Short communication

Expression of microRNA-like RNA-2 (Fgmil-2) and bioH1 from a single transcript in Fusarium graminearum are inversely correlated to regulate biotin synthesis during vegetative growth and host infection

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SUMMARY

MicroRNA-like RNAs (milRNAs) post-transcriptionally down-regulate target genes. We investigated Fusarium graminearum (Fg) miRNA expression during fungal vegetative growth and infection of wheat. Small RNA sequencing identified 36 milRNAs from Fg, one of which, Fgmil-2, had >100 transcripts per million in conidia, mycelia and infected wheat, with the highest expression in conidia and the lowest expression in colonized wheat tissue. Fgmil-2 displays perfect homology to the 3′-untranslated region (3′-UTR) of an FgbioH1 messenger RNA that is involved in biotin biosynthesis. Poly(A) polymerase-mediated rapid amplification of cDNA ends combined with sequencing analysis demonstrated that cleavage at a specific site by FgDicer2 in the 3′-UTR of FgbioH1 transcripts generated the Fgmil-2 precursor with a typical hairpin structure. Deletion of FgbioH1 or FgDicer2 genes abolished Fgmil-2 biogenesis. FgbioH1 had an inversely correlated pattern of expression to that of Fgmil-2 and FgDicer2. Deletion of FgbioH1 also showed that it is required for mycelial growth, virulence, mycotoxin biosynthesis and expression of biotin-dependent carboxylase genes. This study reveals in Fg a novel mode of inversely correlated post-transcriptional regulation in which Fgmil-2 originates from its own target transcript, FgbioH, to govern biotin biosynthesis.

Keywords: biotin biosynthesis, Fusarium graminearum, microRNA-like RNAs, mycotoxin, post-transcriptional regulation, small RNA sequencing.

The ascomycete fungal phytopathogen Fusarium graminearum (Fg) is the globally distributed causal agent of fusarium head blight (FHB) of wheat, costing billions of dollars in lost agricultural productivity (Goswami and Kistler, 2004; Qu et al., 2008). Fg produces trichothecene mycotoxins in infected grains, which are highly toxic to humans and domestic animals (Pestka and Smolinski, 2005). Elucidation of the regulatory mechanisms of Fg pathogenesis is thus essential for FHB control strategies. To this end, we sought to identify the potential roles of microRNAs (miRNAs) in fungal pathogenesis. miRNAs or microRNA-like RNAs (milRNAs) post-transcriptionally down-regulate genes in eukaryotes (Bartel, 2004; Torresmartínez and Ruizvázquez, 2017). In animals, primary transcripts (pri-miRNAs) are initially cleaved by the ribonuclease (RNase) III Drosha, then transported to the cytoplasm and cleaved into 22 nt (nucleotide) miRNA duplexes by Dicer, a cytoplasmic RNase III (Court et al., 2013; Havens et al., 2014; Kurzynska-Kokorniak et al., 2015).

In contrast, fungal milRNAs are produced by at least four different pathways, but lack Drosha (Lee et al., 2010). In Fg, FgDicer2 was shown to be involved in hairpin-induced gene silencing during vegetative growth (Chen et al., 2015). Identification of the targets of miRNA regulation is also important for understanding the mechanisms of Fg development and pathogenesis. In particular, biotin, a highly conserved vitamin cofactor for biotin-dependent carboxylases (Pirner and Stolz, 2006), has been shown to be essential for development in yeast and other eukaryotes. Furthermore, biotin plays a role in the epigenetic regulation of gene expression and cell signalling in mammals (Zempleni et al., 2009).

In this study, RNA deep sequencing identified 36 milRNAs in Fg, one of which, Fgmil-2, displayed a differential pattern of
high expression in conidia and mycelia but substantially lower expression during infection of wheat. We found that this pattern of expression was inversely correlated with transcription of \( \text{Fg} \text{bioH1} \) mRNA, the translated product of which catalyses the hydrolysis of pimeloyl-[ACP] methyl ester to pimeloyl-[ACP], an essential step in the biotin biosynthesis pathway. To explore the roles of \( \text{Fg} \text{bioH1} \) and \( \text{Fgmil-2} \) in the infection process, we generated deletion mutants of \( \text{Fg} \text{bioH1} \) and \( \text{FgDicer2} \). In the absence of \( \text{Dicer2} \), only \( \text{FgbioH1} \) mRNAs were produced. In the absence of \( \text{FgbioH1} \), fungal growth, successful infection of spikelets and mycotoxin synthesis all decreased. We thus propose that a \( \text{Dicer-2} \)-mediated post-transcriptional regulatory switch determines the spatiotemporal and inversely correlated expression of \( \text{Fgmil-2} \) and \( \text{bioH1} \) from a single transcript during the transition from vegetative growth to host colonization by \( \text{Fg} \). These results provide foundational insights into the mechanisms and regulation of miRNA biogenesis as well as a putative role for biotin in fungal vegetative growth and infection of plants.

To systematically identify \( \text{Fg} \) miRNAs transcribed during vegetative growth and infection of wheat, two small RNA (sRNA) libraries were constructed from conidia and from mycelia of \( \text{Fg} \) strain 5035, a deoxynivalenol (DON)-producing strain isolated from a spike of scabby wheat in China (Method S1, see Supporting Information), and four sRNA libraries were constructed from wheat spikes at 0, 48, 72 and 96 h after inoculation (hai) with \( \text{Fg} \). The total number of clean reads from each sample that matched the \( \text{Fg} \) genome are listed in Table S1 (see Supporting Information) and four sRNA libraries were constructed from wheat spikes at 0, 48, 72 and 96 hai (479–735 TPM). Further northern blot experiments confirmed that \( \text{Fgmil-1} \) and \( \text{Fgmil-2} \) were both expressed at high levels in conidia and mycelia while \( \text{Fgmil-3} \) was undetectable in these samples. In contrast, only \( \text{Fgmil-2} \) was detected in infected wheat spikes, with the highest expression at 48 hai (Fig. 1D and Table S3, see Supporting Information), thus confirming the reliability of the sRNA sequencing results. Interestingly, \( \text{Fgmil-2} \) was found to have the same sequence as \( \text{Fg-miRNA-5} \) identified by Chen et al. (2015) in mycelia of \( \text{Fg} \) strain HN9-1. The transcript of origin and its expression in conidia and infected plants were not examined in that work. These patterns of expression and miRNA characteristics led us to more closely examine the origin and role of \( \text{Fgmil-2} \) in \( \text{Fg} \) development.

Unexpectedly, no predicted target sequences aligning with \( \text{Fgmil-2} \) were identified in wheat or other crops. This finding prompted us to search the \( \text{Fg} \) genome for potential precursor mRNAs. We found that \( \text{Fgmil-2} \) aligned with the \( \text{3'-UTR} \) of \( \text{FGSG}_01659 \), within which NCBI blastp revealed a pimeloyl-[ACP] methyl ester carboxylesterase (bioH) domain (Fig. S4, see Supporting Information). This \( \text{Fg} \) gene was thus designated \( \text{FgbioH1} \). Given that \( \text{Dicer2} \) is involved in miRNA biogenesis in \( \text{Fg} \) (Chen et al., 2015), we generated \( \text{FgbioH1} \) and \( \text{FgDicer2}-\text{deletion mutant strains}, \Delta \text{FgbioH1} \) and \( \Delta \text{FgDicer2} \) (Table S3, see Supporting Information), to experimentally verify that \( \text{Fgmil-2} \) is encoded within \( \text{FgbioH1} \). After confirmation by Southern blot of successful deletion at both loci (Fig. S5, see Supporting Information), \( \Delta \text{FgDicer2}, \Delta \text{FgbioH1} \) and wild-type (WT) \( \text{Fg} \) strain 5035 were subjected to sRNA HiSeq platform sequencing. While WT 5035 was shown to contain 1495 TPM of \( \text{Fgmil-2} \), no \( \text{Fgmil-2} \) transcripts were detected in the \( \Delta \text{FgDicer2} \) or \( \Delta \text{FgbioH1} \) strains (Fig. 2A). These results indicate that both \( \text{FgDicer2} \) and \( \text{FgbioH1} \) genes are essential for \( \text{Fgmil-2} \)-biogenesis.

To obtain the sequence from which \( \text{Fgmil-2} \) is generated, poly(A) polymerase-mediated rapid amplification of cDNA ends (PPM-RACE) was used to amplify transcripts derived from the \( \text{3'-UTR of FgbioH1} \) mRNAs lacking poly(A) tails. Total RNAs were isolated from \( \Delta \text{FgDicer2}, \Delta \text{FgbioH1} \) and WT strain 5035 and used for PPM-RACE and 3' RACE (Table S3, see Supporting Information). Each RNA sample was divided into two parts: one part was used for PPM-RACE and 3' RACE, and the other was used for 3' RACE only.

Simultaneous PPM-RACE/3' RACE of WT 5035 RNA generated two DNA fragments with different sizes, whereas 3' RACE of WT 5035 produced only one larger fragment (Fig. 2B), indicating...
the presence of two species of FgbioH1 transcripts in the WT strain. In contrast, PPM-RACE/3’RACE and 3’RACE alone of ΔFgDicer2 RNAs produced only one large DNA fragment, indicating the presence of only one type of FgbioH1 transcript in the ΔFgDicer2 strain. These results confirm the presence of two types of FgbioH1 transcripts in WT strain 5035: large integral FgbioH1 transcripts and small Dicer2-cleaved FgbioH1 transcripts. However, only the large uncleaved FgbioH1 transcripts were observed in ΔFgDicer2, indicating that FgDicer2 participates in generating smaller FgbioH1-derived transcripts.

To determine the precise location of the Fgmil-2 sequence within the FgbioH1 transcript, we examined the PPM-RACE and 3’RACE amplicons. Sequence analysis revealed that the large fragments from strains WT 5035 and ΔFgDicer2 (Fig. 2B) were all identical 1154 bp FgbioH1 gene sequences with poly(A) tails, whereas the small fragment was a 972 bp subfragment of FgbioH1 with a stretch of poly(A), indicating a 182 bp size difference (Fig. 2C). The 3’-UTR of the FgbioH1 mRNA was 295 nt and contained the Fgmil-2 precursor (105 nt), as predicted by MIREAP software; thus, the Fgmil-2 precursor starts 103 nt downstream of the UGA stop codon. Sequence analysis showed a guanine base at the 3’ end of the PPM-RACE-derived small fragment, indicating that the FgDicer2 cleavage site is located between guanine (113) and uracil (114) to generate the Fgmil-2 miRNA (Fig. S6, see Supporting Information). The Fgmil-2 precursor has a typical stem-loop structure and a free energy of folding of −62.20 kcal/mol (Fig. 2D). Taken together, these results demonstrate that the Fgmil-2 precursor is located in the 3’-UTR of FgbioH1 mRNA, from which Fgmil-2 is generated by FgDicer2 cleavage.
Expression of microRNA-like RNA-2 (Fgmil-2) and bioH1

Typically, miRNAs or milRNAs are located within protein-coding regions (intragenic miRNAs) and target other genes for down-regulation (Baskerville and Bartel, 2005; Rodriguez et al., 2004). Recently, a primate-specific exonic miR-198 hairpin was identified in the 3′-UTR of FSTL1 (Follistatin-like 1) mRNA, for which Drosha served as a transcriptional switch by cleavage of the miRNA hairpin, ultimately generating two alternative gene products from a single transcript (Ha and Kim, 2014; Sundaram et al., 2013). To our knowledge, no study has reported finding two such transcriptional products generated from a single mRNA in fungi, where Drosha is absent.

To determine the impact of FgDicer2 on FgbioH1, we compared the relative expression levels of these two genes in conidia, mycelia and infected wheat at 24, 48, 72, 96 and 120 hai in both WT 5035 and ΔFgDicer2 strains. FgDicer2 was most highly expressed in WT conidia but sharply declined in mycelia, while its lowest expression was during the infection process (Fig. 3A). Conversely, FgbioH1 expression was lowest in WT conidia and highest during the infection of wheat, with intermediate expression levels in mycelia. Expression of these two genes is thus inversely correlated. Moreover, in the ΔFgDicer2 strain the FgbioH1 gene showed high constitutive expression across all the samples and timepoints. These results strongly indicated that FgDicer2 negatively regulated FgbioH1 expression, and that functional loss of the FgDicer2 gene resulted in constitutive expression of FgbioH1.

We thus propose that FgDicer2 plays a decisive role in post-transcriptional regulation of FgbioH1 and displays a development-dependent expression pattern (Fig. 3A) that is perfectly...
consistent with that of Fgmil-2 (Fig. 1). FgDicer2 cleaves the Fgmil-2 hairpin structure in the FgbioH1 3'-UTR, causing the mRNA to become vulnerable to degradation by other ribonucleases. Furthermore, the 3' poly(A) tail is an important determinant of translational efficiency. Thus, FgDicer2-deletion clearly correlates with increased FgbioH1 expression. This activity by FgDicer2 differs from its homologues in that Drosha or Dicer enzymes canonically cleave miRNAs that target other gene sequences for regulation; in those cases Drosha or Dicers directly regulate the target genes, whereas FgDicer2 directly effects bioH1 expression because Fgmil-2 targets the same transcripts from which it is generated.

To determine the role of FgbioH1 in fungal growth and biotin synthesis, we cultured three Fg strains, WT 5035, ΔFgbioH1 and an FgbioH1-complemented mutant FgBIOH1C (Fig. S5 and Table S3, see Supporting Information) in the presence and absence of biotin. Deletion of FgbioH1 significantly reduced mycelial growth and biomass (Fig. 3B,C), resulting in a dry weight (DW) of 0.045 g for ΔFgbioH1, which was 53% lower (P < 0.01) than WT 5035 (DW = 0.097 g); FgBIOH1C restored fungal growth and biomass to levels comparable with WT (Fig. 3B,C). To verify that the reduced mycelial growth was due to the lack of biotin, ΔFgbioH1 was cultured in media supplemented with biotin. In the presence of biotin, ΔFgbioH1 growth and biomass (DW = 0.078 g) were restored to levels comparable with (i.e. not significantly different from) the WT. Thus, biotin biosynthesis depends on FgbioH1 and is required for vegetative growth of Fg.

In order to compare biotin production among WT 5035, ΔFgbioH1, ΔFgDicer2 and FgBIOH1C, HPLC was used to measure biotin in samples of each strain (Fig. 3D). WT strain 5035 produced 7.87 μg/g (dry weight) biotin, whereas ΔFgbioH1 had 2.58 μg/g, a 67% reduction (P < 0.01) relative to WT. The FgBIOH1C complementation strain produced 5.15 μg/g biotin, while ΔFgDicer2 produced 8.75 μg/g, a significant (P < 0.05) 11% increase over production in WT. These results indicate that the FgbioH1 gene is a predominant contributor to biotin synthesis.
Expression of microRNA-like RNA-2 (Fgmil-2) and bioH1 biosynthesis, while FgDicer2 down-regulates biotin biosynthesis, the deletion of which increases biotin content in Fg. Notably, biotin synthesis was not completely abolished by FgbioH1 deletion. This phenomenon is likely attributable to functional redundancy by another bioH, FGSG_03039, with 38% amino acid similarity to FgbioH1, but lacking homology to the Fgmil-2 3’-UTR sequence (Fig. S4).

Fgmil-2 accumulated to its highest level in resting conidia and so we are inclined to speculate that this relationship may be part of a regulatory state necessary for maintaining low biological activity in conidia. In Aspergillus niger, resting conidia were found to exhibit a low level of respiratory metabolism in aqueous suspension (Novodvorska et al., 2016). However, regardless of low metabolic activity that may or may not also occur in Fg conidia, Fgmil-2 comprises the predominant FgbioH1 transcript species and biotin does not appear to be required for biomass production and mycelial growth until germination and subsequent host colonization. As conidia germinate to form mycelia, many enzymes are synthesized that require biotin as a cofactor to execute their function; at this point, FgbioH1 transcripts accumulate to higher levels to ensure adequate biotin synthesis (Fig. 3A). Expression of either form of the original transcript therefore depends on the functional requirements of each Fg developmental stage (Figs 1 and 3).

To further characterize the role of the FgbioH1 gene in host colonization in planta, we inoculated wheat with ΔFgbioH1, FgBIOH1C and WT 5035, and compared the differences in virulence between the strains. We found that WT 5035 infected 29% of the spikelets at 14 dai, similar to that of FgBIOH1C (Fig. 4A,B). In contrast, ΔFgbioH1 showed an 8% spikelet infection rate, a 78% reduction compared to WT. These results demonstrate that loss of FgbioH1 function results in a substantially decreased

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Fig. 4 Impacts of FgbioH1 on virulence, mycotoxin biosynthesis and gene expression. (A) Representative wheat spikes 14 days after inoculation (dai) with Fusarium graminearum wild-type (WT) strain 5035, ΔFgbioH1 and complementation strain FgBIOH1C. (B) Percentage of infected wheat spikelets at 14 dai. Percentages were calculated as means ± SD for each strain. Different letters indicate significant difference at P < 0.05. (C) Contents of mycotoxin deoxynivalenol:ergosterol. Data from three biological replicates are averages ± standard errors. Different letters indicate a significant difference at P < 0.05. (D) Fold changes in transcript levels from five genes in ΔFgbioH1 relative to that of WT. Histograms represent fold increase in transcripts from five genes BPL, DUR1,2, ACC, PC and PCCA that use biotin as a cofactor. Line bars denote standard errors of three biological replicates.
ability to colonize plant hosts. As fungal hyphae extend into plant tissues, biotin-dependent enzymes deplete available biotin, and to subsequently accommodate this need the highest expression of bioH1 transcripts was then observed.

Given the importance of up-regulating FgbioH1 expression for successful host colonization, it was also important to test if this gene participated in mycotoxin biosynthesis, an important component of pathogenesis. We evaluated mycotoxin production in the ΔFgbioH1, FgbioH1C and WT 5035 strains by calculating their DON:ergosterol ratios. The ΔFgbioH1 strain showed a significantly lower DON:ergosterol ratio (0.45) than WT 5035 (1.69), a 47% reduction (Fig. 4C). Thus, the FgbioH1 gene is also required for biosynthesis of DON in Fg at levels consistent with successful colonization of wheat.

To probe if FgbioH1 expression also affects the expression of biotin-dependent carboxylase-encoding genes in Fg, we used qPCR assays to compare the transcriptional levels of five biotin-dependent carboxylase genes between the WT and ΔFgbioH1 strains. From studies in humans and yeast showing regulation by or dependence on biotin as a co-factor, we selected BPL (FGSG_08329), urea amidohydrolase DUR1,2 (FGSG_10913), an acetyl-CoA carboxylase (ACC) (FGSG_06580), pyruvate carboxylase (PC) (FGSG_07073) and a propionyl-CoA carboxylase α subunit (PCCA) (FGSG_08688) (Solórzano-Vargas et al., 2002) (Table S3, see Supporting Information). Expression levels of all five genes assayed in the ΔFgbioH1 strain were increased 2.3- to 2.8-fold compared with that of the WT strain 5035 (Fig. 4D).

Thus, FgbioH1 expression decreases expression of other biotin-dependent carboxylase-type genes in Fg, similar to what has been reported in yeast (Pirner and Stolz, 2006). Down-regulation of carboxylase genes may directly impact fatty acid biosynthesis and gluconeogenesis, which are essential for primary metabolism (Magliano et al., 2011).

Recently Zhang et al. (2016) showed that during Fg infection the bioH1 gene displayed enhanced expression whereas, in contrast, biotin-dependent enzymes such as DUR1,2, PC and PCCA all had reduced expression compared to their expression in mycelia. This study is in agreement with our results in that both demonstrate an important function for the bioH1 gene during plant pathogenesis. Importantly, the inversely correlated expression pattern of Fgmil-2 and FgbioH1 transcripts revealed by this study provides insight into the post-transcriptional regulation governing biotin biosynthesis during fungal colonization of plants.

In addition, this study reveals the sub-pathway branch for production of the intermediate compound pimeloyl-[ACP] in Fg. The biotin biosynthesis pathway was originally proposed based on studies in Escherichia coli because of a dearth of studies regarding biotin biosynthesis in fungi (Fig. 57). In E. coli, three sub-pathways were found for the synthesis of the two intermediate compounds, pimeloyl-[ACP] and pimeloyl-CoA, which are substrates for subsequent reactions. Two bioH genes in Fg, FgbioH1 and FgbioH2 are both likely involved in the same sub-pathway: both genes encode proteins predicted to catabolize the hydrolysis of pimeloyl-[ACP] methyl ester to form pimeloyl-[ACP]. Analysis of the Fg genome sequence indicated that Fg does not carry the genes involved in the other two sub-pathways (bioI and bioW). Thus, the biosynthesis of pimeloyl-[ACP] is the only sub-pathway in Fg to synthesize this intermediate compound for subsequent biotin production.

This study can serve as the foundation for further characterization of the regulatory mechanisms for genes in the biotin biosynthesis pathway in fungi.

In conclusion, we show that a microRNA-like RNA in Fg, Fgmil-2, was derived from the 3′-UTR of an FgbioH1 messenger RNA and that the Dicer2-dependent biogenesis of Fgmil-2, through truncation of FgbioH1 transcripts, led to FgbioH1 degradation and subsequent down-regulation. Inversely correlated expression of Fgmil-2 and FgbioH1 from the single bioH1 transcript was dependent on developmental stage, while Fgmil-2 had the highest expression in resting conidia and the lowest expression during infection of wheat, in contrast to FgbioH1 expression, which was lowest in conidia and highest during infection. FgbioH1 is involved in the biosynthesis of biotin, which is required for vegetative growth, virulence, mycotoxin biosynthesis and expression of various genes in Fg. This study provides insight into the mechanisms of miRNA biogenesis and regulation in pathogenic fungi during development and interaction with host plants. Given the essential nature of biotin for Fg development, FgbioH1 may serve as a target for RNA interference to control FHB and mycotoxins in agricultural production.

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Fig. S1 Relative proportions of total sRNA sequences in six samples of *Fusarium graminearum* (Fg) and wheat. (A) Conidia. (B) Mycelia. (C) Wheat spikes at 0 h after inoculation with *Fg*. (D) Wheat spikes at 48 h after inoculation with *Fg*. (E) Wheat spikes at 72 h after inoculation with *Fg*. (F) Wheat spikes at 96 h after inoculation with *Fg*.

Fig. S2 Nucleotide frequency of the 5’ ends of sRNAs in six samples of *Fusarium graminearum* (Fg) and infected wheat. (A) Conidia. (B) Mycelia. (C) Wheat spikes at 0 h after inoculation with *Fg*. (D) Wheat spikes at 48 h after inoculation with *Fg*. (E) Wheat spikes at 72 h after inoculation with *Fg*. (F) Wheat spikes at 96 h after inoculation with *Fg*.

Fig. S3 Clustering analysis of predicted miRNAs in *Fusarium graminearum* (Fg). The heatmap shows the 36 Fg miRNAs from six samples from conidia, mycelia and wheat spikes 0, 48, 72 and 96 h after inoculation with *Fg*.

Fig. S4 Schematic diagrams of amino acids from bioH1 and bioH2 and alignment of amino acid sequences of FgbioH1 and FgbioH2 with other bioH members from bacteria. (A) Yellow boxes indicate pimeloyl-ACP methyl ester carboxylesterase (bioH) domains. (B) The alignment was made using the PROMALS3D multiple sequence and structure alignment server (prodata.swmed.edu/promals3d). Representative sequences are coloured according to predicted secondary structures (red: alpha-helix, blue: beta-strand). Consensus predicted secondary structure symbols: alpha-helix, h; beta-strand, e. Consensus amino acid symbols: conserved amino acids are represented by bold and uppercase letters; aliphatic (I, V, L): I, aromatic (Y, H, W, F): @, hydrophobic (W, F, Y, M, L, I, V, A, C, T, H): h, hydrophilic (S, T): o, polar residues (D, E, H, K, N, Q, R, S, T): p, tiny (A, G, C, S): t, small (A, G, C, S, V, N, D, T, P): s, bulky residues (E, F, I, K, L, M, Q, R, W, Y): b, positively charged (K, R, H): +, negatively charged (D, E); –, charged (D, E, K, R, H).

Fig. S5 Southern blot analyses of gene-deletion strains. For analyses of ∆FgbioH1 and ∆FgbioH2 mutant strains, a NEO fragment was used as a probe. For analyses of complementation strains FgbioHTC, a hygromycin fragment was used as a probe.

Fig. S6 Sanger sequencing chromatograms displaying PPM-RACE products of large and small fragments including the G-U junction uncleaved region.

Fig. S7 Proposed biotin biosynthesis pathway. Numbers in circles indicate three sub-pathways for the production of two intermediate compounds, pimeloyl-[ACP] (1 and 2) and pimeloyl-CoA (3). The dashed arrow represents multiple steps.

Table S1 Summary statistics of small RNA sequence mapping result (mapping to the *Fusarium graminearum* reference genome).

Table S2 Summary statistics of sRNA libraries of miRNA.

Table S3 PCR primers and sequences used in this study.

Method S1 Experimental procedures.