A Conserved Homeobox Transcription Factor Htf1 Is Required for Phialide Development and Conidiogenesis in *Fusarium* Species

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Abstract

Conidia are primary means of asexual reproduction and dispersal in a variety of pathogenic fungi, and it is widely recognized that they play a critical role in animal and plant disease epidemics. However, genetic mechanisms associated with conidiogenesis are complex and remain largely undefined in numerous pathogenic fungi. We previously showed that Htf1, a homeobox transcription factor, is required for conidiogenesis in the rice pathogen *Magnaporthe oryzae*. In this study, our aim was to characterize how Htf1 homolog regulates common and also distinctive conidiogenesis in three key *Fusarium* pathogens: *F. graminearum*, *F. verticillioides*, and *F. oxysporum*. When compared to wild-type progenitors, the gene-deletion mutants in *Fusarium* species failed to form conventional phialides. Rather, they formed clusters of aberrant phialides that resembled elongated hyphae segments, and it is conceivable that this led to the obstruction of conidiation in phialides. We also observed that mutants, as well as wild-type Fusaria, can initiate alternative macroconidia production directly from hyphae through budding-like mechanism albeit at low frequencies. Microscopic observations led us to conclude that proper basal cell division and subsequent foot cell development of macroconidia were negatively impacted in the mutants. In *F. verticillioides* and *F. oxysporum*, mutants exhibited a 2- to 5- microconidia complex at the apex of monophialides resulting in a floral petal-like shape. Also, prototypical microconidia chains were absent in *F. verticillioides* mutants. *F. graminearum* and *F. verticillioides* mutants were complemented by introducing its native HTF1 gene or homologs from other *Fusarium* species. These results suggest that *Fusarium* Htf1 is functionally conserved homeobox transcription factor that regulates phialide development and conidiogenesis via distinct signaling pathways yet to be characterized in fungi.

Introduction

Asexual sporulation is the preferred mode of reproduction in most pathogenic fungi [1,2]. More importantly, these asexual spores, commonly known as conidia, are used as a primary dissemination tool as well as for initiating infection [3–7]. Under favorable conditions, fungal pathogens can rapidly propagate and spread to cause diseases in economically important crops as well as humans and animals. Significantly, recent studies have shown that fungal pathogens responsible for plant diseases can also cause opportunistic mycosis in humans [8,9]. However, the mechanisms of asexual sporulation are diverse and complex and remain largely undefined in numerous pathogenic fungi. In model organisms, namely *Neurospora crassa* and *Aspergillus nidulans*, signaling pathways that regulate conidiation have been extensively studied, and excellent reviews are available [2,10,11]. Briefly, there are key transcriptional regulators known to be involved in this process. One important gene that plays a critical role in the transition from conidiophore to conidia formation is *braA* in *A. nidulans* [12]. Further genetic and biochemical studies led to the discovery of *abaA* and *wetA* [13,14]. These three genes (*braA-abaA-wetA*) have been proposed to constitute a central regulatory pathway that acts in concert with other genes to control conidiation in *Aspergillus* [15,16]. However, we also need to recognize that different fungal species may have developed different regulatory mechanisms for producing various types of conidia.

The genus *Fusarium* is considered the most important and diverse genera of plant pathogenic fungi and causes a wide range of diseases in every economically important crop species [17,18]. Several species within the genus are also associated with the production of mycotoxins which poses a significant threat to food safety and human health [19–22]. Moreover, once considered a relatively uncommon cause of ocular disease, *Fusarium* species have emerged as one of the leading causes of human keratomycosis outbreaks, along with *Aspergillus* and *Candida* species [23,24].
genomes of closely related Fusarium species, F. graminearum, F. verticillioides, and F. oxysporum, have been sequenced mainly due to their economic and scientific importance [18,25,26]. In addition, these Fusarium species offer a unique opportunity to investigate numerous biological features, including distinct asexual sporulation modes. In recent years, a number of conidial-related genes in Fusarium species have been identified by insertion mutagenesis or targeted gene deletion approaches. Several genes are important transcriptional regulators, such as FeSTUA, FaSTUA and REN1, which are conserved in filamentous fungi and essential for conidiogenesis [27–29]. Genes such as FgVEA, FvVEI and FgTEP1 are involved in multiple signaling pathways, regulating virulence, secondary metabolism and conidiation [30–32]. Some important signal transduction related genes, e.g., GPARK1, GzSNF1 and FACS1, which encode various protein kinases are also required for conidiation in Fusarium species [33–37]. However, molecular mechanisms underlying conidiogenesis in Fusarium species is complex and does not seem to adhere to the regulatory pathway established in A. nidulans and N. crassa [2,10,11,38]. Therefore, it is currently difficult to unambiguously define genetic mechanisms or signaling pathways required for this important biological process in Fusaria.

Although F. graminearum, F. verticillioides and F. oxysporum are defined into the same genus, they exhibit distinct features in asexual sporulation. For instance, F. graminearum only produces macroconidia on solitary phialides or on multiple phialides borne on conidiophores [27]. In F. verticillioides, the fungus grows as haploid mycelia and propagates vegetatively via hyphal elongation and produces two types of asexual spores, macroconidia and microconidia [39]. Macroconidia emerge from macroconidiophores, which are branched and unbranched monophialides [40,41]. Similarly, unicellular and unicinucleate microconidia also arise from branched and unbranched monophialides, frequently forming long conidial chains and false heads. When compared to the other two, F. oxysporum is unique in the fact that it can only reproduce asexually, but through three different types of conidia: microconidia, macroconidia, and chlamydospores [40–42]. Microconidia are ellipsoid and have no or one septum, macroconidia are falcate and have three or four septa, and chlamydospores are ellipsoidal and have no or one septum, macroconidia reproduce asexually, but through three different types of conidia: asexual sporulation. For instance, in Fusaria. Currently, it is difficult to unambiguously define genetic mechanisms or signaling pathways required for this important biological process in Fusaria.

FgHTF1 is dispensable for vegetative growth and fertility but essential for conidiogenesis

In order to study the function of Htf1 ortholog in Fusarium conidiogenesis, we first deleted FgHTF1 in F. graminearum using gene replacement approach (Table S1, Figure S2A). Transformants were selected on hygromycin-amended medium, and gene deletion was confirmed by polymerase chain reaction (PCR) and Southern blot analyses (Figure S2B and S2C). FgHTF1 deletion mutant (Fghtf1) showed no discernible difference in vegetative growth and sexual reproduction when compared to the wild-type strain PH-1 on complete medium (CM) and wheat kernels medium, respectively (Table 1, Figure S3A and S3B). However, Fghtf1 mutant showed significantly reduced macroconidia production in liquid carboxymethylcellulose (CMC) medium [52]. After three days, only 1.00±0.87×10⁴ macroconidia were observed in Fghtf1, whereas 51.44±6.64×10⁴ macroconidia were in PH-1 (Table 1). Even after fifteen days of incubation, Fghtf1 mutant did not recover the production of macroconidia compared to PH-1, indicating that this reduction in conidiation was not related to the duration of incubation (Table 1). The defect in conidial production was fully recovered to the wild-type level in the complemented strain AFGhtf1-Com, where the native promoter-
conidiogenesis in phialides and is continuously required for maintenance of uncontrolled proliferation and the loss of conidiation capacity.

PLOS ONE | www.plosone.org 3 September 2012 | Volume 7 | Issue 9 | e45432

Figure 1. Comparative analysis of Htf1 protein in three Fusarium species. (A) Sequence alignment of the homeodomain of F. graminearum (FgHtf1), F. verticillioides (FvHtf1), F. oxysporum (FoHtf1) and M. oryzae (MoHtf1) was performed using Clustal W and Boxshade (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html). The conserved amino acid residues are shaded black, whereas similar residues are shown in gray. Consensus amino acids are marked with asterisk (*). (B) Homology matrix analysis of Htf1 homeodomain (left box) and whole protein (right box) in three Fusarium species and M. oryzae by DNAMAN software. Numbers (%) indicate protein identity. doi:10.1371/journal.pone.0045432.g001

driven FgHTF1 gene cassette was re-introduced into ΔFghtf1 (Figure S2B and S2C, Table 1). These results indicate that FgHTF1 is critical for macroconidia production in F. graminearum.

FgHTF1 specifically regulates phialidegenesis and subsequent conidiation

In order to investigate the reason for significantly reduced conidiation in ΔFghtf1, we microscopically observed fungal tissues grown in CMC, a medium that promotes fungal conidiation. Under the same culture condition, PH-1 and ΔFghtf1-Com produced typical conidiogenous cells, i.e., phialides, which divide to produce incipient macroconidia. The morphology of phialides in PH-1 and ΔFghtf1-Com assumes a bottle-like shape (Figure 2A). The mutants, however, did not produce these structures on its conidiophores, but rather formed clusters consisting of hyphal segments (Figure 2A). Fluorescence staining of nuclei with 4'-6-diamidino-2-phenylindole (DAPI) showed that a phialide in the wild type was uninucleate and harbors a macroconidium (Figure S2B). However, in ΔFghtf1 it appeared that multiple phialide-like structures were disorderly formed on a conidiophore with no macrocondia development (Figure 2B). Presumably, the mutation in FgHTF1 led to abnormal conidiogenous cells with uncontrolled proliferation and the loss of conidiation capacity. These results suggest that FgHTF1 governs proper differentiation of phialides and is continuously required for maintenance of conidiogenesis in F. graminearum.

FgHTF1 regulates macroconidia basal cell division and foot cell development

While ΔFghtf1 deletion mutant lost its ability to produce macroconidia from phialides, which is the main conidiogenesis structure in F. graminearum, we still observed some conidia produced in CMC medium, suggesting that alternative conidiation mechanisms exist. After further microscopic observation, we detected ΔFghtf1 and PH-1 producing conidia directly from hyphae, similar to budding observed in Saccharomyces cerevisiae, albeit at low frequencies (Figure 3A). However there was also a significant difference in conidiogenesis between ΔFghtf1 and PH-1. In the early stages of culturing PH-1 (within 48 h), incipient conidium broke off from intercalary or terminal hyphae without distinct septation, whereas in ΔFghtf1 no conidium was observed at the early stages of culturing in CMC medium. Only after five days of incubation, ΔFghtf1 produced some matured conidia with evident septation at the tip of hyphae (Figure 3A). In addition, we noticed that there was no recognizable narrow region that allows conidium to detach easily from hyphae, and hence, these conidia lack the typical enteroblastic phenomenon associated with PH-1 (Figure 3A). In PH-1, the narrow region serves as the site for the production of macroconidium by cell division. Therefore, we inferred that the dissociative spores may be ruptured away from ΔFghtf1 hyphae by mechanical force during shaking incubation (Figure 3B), and this may explain why macroconidia produced by ΔFghtf1 were morphologically aberrant (Figure 3C). The wild-type macroconidia were moderately curved on the dorsal side and straight on the ventral surface with papillate apical cells and distinct foot-shaped basal cells (Figure 3C). However, ΔFghtf1 conidia were grotesque without proper foot-shaped basal cells (Figure 3C). These observations indicate that FgHTF1 is important for proper basal cell division and subsequent foot cell development in macroconidia produced directly from hyphae.

Aberrant macroconidia of Δfghtf1 mutant still can germinate properly and be pathogenic on hosts

While ΔFghtf1 produced a limited number of macroconidia by budding and have a defect in the foot cell, these spores were still able to germinate like wild type at 25°C in liquid CM with gentle agitation. After 1 h incubation, approximately 70% of macroconidia in ΔFghtf1 and PH-1 looked swollen (Figure 4A). After 2 h, over 95% of macroconidia had at least one germ tube from terminal cells, intercalary cells, or both in the mutant (Figure 4A, Table 1). To determine whether FgHTF1 has a role in pathogenicity, we inoculated wheat heads and wheat coleoptiles with conidia from PH-1 and ΔFghtf1. At 14 days post inoculation (dpi), typical ear rot symptoms were observed on wheat head inoculated with PH-1 and the ΔFghtf1 (Figure 4B, Table 1). Similar brown lesions on coleoptiles and corn stalks infected by PH-1 and ΔFghtf1 were observed (Figure 4C and 4D). These results showed that the aberrant ΔFghtf1 macroconidia can germinate properly and be pathogenic on hosts.
Expression of FgHTF1 correlates with conidiophore development in F. graminearum

In order to investigate the temporal and spatial pattern of FgHTF1 expression during conidiogenesis, FgHTF1 gene with its native promoter was fused in-frame to the green fluorescent protein (GFP)-encoding gene. The construct was then transformed into Dfghtf1 protoplasts. Subsequently, we isolated three transformants expressing GFP in hyphae, and the presence of the FgHtf1-GFP construct was confirmed by PCR (data not shown). All positive transformants (Dfghtf1-GFP) produced a similar number of conidia when compared to the wild-type progenitor. To investigate the expression patterns of FgHtf1 during conidia germination in F. graminearum, we followed GFP expression by fluorescence microscopy at different time points (24 h, 36 h, 48 h, 60 h and 72 h) after inoculating Dfghtf1-GFP mycelia into CMC medium, which is conducive to spore production. GFP signals were not detectable or extremely weak from 24 h to 36 h when Dfghtf1-GFP strain was incubated in CMC (data not shown). However, at 48 h GFP signal spiked, and the localization of FgHtf1 to nucleus was verified by GFP and ethidium bromide (EB) stain (Figure 5A).

To study expression patterns of FgHTF1 in PH-1, we extracted total RNA from PH-1 cultured in CMC medium at 24 h, 36 h, 48 h, 60 h and 72 h. Real-time PCR detected a high-level expression of FgHTF1 at 48 h during the sporulation-induced

Table 1. Characterization of Dfghtf1 and complementation transformants.

| Strain     | Growth (cm) | Conidiation 3 d (10^5/ml) | Conidiation 6 d (10^5/ml) | Conidiation 15 d (10^5/ml) | Foot cell of conidium | Conidium 3 d (10^5/ml) | Conidium 6 d (10^5/ml) | Conidium 15 d (10^5/ml) | Phialide cell | Germination (%) | Wheat disease level |
|------------|-------------|---------------------------|---------------------------|---------------------------|-----------------------|------------------------|------------------------|------------------------|---------------|-----------------|-------------------|
| PH-1       | 6.12±0.07   | 5.14±0.64                 | 15.00±6.6                 | 1.00±0.38                 | normal                | 1.41±0.45              | 4.11±0.45              | 14.56±6.82             | normal        | 98.9±1.39       | 8.11±1.90          |
| Dfghtf1    | 6.10±0.04   | 6.87±6.82                 | 18.45±6.82                | 1.00±0.38                 | normal                | 1.41±0.45              | 4.11±0.45              | 14.56±6.82             | normal        | 98.8±1.35       | 8.11±1.90          |
| Dfghtf1-Com| 6.12±0.07   | 5.14±0.64                 | 15.00±6.6                 | 1.00±0.38                 | normal                | 1.41±0.45              | 4.11±0.45              | 14.56±6.82             | normal        | 98.9±1.39       | 8.11±1.90          |
| Dfghtf1-Fv | 6.14±0.04   | 5.56±5.56                 | 14.65±6.56                | 1.00±0.38                 | normal                | 1.41±0.45              | 4.11±0.45              | 14.56±6.82             | normal        | 98.9±1.39       | 8.11±1.90          |
| Dfghtf1-Fo | 6.16±0.05   | 5.86±5.56                 | 14.75±6.56                | 1.00±0.38                 | normal                | 1.41±0.45              | 4.11±0.45              | 14.56±6.82             | normal        | 98.9±1.39       | 8.11±1.90          |

Radial growth was measured as the diameter of colonies after 3 days incubation on complete agar medium. Means and standard errors were calculated from three independent experiments. Growth was measured as the number of spores/ml after given days of growth on carboxymethylcellulose media. Conidiation was measured as the number of phialides and subsequent macroconidia borne on terminal phialides while the ΔFghtf1 mutant produced aberrant terminal phialides but failed to form macroconidia. tp, terminal phialides; ma, macroconidia; cp, conidiophore; h, hyphae. Bar = 20 μm.

Expression of FgHTF1 correlates with conidiophore development in F. graminearum

In order to investigate the temporal and spatial pattern of FgHTF1 expression during conidiogenesis, FgHTF1 gene with its native promoter was fused in-frame to the green fluorescent protein (GFP)-encoding gene. The construct was then transformed into ΔFghtf1 protoplasts. Subsequently, we isolated three transformants expressing GFP in hyphae, and the presence of the FgHtf1-GFP construct was confirmed by PCR (data not shown). All positive transformants (ΔFghtf1-GFP) produced a similar number of conidia when compared to the wild-type progenitor. To investigate the expression patterns of FgHtf1 during conidia germination in F. graminearum, we followed GFP expression by fluorescence microscopy at different time points (24 h, 36 h, 48 h, 60 h and 72 h) after inoculating ΔFghtf1-GFP mycelia into CMC medium, which is conducive to spore production. GFP signals were not detectable or extremely weak from 24 h to 36 h when ΔFghtf1-GFP strain was incubated in CMC (data not shown). However, at 48 h GFP signal spiked, and the localization of FgHtf1 to nucleus was verified by GFP and ethidium bromide (EB) stain (Figure 5A). To study expression patterns of FgHTF1 in PH-1, we extracted total RNA from PH-1 cultured in CMC medium at 24 h, 36 h, 48 h, 60 h and 72 h. Real-time PCR detected a high-level expression of FgHTF1 at 48 h during the sporulation-induced

Figure 2. FgHtf1 regulates the differentiation of phialides and subsequent macroconidiation. (A) Wild-type strain PH-1 and complementation strain ΔFghtf1-Com produced abundant macroconidia borne on terminal phialides while the ΔFghtf1 mutant produced aberrant terminal phialides but failed to form macroconidia. tp, terminal phialides; ma, macroconidia; cp, conidiophore; h, hyphae. Bar = 20 μm. (B) Fluorescence staining of nuclei with DAPI demonstrated that phialide-like structures in wild type were uninucleate (white arrow). Bar = 20 μm. (C) Fluorescence staining of nuclei with 4′,6-diamidino-2-phenylindole (DAPI) observes clumps consisting of hyphal segments in ΔFghtf1 mutant. Bar = 20 μm.

doi:10.1371/journal.pone.0045432.g002
strain indicated the position of cell division. s, septa, n, nucleus. Black arrows in PH-1 with DAPI and calcofluor white (CFW) to visualize nuclei and septa, resulting cells were observed with a DIC microscope and also stained matured conidium with clear septation from the tip of hyphae. The septation directly from hyphae.

The Htf1-regulated macroconidiation is conserved in F. verticillioides and F. graminearum

While macroconidia are the predominant form of asexual spores in F. verticillioides, it also produces macroconidia in nature and in certain laboratory conditions [36,53]. When we monitored the development process of macroconidiation in F. verticillioides, we observed the mechanism similar to F. graminearum, in which macroconidia are produced on solitary phialides or on multiple phialides borne on conidiophores (Figure 7A). In AΔfhtf1, we found that the macroconidiogenesis from phialide was impaired (Figure 7A), however, the mutant used budding pattern to produce foot cell-defective macroconidia, which is identical to what we observed in F. graminearum AΔfhtf1 mutant (Figure 7A).

In addition, we assayed for the amount of macroconidia in mung bean liquid medium under continuous dark and UV light conditions. In continuous dark condition, the wild-type and complemented strains produced a similar level of macroconidia, which typically accounts for approximately 5% of the total conidia harvested from F. verticillioides cultures after 7 days. Under the same conditions, however, only 2% of the conidia were macroconidia in the AΔfhtf1 mutant (Figure 7B). These data suggested that FvHTF1 plays an important role in macroconidiation development. UV light is known to stimulate macroconidia production in Fusarium species [36,53], and under UV light approximately 17% of the total conidia harvested after 7 days of incubation were macroconidia in wild-type and complemented strains. This is a significant increase when compared to the continuous dark condition. However, in AΔfhtf1 mutant the percentage of macroconidia (2%) was consistent with that which are customary taxonomic features of F. verticillioides.
produced under continuous dark condition (Figure 7C). These results suggested that FvHTF1 is important for macroconidia production and that it may play a role in cellular responses to UV light stimulus.

FoHTF1 is also required for the development of microconidia and macroconidia, but not for chlamydospores

The results we obtained from F. graminearum and F. verticillioides studies led us to further explore F. oxysporum conidiogenesis. This fungus produces three types of asexual spores: microconidia, macroconidia, and chlamydospores. To determine whether the function of Htf1 is conserved in F. oxysporum conidiogenesis, we generated a gene-replacement mutant of FoHTF1 (Figure S6). The mutant (DFOhtf1) was normal in vegetative growth and microconidia morphology (Figure S7), but when assayed for conidiation on SNA medium we found that DFOhtf1 had a significant reduction in macroconidia and a slight reduction in microconidia when compared to the wild type (Figure 8A and 8B). The DFOhtf1 and the wild-type strain were examined under an optical microscope, and we noticed that the mutants lacked normal false head microconidia and formed windmill-shaped structure, which was congruent with microconidiogenesis in DFvhtf1 mutant (Figure 8C). However, DFOhtf1 produced normal chlamydospores acrogenously from hyphae or by the modification of hyphal cells, as the wild type (Figure 8D). Phialides of macroconidia also redundantly proliferated and developed constant extension very similar to DFghtf1 and DFvhtf1 mutants (Figure 8E). These results indicate that FoHTF1 is important for conidiophore and phialide development, and ultimately microconidia and macroconidia production. However, we concluded that FoHTF1 is not involved in hyphal differentiation that leads to chlamydospores in F. oxysporum.

The function of Htf1 is conserved in three Fusarium species

Htf1 in three Fusarium species showed highly conserved functions in macroconidiogenesis and microconidiogenesis. F. graminearum FgHtf1 homeodomain and whole protein sequence
share greater than 95% and 85% amino acid identity, respectively, when compared to FvHtf1 and FoHtf1 (Figure 1B). To test whether Htf1 homologs from three Fusarium species are functional orthologs, we transformed FvHTF1 and FoHTF1 genes with their respective promoter regions into Dfghtf1 mutant. Positive transformants were identified by PCR with respective specific primers (Table S2), and these showed rescued conidiogenesis in Dfghtf1 mutant when incubated in CMC medium (Figure 9A, Table 1). In addition, we also transformed FgHTF1 gene into the DfVhtf1 mutant. Significantly, the complemented strain DfVhtf1-Fg produced abundant microconidia in chain and false head shape although F. graminearum species does not produce microconidia (Figure 9B). These results suggest that Htf1 is conserved in three Fusaria and that FgHtf1 can transcriptionally regulate F. verticillioides proteins that are involved in microconidiogenesis.

Discussion

Conidiation is an important characteristic in fungi that requires spatial and temporal regulation of gene expression that leads to specialized cellular differentiation and intercellular communications [1,2,10]. In our previous study, we found that HTF1 is essential for conidiation in M. oryzae. Further observation revealed that ΔMohtf1 mutant produces greater amounts of conidiophores, which showed curvature slightly near the tip but could not develop into sterigmata-like structures (Figure 10) [50]. This led us to conclude that MoHTF1 is an essential positive regulator responsible for switching from conidiophore maturation to the initiation of conidia development in M. oryzae. Concurrently, we also proposed that MoHTF1 functions as a negative regulator of conidiophore development. In other filamentous fungi, homeodomain transcription factors have been linked to the shaping of fruiting body structure, sexual reproduction, and mycelial branch formation [49,54–56]. In this study, we hypothesized that, while there are similarities and conservation in HTF1 gene function between M. oryzae and Fusarium species, there are divergent biological features exhibited by Htf1 in Fusarium species. In three Fusarium species, we found that the deletion of HTF1 also abolished macroconidia development from conidiophores.
Figure 7. Macroconidiation in \(Dvhtf1\) mutant. (A) In wild-type strain (A149) and the complementation strain (\(Dvhtf1-\text{Com}\)), incipient macroconidia without septation are produced on terminal phialides or hyphae. The gene-deletion mutant (\(Dvhtf1\)) failed to form macroconidia from aberrant terminal phialides. \(Dvhtf1\) produced mature conidium with clear septation from the tip of the hyphae. The macroconidia of \(Dvhtf1\) deletion mutant showed a morphological defect. These observed phenotypes in \(Dvhtf1\) are consistent with what we witnessed in \(F.\) graminearum mutant (\(Dghtf1\)). tp, terminal phialides; ma, macroconidia; nr, narrow region, ac, apical cell; bc, basal cell (foot cell). Bar = 20 \(\mu\)m. (B) Macroconidia production by A149, \(Dvhtf1\), and \(Dvhtf1-\text{Com}\) in mung bean liquid medium under continuous dark conditions. (C) Macroconidia production by A149, \(Dvhtf1\), and \(Dvhtf1-\text{Com}\) in mung bean liquid medium under UV light conditions.

doi:10.1371/journal.pone.0045432.g007

Figure 8. Conidiation in \(Fohtf1\) mutant. (A) The wild-type \(F.\) oxysporum (WT) and the gene-deletion mutant (\(Fohtf1\)) strains were assayed for macroconidia production in SNA medium under continuous UV light. (B) WT and \(Fohtf1\) strains were assayed for macroconidia production in SNA medium under continuous UV light. (C) WT and \(Fohtf1\) strains were grown on SNA medium for 5 days. In the wild-type strain, microconidia were produced from phialides generally in false heads. \(Fohtf1\) lacked prototypical false head microconidia but rather formed a windmill-shaped microconidia head (a–c). Bar = 20 \(\mu\)m. (D) Chlamydospores are formed from hyphae of WT and \(Fohtf1\) strains. Bar = 20 \(\mu\)m. (E) Aberrant terminal phialides and macroconidia produce by \(Fohtf1\) mutant. tp, terminal phialides; ma, macroconidia; ac, apical cell; bc, basal cell (foot cell). Bar = 20 \(\mu\)m.

doi:10.1371/journal.pone.0045432.g008
addition, Ahf1 mutants in these Fusarium species failed to form morphologically discernible phialide, but rather forming “clusters” consisting of hyphal segments (Figure 10). This phenotype was more profound in F. graminearum than F. verticillioides and F. oxysporum, however it was consistently observed in three Fusaria. In addition, the deletion of HTF1 in Fusarium species caused excessive elongation of conidiogenous cell, suggesting that Htf1 is a negative regulator of conidiogenous cell development similar to M. oryzae.

But we also discovered a major difference in conidiogenesis between M. oryzae and Fusarium species. In M. oryzae, the conidiogenous cell usually was deemed as conidiophore, while in Fusarium species it often represent phialides, and therefore it would be reasonable to presume that the function of HTF1 in Fusarium species underwent a further specialization for phialidogenesis. Some genes associated with phialide development have been reported in Fusarium species, such as FgStuA in F. graminearum, FoStuA and REN1 in F. oxysporum. The sequence of FgStuA protein showed a very high level of homology (72%) with FoStuA [27,29]. Not surprisingly, the deletion mutants of FgStuA and FoStuA lacked conidiophores and uninucleate phialides, suggesting a conserved function in two orthologs. In A. nidulans, where phialide conidiation has been extensively studied, stuA mutants produced significantly stunted conidiophores and lacked normal metulae and phialides [57,58]. It is also recognized that stuA affects conidiation through the spatial and temporal modifier of bnlA and abaA expression [39,60]. However, FgStuA regulates sexual development and pathogenicity in addition to conidiogenesis, suggesting that this transcription factor may have a broader and diverse impact on F. graminearum lifestyle [27]. Significantly, in contrast to FgStuA all gene-deletion mutants we studied (ΔMohf1, ΔFght1, ΔFvhf1, ΔFgf1) showed phenotypic deformity only limited to conidiogenesis. Our SAGE data (unpublished) and previously published microarray study in PH-1 and ΔFgstua [27] showed no reciprocal influence in FgHTF1 and FgSTUA, and it is reasonable to hypothesize that FgStuA and FgHtf1 regulate phialide development through different cellular networks.

REN1 encodes a protein analogous to A. nidulans MedA and M. oryzae Acr1, and all of these are involved in conidiogenesis [28]. The ren1 mutant strains lacked normal conidiophores and phialides and formed rod-shaped, conidium-like cells directly from hyphae by acropetal division [28], but maintained pathogenicity on host. These results showed that Ren1 specifically regulates conidiogenesis. In our study, we concluded that FgHTF1 does not directly play a role in conidial germination and pathogenicity, although there were many significant defects in phialidogenesis and macroconidiogenesis in ΔFghtf1 mutant. This similarity in cellular function led us to further analyze the expression of REN1 orthologous gene FGSG_02471 in ΔFghtf1 mutant. However, the result showed no significant change in expression (data not shown), suggesting that these two transcription factors are not epistatic. However, it remains to be tested whether these two genes regulate signaling pathways that converge downstream and impact conidiogenesis in F. graminearum.

In Fusarium species, macroconidia have distinct basal foot cell and pointed distal ends. In this study, we discovered that macroconidia in Ahf1 mutants, those produced through hyphal budding, exhibit significant defect in the foot cell (Figure 10). When we monitored conidiogenesis in microscopic detail, we recognized that the first initial conidium of the wild-type strain is formed within the apical extension of the phialide or hyphae at the early stage of development. Before the macroconidium is released, the characteristic foot cell at the base of macroconidium is formed.
Nevertheless, at late stages of development, the expanding conidium ruptures the conidiophore wall and is released by an abscissional splitting of the basal septum. Internal septation of the conidium normally occurs after it is released. In the \( \text{Afhtf1} \) mutant, it is unlikely that a conidium is released by abscissional splitting of the basal septum (Figure 10). Perhaps this is why we found mature conidium with distinct septum born on hyphae in the \( \text{Afhtf1} \) mutant. This implies that Htf1 play a key role in regulating foot cell division during macroconidiogenesis.

Production of both microconidia and macroconidia is a common phenomenon in most \( \text{Fusarium} \) species, and both conidia are formed from phialides in false heads by basipetal division, the developmental mode from the apex toward the base without catenation of cells [28]. In our study, we learned that Htf1 not only regulated macroconidiogenesis, but also for microconidiogenesis. In \( \text{Afhtf1} \) mutant, long chains of microconidia were completely absent in contrast to the wild-type strain (Figure 10). In \( F. \text{verticillioides} \), the cAMP signaling pathway gene \( \text{EAC1} \) and two hydrophobin genes \( \text{HYD1} \) and \( \text{HYD2} \) have been reported to be important for the production of microconidial chains [39]. In addition, Choi and Xu [36] further showed that \( \text{EAC1} \) positively regulates microconidia production and the expression of two hydrophobin genes, \( \text{HYD1} \) and \( \text{HYD2} \) [36]. Notably, all these reported genes had no discernible effect on false-head pattern of microconidia, suggesting that these gene-deletion mutants still can produce microconidia through basipetal division. Intriguingly, our study revealed that the deletion of \( \text{HTF1} \) in \( F. \text{verticillioides} \) and \( F. \text{oxysporum} \) led to the formation of petal-shaped pattern by sharing the apical branches (Figures 6 and 10) instead of producing typical false heads with characteristic ball-shaped assemblage of microconidia held together apparently by mucilage. As we described earlier, this aberrant microconidiogenesis from monophialide may have interfered with typical microconidia chain development that occurs through basipetal division and ultimately led to significant reduction in microconidia in \( F. \text{verticillioides} \) and \( F. \text{oxysporum} \). Therefore, our results collectively provide evidences that Htf1 regulates microconidial formation from chains and false heads by basipetal division in \( \text{Fusarium} \) species.

Materials and Methods

Strains, media and growth condition

All wild-type and mutant strains used in this study are listed in Table S1. In \( F. \text{graminearum} \), growth and morphology were evaluated by culturing strains on complete medium (CM: 0.6% yeast extract \([\text{w/v}]\), 0.6% casein hydrolysate \([\text{w/v}]\), and 1% sucrose \([\text{w/v}]\) at 28°C for 4 days. Formation of perithecia was assayed on wheat kernels medium as described previously [61]. To assay conidiation, an agar block (3 mm in diameter) carrying mycelia was introduced into 50 ml of liquid CMC medium [52]. The suspension was shaken at 180 rpm for 3–15 days, and the mycelia was introduced into 50 ml of liquid CMC medium [52]. The suspension was shaken at 180 rpm for 3–15 days, and the concentration of conidia was determined with a hemacytometer. For spore germination assays, fresh macroconidia were suspended in CM for 4 h with gentle agitation [62]. Macroconidia of PH-1 and mutants were observed using an Olympus BX51 Microscope. Infection assays on flowering wheat heads, wheat coleoptiles and corn stalks were conducted as previously described [63–65].

\( F. \text{verticillioides} \) strains were grown on CM agar and mung bean agar medium (5% mung bean \([\text{w/v}]\), 1.5% agar \([\text{w/v}]\), pH 6.0) to observe morphology and growth. For macro- and microconidiation assays, a culture block (3 mm in diameter) was inoculated on synthetic low-nutrient agar (SNA) medium, containing (all in \( \text{w/v} \)) 0.1% KH\(_2\)PO\(_4\), 0.1% KNO\(_3\), 0.05% MgSO\(_4\), 7H\(_2\)O, 0.05% KCl, 0.02% glucose, 0.02% sucrose, and 2% agar, and mung bean agar or broth. After incubation at 25°C for 7 days under continuous near-ultraviolet (UV) light or dark condition [66], conidiation was observed under a light microscope (Olympus BX51).

The \( F. \text{oxysporum} \) wild-type and mutant strains were cultured on CM agar to observe morphology and growth. To induce conidiation in \( F. \text{oxysporum} \) strains, SNA and mung bean agar were used as described above. All tests were repeated three times.

Microscopy and histological visualization

To observe conidiogenesis in \( F. \text{graminearum} \), an agar block carrying mycelium was inoculated into CMC as described above and then were imaged at different culture stages with Olympus BX51 Research Microscope. Nuclear visualization in phialides was observed by DAPI staining. Mycelia were collected by centrifugation, washed with PBS buffer (pH 7.2) and then resuspended in PBS containing 0.1% Triton X-100. Cells were then fixed with PBS paraformaldehyde (3.7%, \( \text{w/v} \)) and stained with 10 μg/ml DAPI (Sigma). The cell nuclei were observed with Olympus BX51 Research Microscope at UV excitation wavelength. For spore germination studies, fresh PH-1 macroconidia were suspended in CM for 4 h with gentle agitation. Cell walls and septa of germinating conidia were visualized by staining with Calcofluor White (10 mg/ml, Sigma).

To directly visualize \( F. \text{verticillioides} \) and \( F. \text{oxysporum} \) conidial chains and false heads without immersion in water or buffer, agar squares were removed from actively growing colonies and placed in a slide glass with the fungal colony surface oriented perpendicular to the cover slip. Images were acquired from Olympus BX51 Research Microscope.

For scanning electron microscopy (SEM), blocks of 5-day-old mung bean agar cultures (5 mm\(^2\)) were fixed in 4% glutaraldehyde at 4°C for 16 h. The samples were then dehydrated in a graded ethanol series and dried in a critical point dryer as described [67]. Samples were coated with a thin gold layer and observed with JSM-6360LV (Jeol Ltd., Tokyo) scanning electron microscope.

Fungal transformation and generation of gene-deletion mutants

The \( F. \text{graminearum} \), \( F. \text{verticillioides} \) and \( F. \text{oxysporum} \) protoplast preparation and fungal transformation were performed following standard protocols [29,53,63]. Hygromycin- or neomycin-resistant transformants were selected on media supplemented with 250 g/ml hygromycin B (Roche Applied Science) or 200 g/ml G418 (Invitrogen).

To generate the \( \text{Afhtf1} \) mutant, a 1,291-bp fragment upstream from \( \text{Fghtf1} \) was amplified with primers FG07097AF and FG07097AR (Table S2). An isolate that tested positive with PCR was further verified by Southern blot screening by PCR with primers FG07097UA and H853 and then fixed with PBS paraformaldehyde (3.7%, \( \text{w/v} \)) and stained with 10 μg/ml DAPI (Sigma). The plasmid was transformed into protoplasts of the wild-type PH-1 strain as described [63]. Hygromycin-resistant transformants were screened by PCR with primers FG07097BF and FG07097BR, and cloned into the \( \text{BanHI} \) and \( \text{SacI} \) sites downstream of the \( \text{hph} \) cassette on PCM53 [68]. Then, 1,033-bp fragment downstream from \( \text{Fghtf1} \) was amplified with primers FG07097BF and FG07097BR, and cloned into the \( \text{BanHI} \) and \( \text{SacI} \) sites downstream of \( \text{hph} \) cassette, and this plasmid was transformed into protoplasts of the wild-type PH-1 strain as described [63]. Hygromycin-resistant transformants were screened by PCR with primers FG07097UA and H853 and primers FG07097OF and FG07097OR (Table S2). An isolate that tested positive with PCR was further verified by Southern blot analysis performed with the digoxigenin high prime DNA labeling and detection starter Kit I (Roche, Mannheim, Germany).

We generated \( \text{HTF1} \) gene-replacement constructs in \( F. \text{verticillioides} \) and \( F. \text{oxysporum} \) using the split-marker approach [69,70]. Upstream and downstream fragments were amplified with specific primer pairs that are listed in Table S2. Partial fragments of the hygromycin phosphotransferase (\( \text{hph} \)) gene were amplified with primers HYG/F, HY/R, YG/F, and HYG/R as
were similar to generation of the complementation construct, and further validated by hygromycin were selected, screened by PCR for the presence (pKNTG). Transformants exhibiting resistance to both geneticin constructs were co-transformed into protoplasts of the target fungal strain FV08072CF/FV08072CR, respectively (Table S2). The resulting FvHTF1 (with upstream promoter and downstream terminator) or FgHTF1 was amplified with a set of primers (Table S2), and subsequently co-transformed into the target fungal protoplasts with pKNTG vector. The selected isolates were further analyzed by PCR, using primers (Table S2) to determine the presence of FgHTF1, FvHTF1 or FgHTF1 gene.

Construction of FgHTF1-GFP vector and complementation

The FgHTF1-GFP fusion vector, pGM-FgHTF1-GFP, was constructed by amplification of 2,886-bp fragment including 1,459-bp FgHTF1 coding sequence and a 1,427-bp promoter region using primers FG07097CF3-GFP and FG07097CR3-GFP (Table S2). The 2,886-bp PCR product was then cloned into pGEM-T easy vector to generate pGM-FgHTF1. The 1.5-kb GFP alelle [72] carrying the A. nidulans trpC terminator was amplified using primers HindIII-GFPF and HindIII-GFPFR (Table S2), then cloned into pGEM-T easy vector. It was subsequently digested with HindIII to release the GFP allele with HindIII sticky ends, which was inserted into HindIII site of pGM-FgHTF1 to create pGM-FgHTF1-GFP. We verified the orientation of GFP insertion and in-frame fusion by sequencing the pGM-FgHTF1-GFP vector. To generate FgHTF1-GFP strain, pGM-FgHTF1-GFP vector and pKNTG vector were cotransformed into Afghtf1 mutant. Transformants carrying a single insertion were selected and phenotypic restoration in Afghtf1 mutants was sought. GFP fluorescence was observed using a Leica TCS SP5 inverted confocal laser scanning microscope (Leica, Germany).

Quantitative RT–PCR

Wild-type conidia were harvested at growth stages (24 h, 36 h, 48 h, 60 h and 72 h incubated on CMC medium). RNA was isolated with TRIzol reagent (Invitrogen) and purified with the purelink RNA purification kit (Qiagen). Primers used to amplify selected genes in qRT-PCR reactions are listed in supplemental Table S2. TUB2 (FGSG_06610.3) was used as the endogenous reference gene. The relative quantification of each transcript was calculated by the 2−ΔΔCt method [73]. All qRT-PCR reactions were conducted in triplicates for each sample and the experiment was repeated three times.

Supporting Information

Table S1 Wild-type and mutant strains of fungi used in this study.

(LOC)

Table S2 PCR primers used in this study.

(LOC)

Figure S1 Analysis of putative Htf1 homeobox transcription factors in fungi. (A) Schematic description of HTF1 gene structure, namely intron/exon boundaries, in Fusarium species (FvHTF1, FoHTF1, and FgHTF1) and Magnaporthe oryzae (MoHTF1). Gray blocks and gray lines indicate exons and introns, respectively. Numbers on right indicate deduced protein sequence length in amino acids. (B) Sequence alignment of F. graminearum (FgHtf1), F. verticillioides (FvHtf1), F. oxysporum (FoHtf1) and M. oryzae (MoHtf1). (TIF)

Figure S2 The FgHTF1 gene-replacement construct and mutants. (A) Schematic diagram of the genomic region of the FgHTF1 and hph genes. Primers F1 (FG07097AF), R1 (FG07097AR), F2 (FG07097BF) and R2 (FG07097BR) were used to generate FgHTF1 gene replacement constructs, and OF1 (FG07097OF), OR1 (FG07097OR), F1 (FG07097AF) and R1 (FG07097AR) were used for mutant screening and identification. S, Sal I. (B) DNA gel blots of Sal I-digested genomic DNA were hybridized with FgHTF1 upstream fragment as the probe (shown in Figure S2A). PH-1, wild-type strain; Afghtf1-Com, complementation strain; Afghtf1-7 and Afghtf1-8, null mutants. (TIFF)

Figure S3 Vegetative growth and fertility in F. graminearum wild type (PH-1) and Afghtf1 mutant. (A) Colonies PH-1 and Afghtf1 grown on CM agar for 4 days. (B) PH-1 and Afghtf1 were incubated on wheat kernels medium for 2 weeks to induce formation of perithecia. No significant difference was observed. (TIFF)

Figure S4 F. verticillioides FoHTF1 gene-replacement strategy and confirmation. (A) Schematic diagram of the genomic region of the FoHTF1 and hph genes. Primers F1 (FV08072AF), R1 (FV08072AR), F2 (FV08072BF) and R2 (FV08072BR) were used to generate FoHTF1 gene replacement constructs. Probe 1 and probe 2 were used to screen and verify gene replacement mutants. K, Kpn I. (B) DNA gel blots of KpnI-digested genomic DNA were hybridized with probe 1 and probe 2. A149, wild-type F. verticillioides; Afohtf1-Com, complementation strain; Afohtf1-9 and Afohtf1-15, null mutants. (TIFF)

Figure S5 Colony and microconidia morphology of F. verticillioides wild type (A149) and Afohtf1 mutant. (A) Colony morphology of A149 and Afohtf1 mutant grown on CM and MB agar for 6 and 7 days, respectively. (B) Microconidia stained with 4′,6-diamidino-2-phenylindole (DAPI) observed under a fluorescence microscope. Bar = 10 μm. (TIFF)

Figure S6 F. oxysporum FoHTF1 gene-replacement strategy and confirmation. (A) Schematic diagram of the genomic region of the FoHTF1 and hph genes. Primers F1
(FO01706AF), R1 (FO01706AR), F2 (FO01706BF) and R2 (FO01706BR) were used to generate FoHT1 gene replacement constructs. Probe was used to screen and verify gene replacement mutants. N, noN. (B) DNA gel blots of noN-digested genomic DNA were hybridized with probe. WT, wild-type F. oxysporum. FoHT1-3, FoHT1-6, FoHT1-9 and FoHT1-11, null mutants. FoHT1-Ec, ectopic strain. (TIFF)

Figure S7 Colony and microconidia morphology of F. oxysporum wild type (WT) and FoHT1 mutant. (A) Colony morphology of WT and FoHT1 mutant grown on CM agar for 6 days. (B) Microconidia stained with 4’6-diamidino-2-phenylindole (DAPI) observed under a fluorescence microscope. Bar = 10 μm.

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Acknowledgments

We would like to thank Drs Wende Liu (Chinese Academy of Agricultural Sciences, Beijing, China), Jin-Rong Xu (Purdue University, USA), and Daniel Ebbole (Texas A&M University, USA) for helpful discussion.

Author Contributions

Conceived and designed the experiments: WZ, GL, WBS, ZW. Performed the experiments: WZ XZ QX QH CZ HZ. Analyzed the data: WZ LX GL WBS ZW. Wrote the paper: WZ ZW. Originated research leading up to this paper and provided guidance and review: LX GL WBS ZW.

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