from a 6-day-old colony of wild-type and all mutant
371 strains grown on 0.2×PDA were inoculated on 0.2×PDA plate containing 0.05% Congo red
372 (w/v) or NaCl (1.2 M). Carbon source utilization assays were performed on minimal medium
373 (MM) supplemented with different carbon sources at concentrations of 1% (w/v). All
374 experiments were repeated three times independently.
375

376 **RNA-Seq data analysis.** RNA-seq data were obtained through sequencing platform,
377 Illumina HiSeq 2500, from Texas A&M AgriLife Genomics and Bioinformatics Services
378 (College Station, Texas). The platform processed fourteen independent samples composed of
379 seven biological replicates and seven technical replicates at four distinct time points (2 dpi, 4
380 dpi, 6 dpi, and 8 dpi). The sequencing data were generated as paired-end reads with 125-bp for
381 two different maize kernels (hybrid 33k44 vs. inbred B73), where *F. verticillioides* inoculated.
382 We first aligned and quantified the RNA-seq by TopHat2 (51) along with HTSeq (53).

383 Subsequently, we filtered scarcely expressed genes out and normalized the remaining quantity
384 by two steps, TPM (54) and TMM (55). Our dataset is publicly available at NCBI Gene
385 Expression Omnibus (Accession GSE146208).

386 For those selected genes, we analyzed whether their expression levels were
387 directly/indirectly associated with the virulence by comparing expression levels of 33K44 and
388 those of B73 at all the time points. *P value* for comparison was computed by the non-parametric
389 Van der Waerden (VDW) test while not having any assumptions for normality or equality of
390 variance between 33K44 and B73. The bar plots of their mean expression levels with *P values*
391 for the comparison are shown in Fig. 1 and Fig. S1.

392

393 **Strains construction.** All gene deletion mutants were constructed using our standard split-
394 marker gene knock out protocol as previously described (34). Hygromycin B
395 phosphotransferase gene (*HPH*) was split into two fragments *PH* (929 bp) and *HP* (765 bp)
396 and fused with left and right flanking sequence fragments of each targeted gene, respectively
397 (Fig. S2). As the result of this homologous recombination, the open reading frame (ORF) of
398 each gene was replaced with *HPH* gene. We confirmed targeted gene replacement by PCR,
399 and further tested the absence of gene transcription by qPCR. FvMed1-GFP fusion cassette
400 was constructed and transformed into *F. verticillioides* following our standard protocol
401 described previously (34). All transformants were verified by PCR and observed under an
402 Olympus BX51 microscope (Olympus America, Melville, NY, USA). A detailed description
403 of features used for imaging fluorescence with this microscope and the image processing has
404 been described previously (34).

405

406 **Sensitivity assay against ketoconazole and fluconazole.** To determine the sensitivity to
407 ketoconazole and fluconazole, a mycelial plug of all strains from a 6-day-old 0.2×PDA plate

408 was transferred to the center of 0.2×PDA plates amended with ketoconazole and fluconazole
409 at different concentrations. Three replicates for all concentrations were tested. After incubation
410 at 28˚C for 6 days, the colony diameter in all plates were measured. For each plate, the average
411 colony diameter was used to calculate the fungicide concentration that resulted in 50% mycelial
412 growth inhibition (EC_{50}). The EC_{50} values were calculated with the Data Processing System
413 (DPS) software. Ketoconazole was added to 0.2×PDA plates at 0, 0.01, 0.04, 0.2, 1.0 and 5.0
414 μg/mL. Fluconazole was added to 0.2×PDA plates at 0, 0.2, 1.0, 5.0, 10.0 and 50.0 μg/mL. The
415 assays were repeated three time.
416
417 **RNA preparation and qPCR assays for Mediator subunit genes.** To analyze relative
418 gene expression of Mediator subunits, fungal mycelia were harvested from 3-days-old YEPD
419 or 3-days-old Myro cultures and used for RNA isolation with the GeneJet RNA Purification
420 Kit (Thermo Scientific). First-strand cDNA was synthesis by Verso cDNA synthesis kit
421 (Thermo Scientific) and subsequently used for qPCR assays with DyNAmo ColorFlash SYBR
422 Green qPCR Kit (Thermo Scientific). The β-tubulin gene of *F. verticillioides* was used as the
423 internal control. The relative quantification of all genes was calculated with the $2^{-\Delta\Delta Ct}$ method
424 (56). All qPCR assays were conducted with a StepOne plus realtime PCR system. All results
425 were calculated with the data from three biological replicates.
426
427 **Fumonisin B_1 assay and Maize seedling infection assay.** To determine FB_1 and
428 ergosterol production, we followed the standard method described previously (34, 57). FB_1
429 levels were normalized with ergosterol levels. All results were calculated with the data from
430 three biological replicates. Stalk rot virulence assays was conducted using silver queen hybrid
431 seeds as previously described (34). After growth on V8 plates for 6 days, conidia of each mutant
432 were collected and re-suspended in sterile distilled water to a concentration of 1×10^7 mL^{-1}.

Seedlings were inoculated with 10 μL conidia suspension. After 10 days inoculation in the dark room, the infected seedlings were recorded and imaged. For all mutants, 15 seedlings were inoculated.

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603 **TABLE 1** Comparisons of Mediator modular organization and subunit composition between *Fusarium*
604 *verticillioides* and *Saccharomyces cerevisiae*.

		S. cerevisiae		*F. verticillioides*	
Module	Subunit	Gene ID	Essential	Gene ID	Essential
Scaffold	Med14	Rrg1	Yes	FVEG_01411	Yes
Head	Med6	Med6	Yes	FVEG_04734	Yes
	Med8	Med8	Yes	FVEG_09579	Yes
	Med11	Med11	Yes	FVEG_07465	Yes
	Med17	Srb4	Yes	FVEG_02224	Yes
	Med18	Srb5	No	FVEG_03198	No
	Med20	Srb2	No	-	-
	Med22	Srb6	Yes	FVEG_05568	Yes
Middle	Med1	Med1	No	FVEG_02872	No
	Med4	Med4	Yes	FVEG_06276	Yes
	Med7	Med7	Yes	FVEG_04284	Yes
	Med9	Cse2	No	FVEG_04688	No
	Med10	Nut2	Yes	-	-
	Med19	Rox3	Yes	-	-
	Med21	Srb7	Yes	FVEG_06141	Yes
	Med31	Soh1	No	FVEG_02237	No
Tail	Med2	Med2	No	-	-
	Med3	Pgd1	No	FVEG_07603	No
	Med5	Nut1	No	FVEG_07865	No
	Med15	Gal11	No	11 copies	N/A
	Med16	Sin4	No	FVEG_01083	No
Kinase	Med12	Srb8	No	FVEG_02030	No
	Med13	Ssn2	No	FVEG_02107	No
	Cdk8	Ssn3	No	FVEG_11159	No
	CycC	Ssn8	No	FVEG_11306	No

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612 **TABLE 2** Hyphal growth, conidiation and conidial germination of the Mediator deletion mutants.
613

Strain	Colony diameter (mm)[a]			Conidiation ($10^5/cm^2$)[b]	Percentage of germinated conidia (%)[c]
	V8	PDA	Myro		
WT	80.0±1.4a	63.0±0.8c	66.8±0.9b	104.1±3.9c	89.0±2.6a
ΔFvMed18	44.7±1.2g	38.2±0.8h	29.5±1.4i	100.2±5.0c	-
ΔFvMed1	78.0±1.4a	59.8±1.1e	54.5±0.8e	87.8±2.9d	55.8±9.1c
ΔFvMed9	66.3±0.9d	59.0±1.2e	61.3±1.4d	182.2±6.2a	62.5±9.3bc
ΔFvMed31	70.5±1.3c	61.0±1.0de	62.8±0.9cd	73.7±3.7e	74.8±8.4bc
ΔFvMed3	78.4±1.3a	70.1±1.1a	71.2±0.9a	66.6±3.9e	88.6±1.8a
ΔFvMed5	74.0±1.0b	62.8±1.2cd	68.0±1.4b	115.4±3.8b	92.2±2.8a
ΔFvMed16	75.0±1.0b	65.2±0.9b	64.3±1.3c	85.2±3.8d	70.2±4.7b
ΔFvMed12	64.1±1.2e	54.8±1.2f	40.5±1.2g	26.4±1.4g	31.8±1.2d
ΔFvMed13	69.0±1.1c	60.0±0.9e	43.6±1.4f	47.6±2.4f	38.2±7.9d
ΔFvFcc1	62.5±1.1ef	53.4±1.2f	35.1±0.8h	42.5±2.1f	17.6±2.1e
ΔFvFck1	61.5±0.9f	50.7±1.4g	41.7±0.8fg	40.0±5.7f	20.0±1.8e

614 [*]Means and standard deviations (SD) were calculated from three independent experiments. Values in table
615 followed by the same letter are not significantly different ($P < 0.05$).
616 [a]Colonies were cultured on V8, 0.2×PDA or Myro plates for 6 days at 28°C.
617 [b]Conidiation inV8 plate was examined after incubation for 6 days at 28°C.
618 [c]Conidial germination was assayed on Water Agar (WA) after 6.5 h of incubation at 28°C.
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624 **Figure legends**

625

626 **Fig. 1** Transcription profile of 6 key Mediator genes in moderately resistant (B73) versus highly
627 susceptible (33K44) maize seeds. *F. verticillioides* was inoculated on surface sterilized B73
628 and 33K44 seeds, and RNA-Seq was performed on 2, 4, 6, 8 days-post-inoculation (dpi)
629 samples. We analyzed whether transcription of Mediator genes show difference in two maize
630 seeds. *P value* for comparison was computed by the non-parametric Van der Waerden (VDW)
631 test while not having any assumptions for normality or equality of variance between 33K44
632 and B73. A complete set of transcriptional comparisons for all *F. verticillioides* Mediator genes
633 is shown in Fig. S1.

634

635 **Fig. 2** Phenotypes of the Mediator deletion mutants in vegetative growth, conidiation, conidial
636 germination and hydrophobicity. (A) Colony morphology of wild-type and Mediator mutants
637 grown on V8 plate at 28°C for 6 days. (B) Deletion of Mediator subunits on conidia production.
638 Conidia of all strains were quantified with a hemacytometer after incubation on V8 plate for 6
639 days. (C) Conidia of each strain were cultivated in WA medium. After incubation at 28°C for
640 6.5 h, conidial germination of 200 conidia was examined. (D) Hydrophobicity assays of wild-
641 type and all mutants. Bars denote standard errors from three repeated experiments. Values on
642 the bars followed by the same letter are not significantly different at P = 0.05, according to
643 Fisher's LSD test.

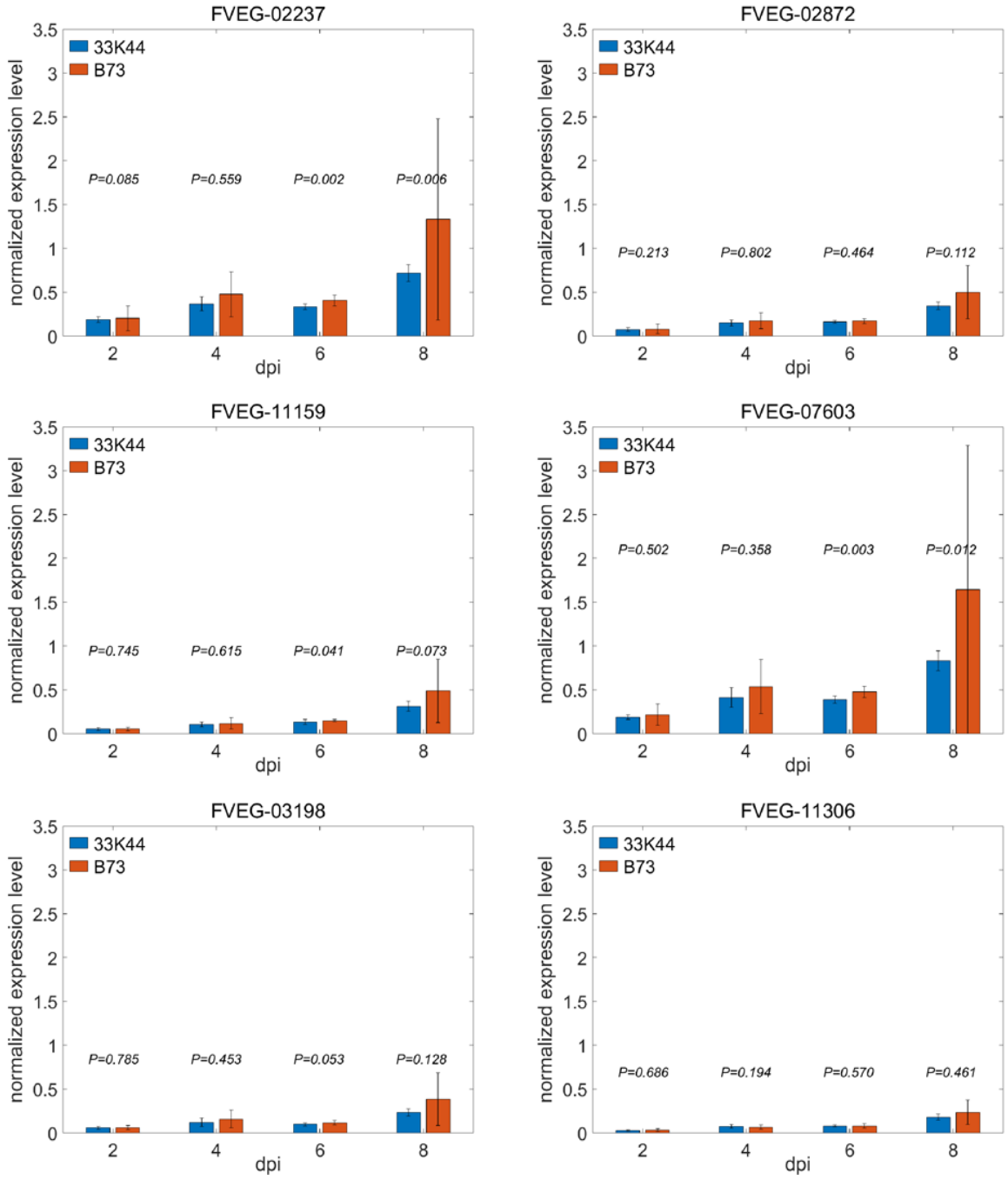
644

645 **Fig. 3 Impact of Mediator gene mutation in virulence when tested on maize seedlings**. (A)
646 Maize seedlings were grown in the dark and inoculated with conidia suspension from all strains.
647 The images were taken after 10 days of incubation. Sterile water was used as negative control
648 (NC). (B) The relative lesion size in maize seedling rot assays was measured by image J. (C)

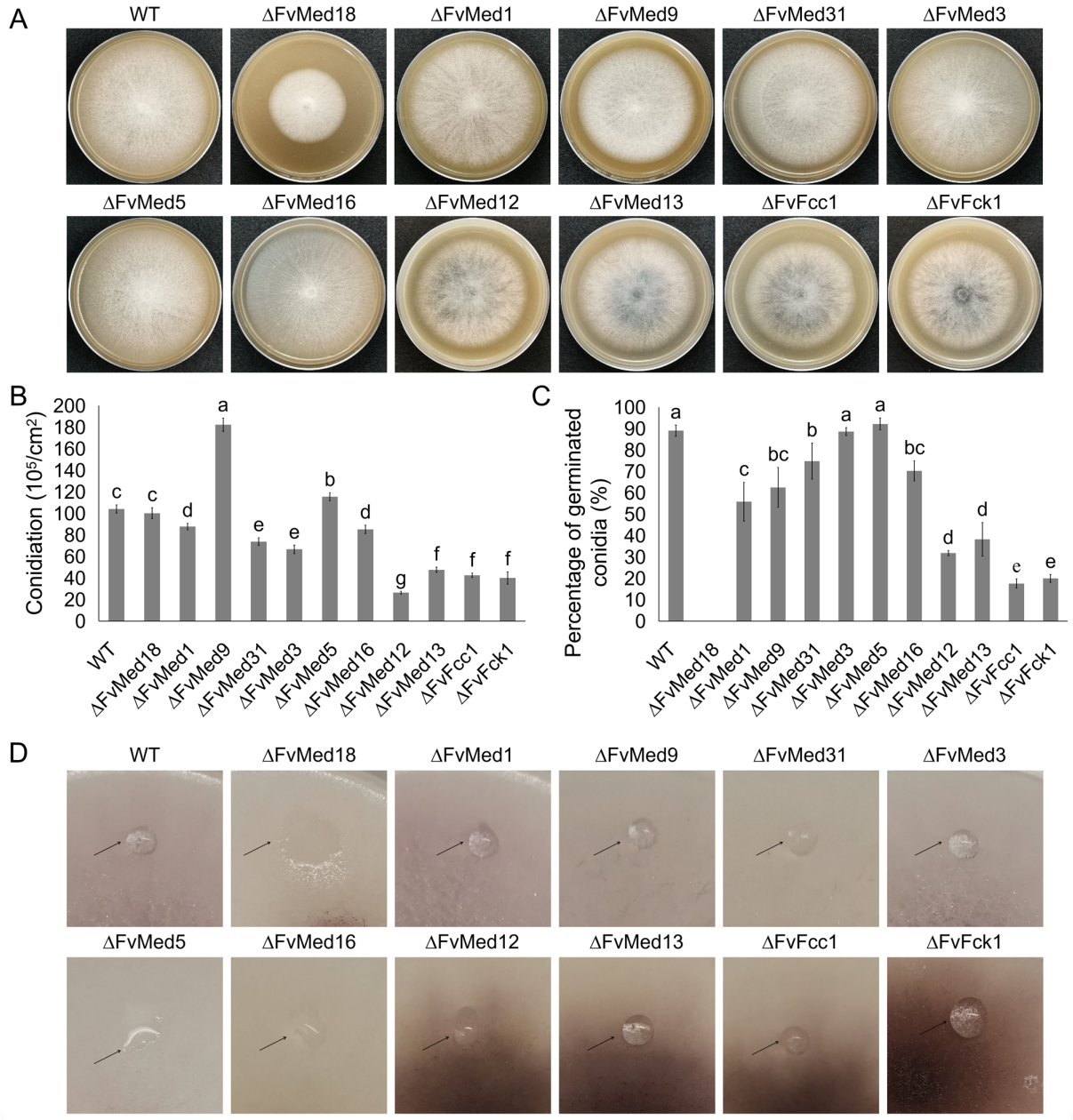
649 The relative lesion size was normalized with strain growth rate on V8 plate. Bars denote
650 standard errors from three repeated experiments. Values on the bars followed by the same letter
651 are not significantly different at P = 0.05, according to Fisher's LSD test.
652
653 **Fig. 4** Gene disruption of Head and Kinase modules of Mediator increases the sensitivity
654 towards cell-wall damage agent. (A) Growth phenotype of the wild type (WT) and mutants
655 with Congo Red amended in 0.2xPDA plates after 6 days of incubation at 28°C. (B) Statistical
656 analysis of the growth inhibition rate of each strain with Congo Red. Bars denote standard
657 errors from three repeated experiments. Values on the bars followed by the same letter are not
658 significantly different at P = 0.05, according to Fisher's LSD test.
659
660 **Fig. 5** Mediator complex is involved in acetate utilization. (A) All strains were cultivated on
661 MM with glucose or acetate, and incubated at 28°C for 6 days. (B) The growth inhibition rate
662 of each strain was analyzed by comparing the growth with acetate against glucose. Bars denote
663 standard errors from three repeated experiments. Values on the bars followed by the same letter
664 are not significantly different at P = 0.05, according to Fisher's LSD test.
665
666 **Fig. 6** Roles of Mediator complex on FB_1 and secondary metabolite biosynthesis. (A) FB_1
667 production in the wild type (WT) and Mediator mutants grown on autoclaved kernels for 7
668 days at room temperature. FB_1 production levels were normalized with ergosterol production
669 in each strain. (B) Comparisons of secondary metabolite biosynthesis among the WT and
670 Mediator mutants. Images were taken after all strains were grown on liquid Myro for 7 days.
671 Bars denote standard errors from three repeated experiments. Values on the bars followed by
672 the same letter are not significantly different at P = 0.05, according to Fisher's LSD test.
673

674 **Fig. 7** Subcellular localization of Med1-GFP in *F. verticillioides* mycelial cells grown in (A)
675 FB_1-non-inducing medium YEP and (B~D) FB_1-inducing medium Myro. We inoculated *F.*
676 *verticillioides* Med1-GFP conidia in two cultures and took images after 2-day incubation. GFP
677 expression was observedunder an Olympus BX51 microscope and the images were processed
678 by ImageJ. Bar = 10 μm.
679

Zhou et al. Fig 1.



Zhou et al. Fig 2.



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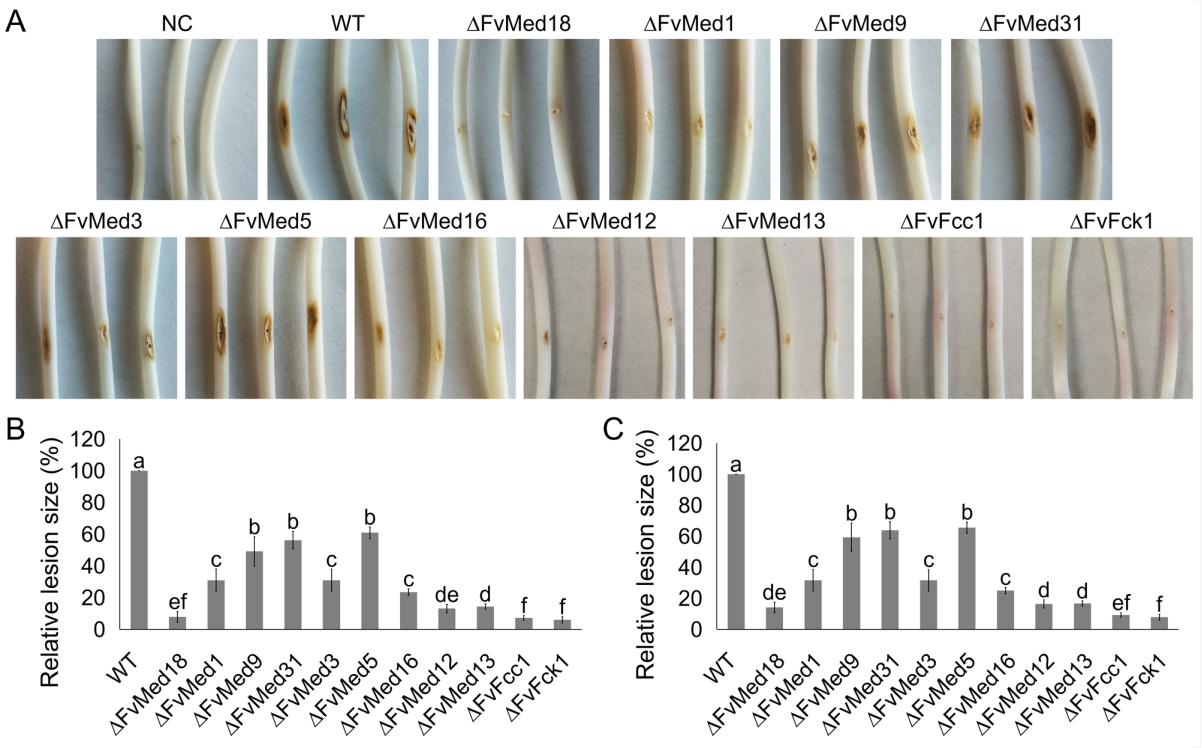

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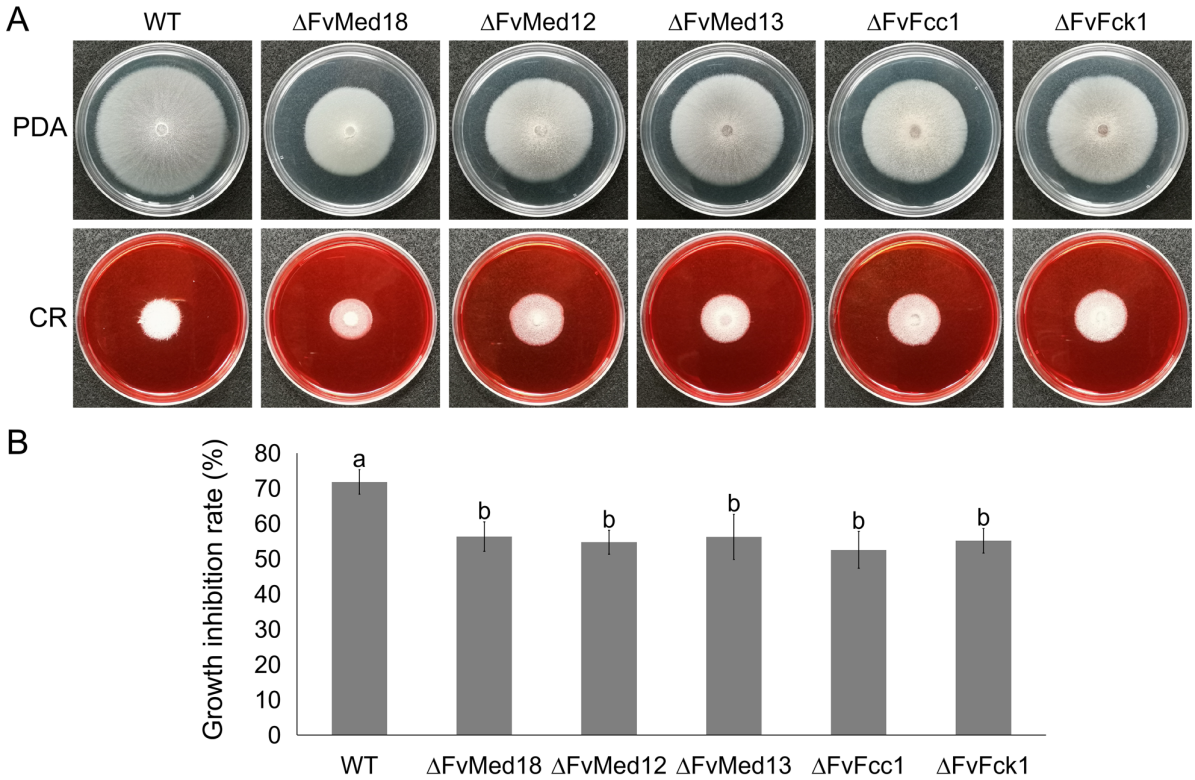

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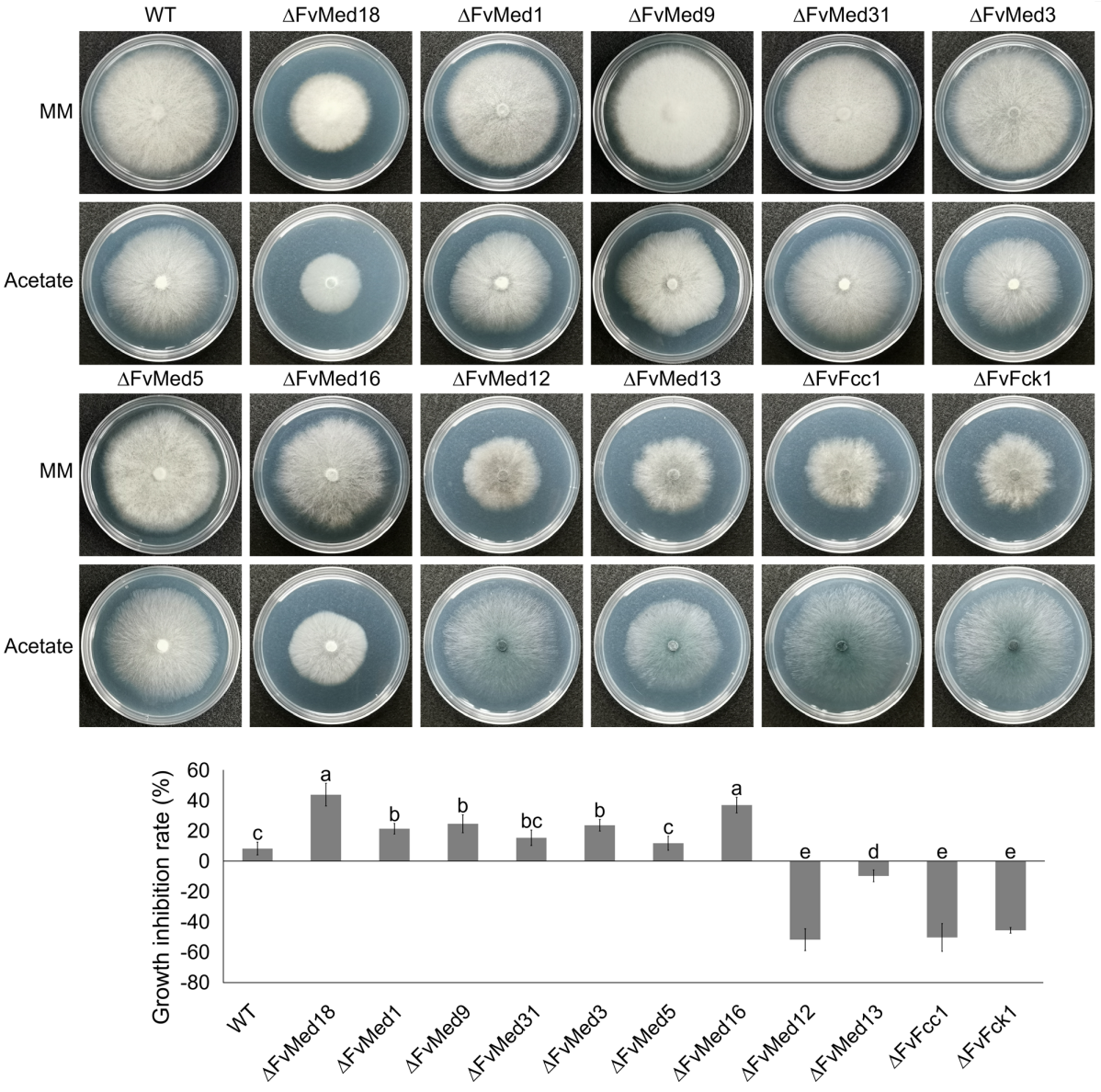

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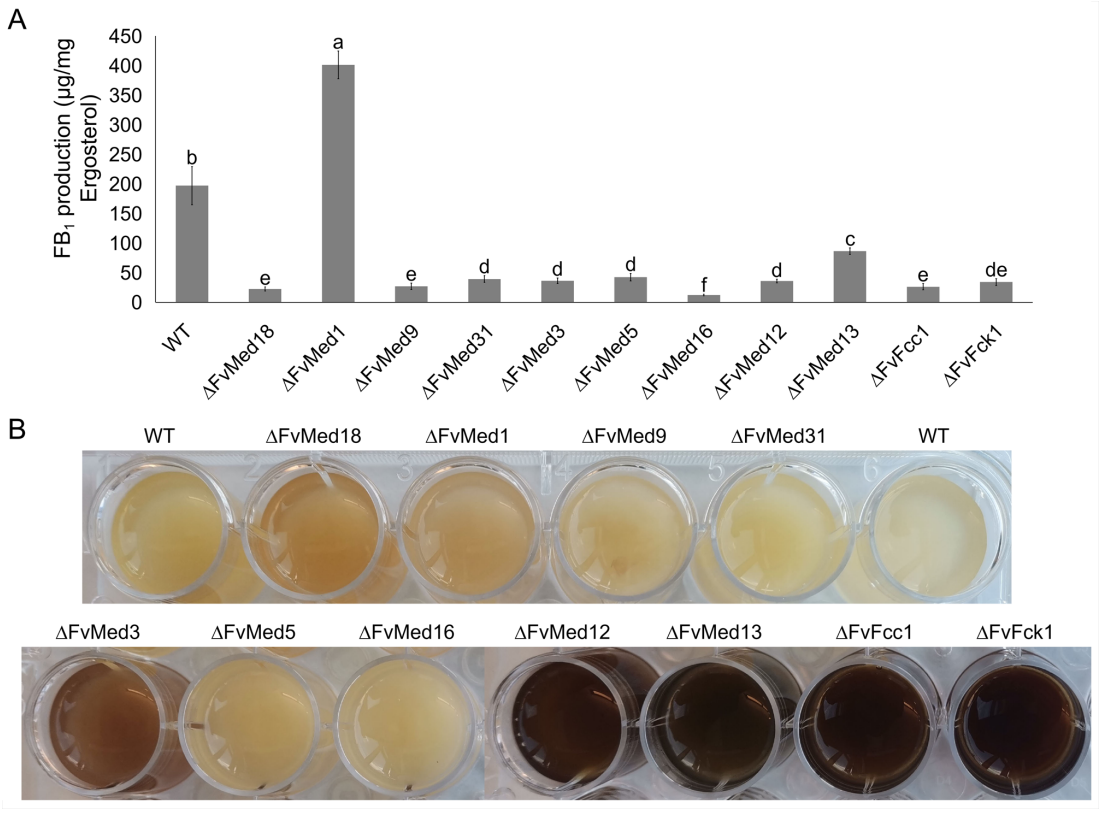

Zhou et al. Fig 7.

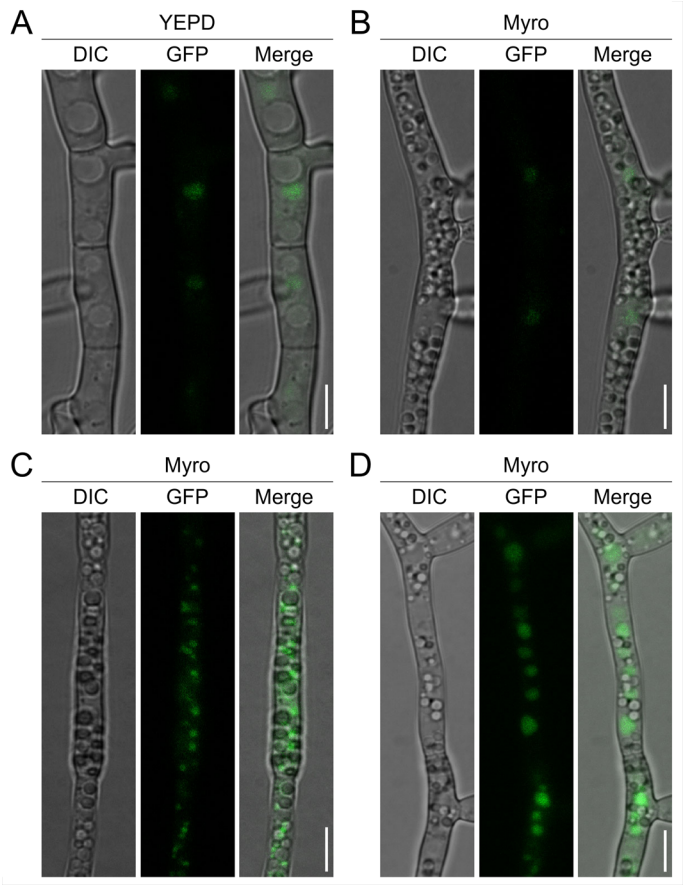

Table S1. Primers used in this study.

Primer	Sequence (5'-3')	Application
Med1_LF/FN	TCCCTAAGGATTTAGACTTC	Amplify *FvMed1* 5' flank sequence, for gene knock out
Med1_LF/R1	TAGATGCCGACCGGGAACGAG CTCCAGGTTGAAAGCAA	
Med1_RF/F1	CCACTAGCTCCAGCCAAGGGC GGAGAAACTGAACGAC	Amplify *FvMed1* 3' flank sequence, for gene knock out
Med1_RF/RN	GCTTCCTCGCTGAGCACTT	
Med4_LF/FN	TTCCTCAAGTCCACCAGAT	Amplify *FvMed4* 5' flank sequence, for gene knock out
Med4_LF/R1	TAGATGCCGACCGGGAACGGT CAATGTGGGTCTTTAGT	
Med4_RF/F1	CCACTAGCTCCAGCCAAGGGT AAACGCGAATTCCAGAT	Amplify *FvMed4* 3' flank sequence, for gene knock out
Med4_RF/RN	ATACTGTCCTCGGCTTCGT	
Med5_LF/FN	GAACGACCACGAACCTATG	Amplify *FvMed5* 5' flank sequence, for gene knock out
Med5_LF/R1	TAGATGCCGACCGGGAACTGG GCTTGGTGTTGACTTT	
Med5_RF/F1	CCACTAGCTCCAGCCAAGGTC AGTCCGTCTATTTTCTTG	Amplify *FvMed5* 3' flank sequence, for gene knock out
Med5_RF/RN	GTCGGTGACACTAATGGTTTG	
Med7_LF/FN	CGTCTTCATCGTATGTGGC	Amplify *FvMed5* 5' flank sequence, for gene knock out
Med7_LF/R1	TAGATGCCGACCGGGAACCGT ACCATCCAACGTCATA	
Med7_RF/F1	CCACTAGCTCCAGCCAAGTCA GTAATCTACGCAAGCATG	Amplify *FvMed5* 3' flank sequence, for gene knock out
Med7_RF/RN	ATGCGAGATAGAGGACGGT	
Med9_LF/FN	CAGTGTAGCCCTTGGAGTC	Amplify *FvMed9* 5' flank sequence, for gene knock out
Med9_LF/R1	TAGATGCCGACCGGGAACGGT CTGTCGTTCTATGATATG	
Med9_RF/F1	CCACTAGCTCCAGCCAAGTATC AGCAGGGTGTTTGGG	Amplify *FvMed9* 3' flank sequence, for gene knock out
Med9_RF/RN	CTCTTTGACTCGGTGCTTC	
Med21_LF/FN	GGAGGGCTCGCTTGACTAA	

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Table S2. Wild-type and mutant strains of *F. verticillioides* used in this study.

Strain	Genotype description	Reference
M3125	Wild-type strain of *F. verticillioides*	Lab preservation
ΔFvMed18	*FvMed18* deletion mutant of M3125	This study
ΔFvMed1	*FvMed1* deletion mutant of M3125	This study
ΔFvMed9	*FvMed9* deletion mutant of M3125	This study
ΔFvMed31	*FvMed31* deletion mutant of M3125	This study
ΔFvMed3	*FvMed3* deletion mutant of M3125	This study
ΔFvMed5	*FvMed5* deletion mutant of M3125	This study
ΔFvMed16	*FvMed16* deletion mutant of M3125	This study
ΔFvMed12	*FvMed12* deletion mutant of M3125	This study
ΔFvMed13	*FvMed13* deletion mutant of M3125	This study
ΔFvFcc1	*FvFcc1* deletion mutant of M3125	Shim and Woloshuk, 2001 and this study
ΔFvFck1	*FvFck1* deletion mutant of M3125	Bluhm and Woloshuk, 2005 and this study

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Table S3. Sensitivity of *F. verticillioides* to ketoconazole and fluconazole.

Strain	EC_{50} (μg/mL)	
	Ketoconazole	Fluconazole
WT	1.41±0.11c	1.32±0.10c
ΔFvMed18	2.76±0.30ab	4.09±0.86a
ΔFvMed1	1.48±0.11c	1.38±0.05c
ΔFvMed9	1.43±0.11c	1.34±0.03c
ΔFvMed31	1.32±0.16c	1.29±0.16c
ΔFvMed3	1.45±0.15c	1.36±0.10c
ΔFvMed5	1.55±0.09c	1.45±0.05c
ΔFvMed16	1.36±0.32c	1.28±0.16c
ΔFvMed12	3.08±0.24a	2.17±0.21b
ΔFvMed13	2.65±0.08b	2.06±0.06b
ΔFvFcc1	2.43±0.19b	2.10±0.07b
ΔFvFck1	2.84±0.22ab	2.17±0.18b

*Means and standard deviations (SD) were calculated from three independent experiments. Values in table followed by the same letter are not significantly different ($P < 0.05$).
EC_{50}: the fungicide concentration resulting in 50% inhibition of mycelial growth.

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Table S4. Fumonisin B_1 (FB_1) and ergosterolproduction of the wild-type and Mediator deletion mutants.

	FB_1 (μg/L)[a]	Ergosterol (mg/L)[b]	FB_1/Ergosterol (μg/mg)[c]
WT	73915.6±12127.6a	375.0±49.8a	197.1±32.3b
ΔFvMed18	4797.6±712.0e	213.1±29.4b	22.5±3.4d
ΔFvMed1	84499.1±4949.8a	210.6±9.9b	401.3±23.5a
ΔFvMed9	6719.5±799.1d	248.3±27.5b	27.1±7.1d
ΔFvMed31	9551.1±1822.6c	234.3±41.0b	39.3±7.5d
ΔFvMed3	13488.1±1790.0b	370.2±53.6a	36.4±4.8d
ΔFvMed5	10845.0±1175.8bc	254.9±24.7b	42.5±6.5d
ΔFvMed16	3270.1±353.0f	264.5±47.0b	12.4±1.3d
ΔFvMed12	2475.8±196.8f	68.7±14.3cd	36.0±2.9d
ΔFvMed13	6349.3±407.3d	73.5±16.7cd	86.4±5.5c
ΔFvFcc1	1636.1±327.9g	62.0±8.8d	26.4±5.3d
ΔFvFck1	2859.8±583.3f	83.5±6.0c	34.2±7.0d

[*]Means and standard deviations (SD) were calculated from three independent experiments. Values in table followed by the same letter are not significantly different ($P < 0.05$).

[a]FB_1 production in wild-type and mutants grown on autoclaved kernels for 7 days at room temperature.

[b]Ergosterol production in wild-type and mutants grown on autoclaved kernels for 7 days at room temperature.

[c]FB_1 levels were normalized with fungal ergosterol levels.

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Table S5. Relative expression level of 15 *PKS* genes in wild-type and Mediator mutants.

Gene	Relative expression level				
	WT	ΔFvMed1	ΔFvMed3	ΔFvMed18	ΔFvFcc1
PKS1	1	2.55±0.33	ND	ND	8.78±2.52
PKS2	1	0.97±0.10	0.98±0.22	0.64±0.06	3.74±0.37
PKS3	1	0.36±0.03	1.93±0.77	8.66±0.84	31.17±3.65
PKS4	1	8.41±1.12	0.24±0.11	0.29±0.11	9.92±1.52
PKS5	1	5.18±0.78	1.00±0.15	ND	1.14±0.17
PKS6	1	2.30±0.21	1.85±0.31	0.02±0.01	3.70±0.26
PKS7	1	0.47±0.08	2.44±0.36	3.18±0.45	0.63±0.16
PKS8	1	3.70±0.35	11.03±1.96	0.03±0.003	0.05±0.01
PKS9	1	0.31±0.05	0.62±0.04	0.56±0.06	0.27±0.02
PKS10	1	0.14±0.04	2.34±0.34	0.07±0.04	0.58±0.04
PKS11	1	2.49±0.27	0.62±0.2	0.04±0.01	0.06±0.01
PKS12	ND	ND	ND	ND	ND
PKS13	1	1.13±0.07	2.25±0.13	1.21±0.07	1.80±0.11
PKS14	1	0.06±0.01	ND	0.24±0.06	0.08±0.01
PKS15	1	0.66±0.2	0.23±0.07	65.78±21.22	1.42±0.33

Mycelia of each mutants were incubated in myro liquid medium for 3 days at 28℃. Each gene expression were normalized with β-tubulin expression level. Gene expressions were calculated using $2^{-\Delta\Delta Ct}$. Gene expression level of wild-type strain was standardized to 1.0. Three replicates were performed for each test. ND: not detected.

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FIG S1. Assessment of the RNA-Seq Data Set of *F. verticillioides* Mediator genes under different conditions. The Mediator genes expression were normalized with β-tubulin expression. Error bars indicate SD of 7 biological replicates.

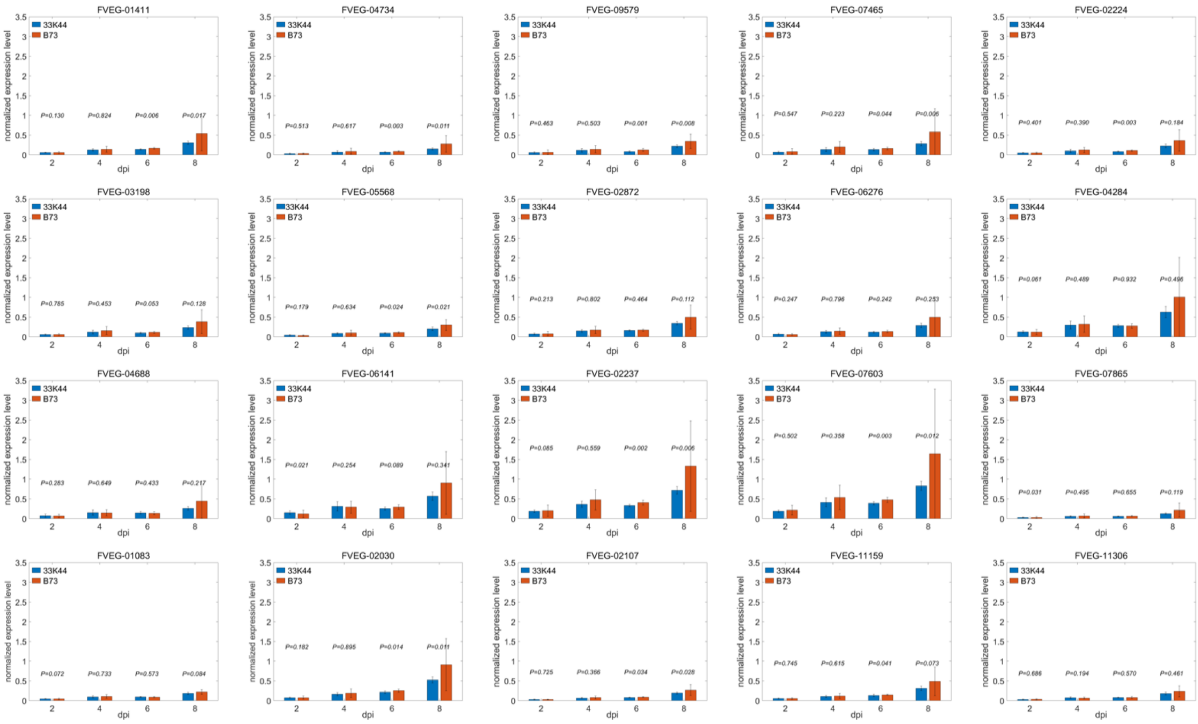


FIG S2 Schematic description of split marker approach for constructing Mediator deletion mutants in *F. verticillioides.*

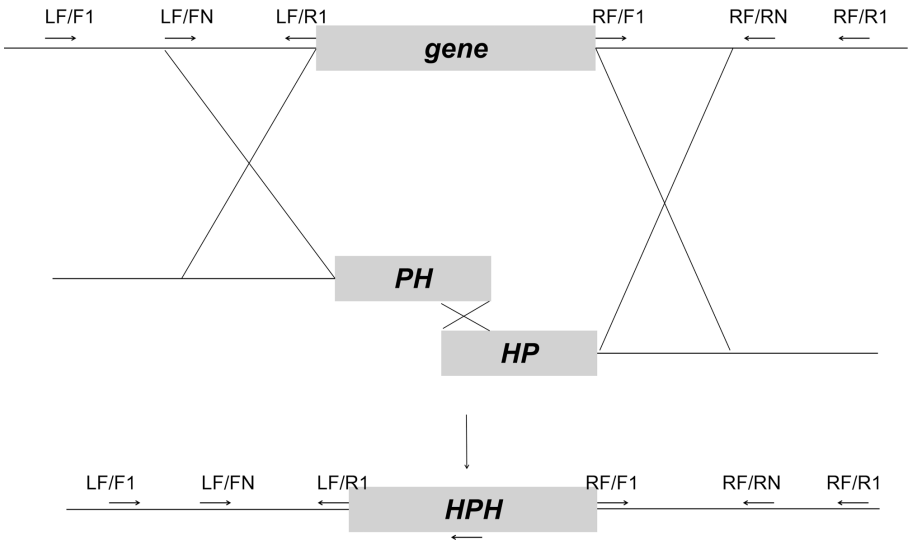
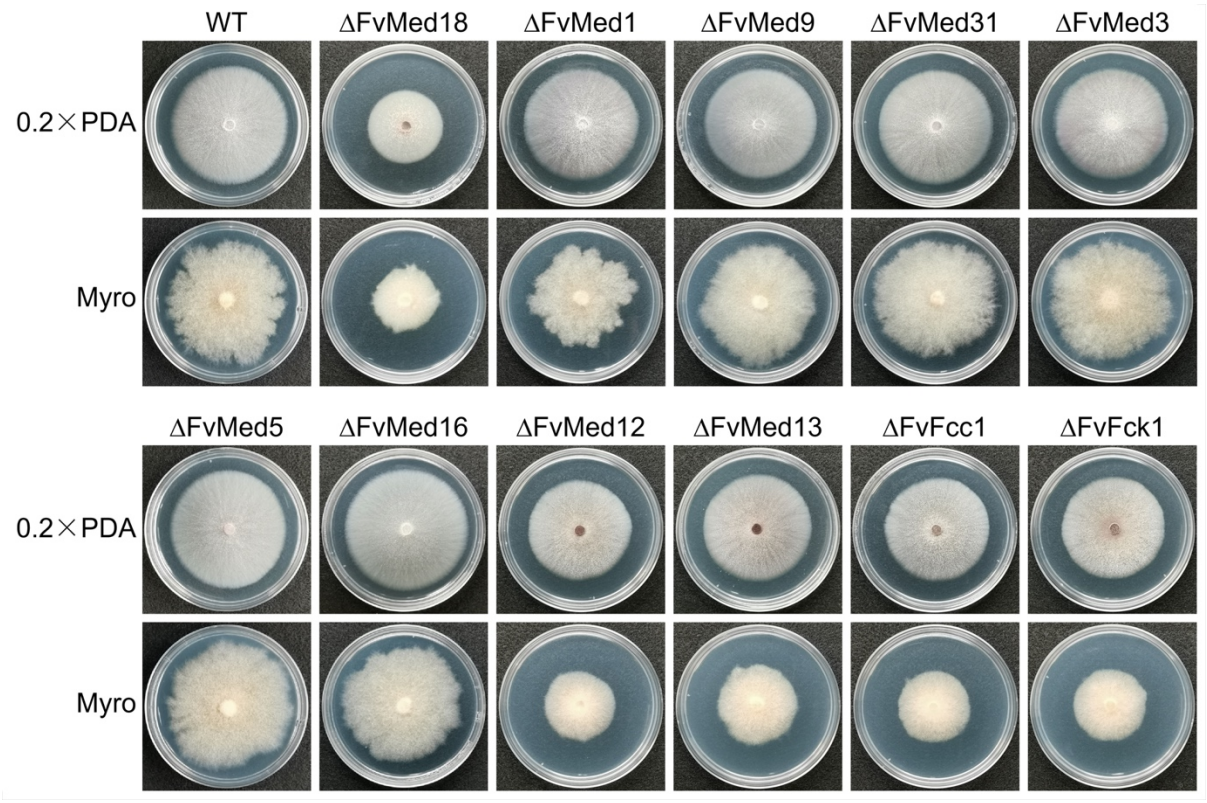


FIG S3 Colony morphology of wild-type and Mediator mutants grown on 0.2×PDA and Myro agar plate at 25℃ for 6 days



742 **FIG S4** Deletion of Mediator subunits on cell-wall damage agent response in *F.*
743 *verticillioides*
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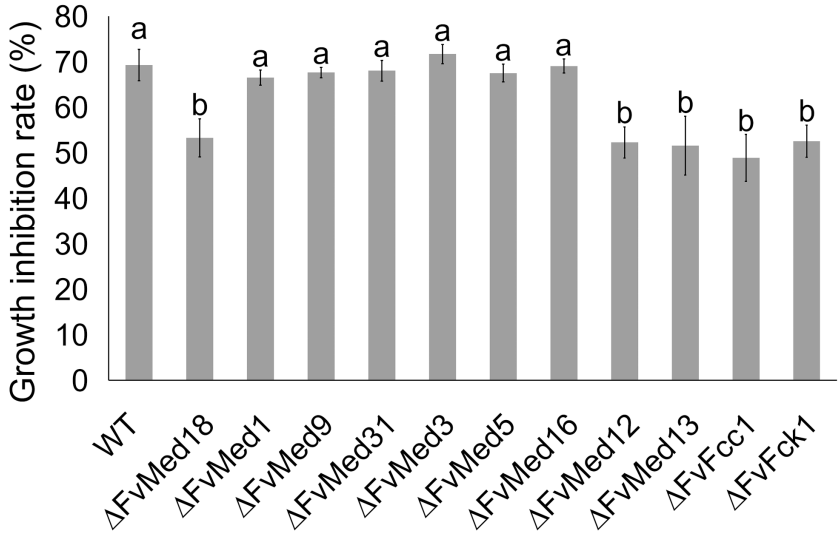

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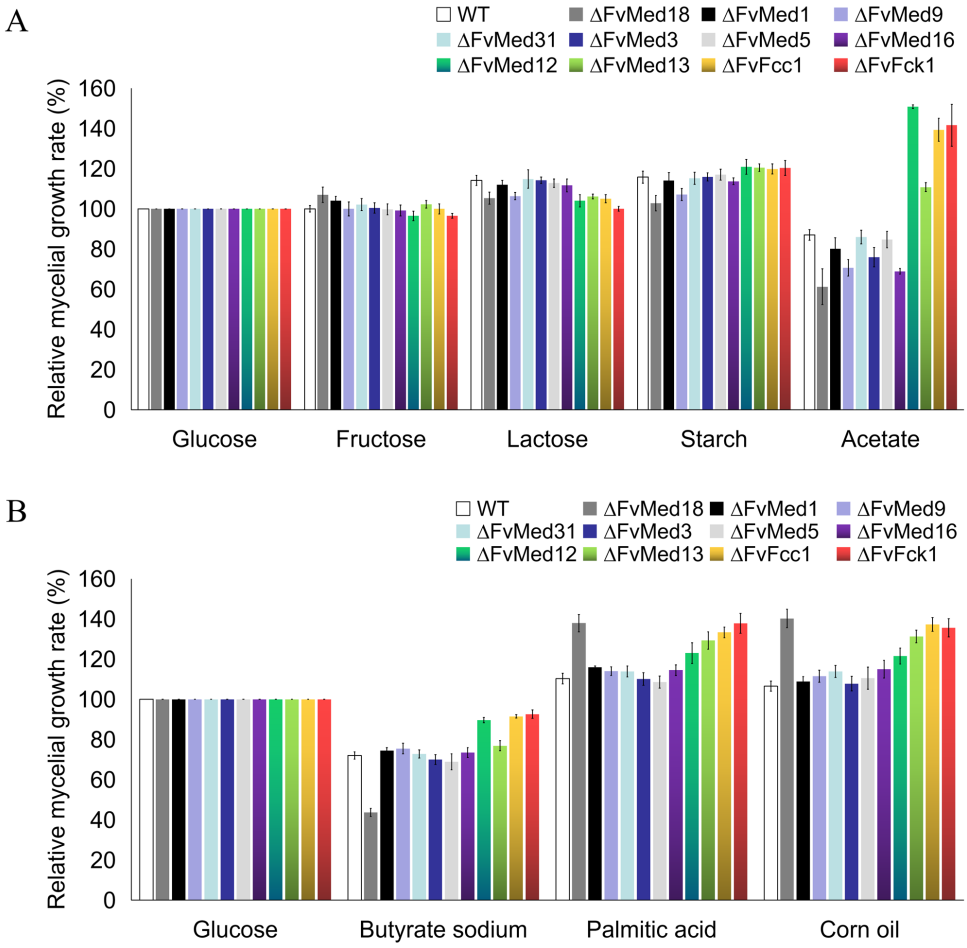
747 **FIG S5** Relative growth rates of wild-type and Mediator mutants on various carbon sources.
748 Values on the bars followed by the same letter are not significantly different at P = 0.05,
749 according to Fisher's LSD test



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