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Research

Stage-specific regulation of purine metabolism during infectious growth and sexual reproduction in *Fusarium graminearum*

Manli Sun[1], Zhuyun Bian[2], Qiaoqiao Luan[1], Yitong Chen[1], Wei Wang[1], Yongrong Dong[1], Lingfeng Chen[1], Chaofeng Hao[1], Jin-Rong Xu[2] and Huiquan Liu[1]

[1]State Key Laboratory of Crop Stress Biology for Arid Areas, NWAFU-Purdue Joint Research Center, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China; [2]Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA

Authors for correspondence
Huiquan Liu
Email: liuhuiquan@nwsuaf.edu.cn

Jin-Rong Xu
Email: jinrong@purdue.edu

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Summary

- Ascospores generated during sexual reproduction are the primary inoculum for the wheat scab fungus *Fusarium graminearum*. Purine metabolism is known to play important roles in fungal pathogens but its lifecycle stage-specific regulation is unclear.
- By characterizing the genes involved in purine *de novo* and salvage biosynthesis pathways, we showed that *de novo* syntheses of inosine, adenosine and guanosine monophosphates (IMP, AMP and GMP) are important for vegetative growth, sexual/asexual reproduction, and infectious growth, whereas purine salvage synthesis is dispensable for these stages in *F. graminearum*.
- Addition of GMP rescued the defects of the *Fgimd1* mutant in vegetative growth and conidiation but not sexual reproduction, whereas addition of AMP rescued all of these defects of the *Fgade12* mutant, suggesting that the function of *de novo* synthesis of GMP rather than AMP is distinct in sexual stages. Moreover, Acd1, an ortholog of AMP deaminase, is dispensable for growth but essential for ascosporogenesis and pathogenesis, suggesting that AMP catabolism has stage-specific functions during sexual reproduction and infectious growth. The expression of almost all the genes involved in *de novo* purine synthesis is downregulated during sexual reproduction and infectious growth relative to vegetative growth.
- This study revealed that *F. graminearum* has stage-specific regulation of purine metabolism during infectious growth and sexual reproduction.

Introduction

Fusarium graminearum is one of the causal agents of Fusarium head blight (FHB), a major fungal disease of wheat and barley worldwide. In addition to causing yield losses, this pathogen produces mycotoxins such as deoxynivalenol (DON) (Desjardins, 2003; De Walle *et al.*, 2010). Infection of wheat heads by *F. graminearum* starts with airborne spores landing on flowering spikelets. After spore germination, *F. graminearum* colonizes the surface of wheat florets with specialized runner hyphas. The epiphytic runner hyphas differentiate into several types of infection structures, including simple foot structures, lobate appressoria and complex infection cushions for plant penetration (Boenisch & Schafer, 2011; Bormann *et al.*, 2014). After penetration, *F. graminearum* develops flat, coralloid invasive hyphas between the cuticle and epidermal cells, and bulbous invasive hyphas within epidermal cells (Pritsch *et al.*, 2000; Rittenour & Harris, 2010). Under favorable conditions, invasive hyphas can spread rapidly to neighboring spikelets via the rachis and cause FHB (Wanjiru *et al.*, 2002; Brown *et al.*, 2010). To date, no wheat germplasm is immune to FHB and the best available resistance is against fungal spreading throughout the spike (Li *et al.*, 2019; Su *et al.*, 2019).

For *F. graminearum*, DON is a virulence factor known to be important for spreading through rachis tissues (Bai *et al.*, 2002; Jansen *et al.*, 2005). Recent studies have identified a number of proteins with various biochemical functions as well as orphan proteins that play critical roles in spreading of invasive hyphas in infected wheat heads (Wang *et al.*, 2011; Son *et al.*, 2011b; Yun *et al.*, 2015; Shin *et al.*, 2017; Lee *et al.*, 2018; Jiang *et al.*, 2019; Jiang *et al.*, 2020). Like many other fungal pathogens, invasive hyphas of *F. graminearum* differ in morphology from vegetative hyphas (Rittenour & Harris, 2010; Zhang *et al.*, 2012; Bormann *et al.*, 2014). Infectious growth (growth of invasive hyphas) *in planta* also is known to be under distinct transcriptional and cell cycle regulation relative to vegetative or saprophytic growth (growth of vegetative hyphas) in cultures in *F. graminearum* (Zhang *et al.*, 2012; Liu *et al.*, 2015; Jiang *et al.*, 2016).

Ascospores are the primary inoculum in *F. graminearum,* a homothallic fungus that produces abundant perithecia on infected plant residues (Lee *et al.*, 2003). Both repeat-induced point mutation and meiotic silencing of un-paired DNA, two sexual stage-specific phenomena, have been reported in

F. graminearum (Cuomo *et al.*, 2007; Son *et al.*, 2011a). A-to-I messenger (m)RNA editing also occurs specifically during sexual reproduction in *F. graminearum* (Liu *et al.*, 2016a). RNA editing of *PUK1*, *FgAMA1* (ortholog of yeast *AMA1*) and *AMD1* is important for ascosporogenesis and ascospore discharge (Liu *et al.*, 2016a; Cao *et al.*, 2017; Hao *et al.*, 2019). A-to-I editing also has been observed in the sexual stage of *Neurospora crassa* and other filamentous ascomycetes (Liu *et al.*, 2017; Teichert *et al.*, 2017; Bian *et al.*, 2019) although fungi lack orthologs of adenosine deaminase acting on RNAs (ADARs) that catalyze mRNA editing in animals (Nishikura, 2010; Savva *et al.*, 2012).

In order to identify the enzymes responsible for A-to-I editing, we identified and characterized all of the 18 *ACD* genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain in *F. graminearum*. Knockout mutants were generated for all but two *ACD* genes (*ACD17* and *ACD18*) that are orthologous to *TAD2* and *TAD3*, two essential genes encoding tRNA-specific adenosine-34 deaminases in *Saccharomyces cerevisiae* (Gerber & Keller, 1999). Among the 16 *acd* mutants characterized, only *acd1* and *acd16* had detectable phenotypes. Although the *acd16* mutant had severe defects in growth, development and pathogenesis, deletion of *ACD1* had no effect on vegetative growth but did block ascosporogenesis and infectious growth. *ACD1* and *ACD16* are orthologous to the yeast *AMD1* AMP deaminase (Sollitti *et al.*, 1993) and *ADE16/ADE17* (Tibbetts & Appling, 2000) genes, respectively, that are involved in purine metabolism. Because the function of purine metabolism in *F. graminearum* is not clear, we then functionally characterized genes involved in purine *de novo* biosynthesis and salvage pathways. Although mutants of four genes involved in purine salvage synthesis had no detectable phenotype, deletion of *ACD16*, *FgADE12* and *FgIMD1* that are involved in *de novo* syntheses of inosine, adenosine and guanosine monophosphates (IMP, AMP and GMP), respectively, resulted in pleiotropic defects. Exogenous GMP rescued the defects of *Fgimd1* in vegetative growth but not sexual reproduction, whereas exogenous AMP rescued all of these defects of the *Fgade12* mutant. More importantly, we showed that *ACD1* had stage-specific functions during sexual reproduction and infectious growth. Interestingly, the expression of almost all of the genes involved in *de novo* purine synthesis was downregulated during sexual reproduction and infectious growth. Taken together, our data provide convincing evidence for stage-specific regulation of purine metabolism in plant pathogenic fungi.

Materials and Methods

Strain culture conditions

The wild-type (WT) strain PH-1 of *Fusarium graminearum* and its transformants were routinely cultured on potato dextrose agar (PDA) at 25°C. Growth on different media and conidiation in carboxymethylcellulose (CMC) liquid cultures were assayed as described (Wang *et al.*, 2011). For sexual reproduction, aerial hyphas of 7-d-old carrot agar (CA) cultures were pressed down with 0.1% Tween 20 for self-fertilization and cultured at 25°C under black light (Wang *et al.*, 2011). For the *Fgimd1* mutant, 10-d-old CA cultures were used because of its growth defect. Perithecia and ascospore cirrhi were examined at 8 d post-fertilization (dpf). For transformation, protoplasts were prepared from germlings collected from 12-h YEPD (1% yeast extract, 2% peptone, 2% dextrose) cultures (Hou *et al.*, 2002; Li *et al.*, 2018). Hygromycin B (MDBio, CAS#101-31282-04-9) and geneticin (DIYIBio, DY80105) were added to the final concentration of 300 μg ml^{-1} and 400 μg ml^{-1}, respectively, for selection. For assaying the effects of exogenous supplements, adenosine monophosphate (AMP) (BBI Life Sciences, A620016), inosine monophosphate (IMP) (Sigma-Aldrich, 57510), guanosine monophosphate (GMP) (Santa Cruz, CAS#85-32-5), adenine (Sigma-Aldrich, A9126), guanine (Sigma-Aldrich, G6779), histidine (BBI Life Sciences, A600806), 8-azaguanine (Runye Biology, S31051) and 8-azaadenine (TGI, A0552) were added to the final concentration of 0.1, 1 or 5 mM.

Targeted gene deletion and complementation assays

In order to generate deletion mutants with the split-marker approach (Catlett *et al.*, 2003), *c.* 1-kb upstream and *c.* 1-kb downstream fragments of the targeted gene were amplified and fused to the N-terminal and C-terminal regions of the hygromycin phosphotransferase (*hph*) gene by overlapping PCR (Wang *et al.*, 2011). After transformation of PH-1 protoplasts, hygromycin-resistant transformants were identified and confirmed by PCR. At least two independent gene replacement mutants with the same phenotypes were identified for each gene (Table 1). For complementation, full-length genes were amplified and co-transformed with *Xho* I-digested vector pFL2 carrying the neomycin/geneticin resistance gene (*neo*) into yeast strain XK1-25 as described (Bruno *et al.*, 2004; Zhou *et al.*, 2011). The complementary constructs were rescued from yeast cells, verified by PCR and Sanger sequencing, and transformed into the corresponding gene deletion mutants. Transformants resistant to both hygromycin and geneticin were screened by PCR and assayed for phenotype complementation. The *ACD1*-green fluorescent protein (GFP) fusion construct was generated using the same approach and transformed into the *acd1* mutant (*ACD*, genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain). The resulting *acd1*/*ACD1*-GFP transformants were screened by PCR and examined for GFP signals. All of the primers used for PCR and sequencing are listed in Table S1.

Targeted replacement of the *ACD1* and *ACD16* genes by their yeast orthologs

The open reading frame (ORF) fragment of yeast *AMD1* was amplified and fused with the promoter region of *ACD1*, and the N-terminal region of *neo* by overlapping PCR. The downstream 1015-bp fragment of the *ACD1* locus was amplified and fused to the C-terminal region of *neo* by overlapping PCR. The two fused fragments were co-transformed into PH-1 protoplasts. The *acd1*/

Table 1 The wild-type (WT) and mutant strains of *Fusarium graminearum* and *Saccharomyces cerevisiae* used in this study.

Strain*	Brief description	Reference
PH-1	WT strain of *F. graminearum*	Cuomo *et al*. (2007)
ACD1-**1**, 6, 8	*acd1* (*Fgamd1*) deletion mutants of PH-1	This study
ACD2-2, 4	*acd2* deletion mutants of PH-1	This study
ACD3-7, 9, 10	*acd3* (*Fgaah1*) deletion mutants of PH-1	This study
ACD4-2, 5, 7	*acd4* deletion mutants of PH-1	This study
ACD5-5, 6	*acd5* deletion mutants of PH-1	This study
ACD6-2, 8	*acd6* deletion mutants of PH-1	This study
ACD7-5, 8	*acd7* (*Fgtad1*) deletion mutants of PH-1	This study
ACD8-1, 2	*acd8* (*Fgcdd1*) deletion mutants of PH-1	This study
ACD9-3, 6	*acd9* (*Fgdcd1*) deletion mutants of PH-1	This study
ACD10-2, 5	*acd10* (*Fgfcy1*) deletion mutants of PH-1	This study
ACD11-3, 4	*acd11* deletion mutants of PH-1	This study
ACD12-4, 6	*acd12* deletion mutants of PH-1	This study
ACD13-2, 5	*acd13* deletion mutants of PH-1	This study
ACD14-1, 2	*acd14* (*Fggud1*) deletion mutants of PH-1	This study
ACD15-17,19	*acd15* deletion mutants of PH-1	This study
ACD16-**1**, 27, 34	*acd16* (*Fgade16/17*) deletion mutants of PH-1	This study
AMC1, AMC2	*acd1/ACD1* transformants	This study
AMG1, AMG2	*acd1/ACD1-GFP* transformants	This study
SC317-1, -2, -3	*acd1/AMD1* transformants	This study
ADC1, ADC2	*acd16/ACD16* transformants	This study
SC16-2, -3, -4	*acd16/ADE16* transformants	This study
SC17-1, -5, -8	*acd16/ADE17* transformants	This study
A12, A15	*Fgade12* deletion mutants of PH-1	This study
ID22, ID24	*Fgimd1* deletion mutants of PH-1	This study
HP1, HP2	*Fghpt1* deletion mutants of PH-1	This study
AP1, AP2	*Fgapt1* deletion mutants of PH-1	This study
IDC1, IDC2	*Fgimd1/FgIMD1* transformants	This study
AC1, AC2	*Fgade12/FgADE12* transformants	This study
BY4741	WT strain of *S. cerevisiae*	Winzeler *et al*. (1999)
D1	*amd1* deletion mutant of BY4741	Winzeler *et al*. (1999)
H1	*aah1* deletion mutant of BY4741	Winzeler *et al*. (1999)
SFD-1, -2, -3	*amd1/ACD1* transformants	This study
SFH-1, -2, -3	*aah1/ACD3* (*FgAAH1*) transformants	This study

*For each gene, only independent mutants with the same phenotype were listed. The mutant strain used for complementation assays are in bold.

AMD1 replacement transformants resistant to geneticin were screened by PCR and verified by sequencing with primers listed in Table S1. Similar approaches were used to generate the *acd16/ADE16* and *acd16/ADE17* replacement transformants.

Heterologous complementation of yeast *amd1* and *aah1* mutants

The ORF fragments of *ACD1* and *ACD3* were amplified from cDNA template of PH-1 and fused with *Kpn*I-digested vector pYES2. The resulting constructs were transformed into the yeast *amd1* and *aah1* mutants (Winzeler *et al*., 1999). Ura3$^+$ transformants were isolated and assayed for sensitivity to 1 mM adenine on YPG (1% yeast extract, 2% peptone and 2% galactose) plates.

Plant infection assays

Flowering wheat heads of cultivar XiaoYan 22 (Kang *et al*., 2008) were inoculated with 10 µl of conidium suspensions (2×10^5 conidia ml^{-1}) at the fifth spikelet from the base as described (Gale *et al*., 2007). Infected heads were examined for head blight symptoms and analyzed for the disease index at 14 d post-inoculation (dpi). Infection assays with corn silks and wheat coleoptiles were performed as described (Hou *et al*., 2002; Zhang *et al*., 2012). Inoculated lemmas were sampled at 48 h post-inoculation (hpi), fixed, dehydrated and coated with gold-palladium before being examined for epiphytic runner hyphas and infection cushion with a Jeol 6360 (Jeol Ltd, Tokyo, Japan) scanning electron microscope (SEM) as described (Kang *et al*., 2008; Hu *et al*., 2014).

For assaying invasive hyphas in plant tissues, samples of infected lemma and rachis tissues were collected at 48 hpi and 5 dpi, respectively, fixed with 4% (v/v) glutaraldehyde, dehydrated with ethyl alcohol and embedded in Spurr resin (Hu *et al*., 2014; Li *et al*., 2015). The thick sections then were prepared and stained with 0.5% (w/v) toluidine blue before examination with an Olympus BX-53 microscope. Infected wheat coleoptiles sampled at 36 or 72 hpi and lemma sampled at 72 hpi were boiled in 1 M KOH for 5 min, washed twice with 0.5 M Tris-HCl, and stained with wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488 (Invitrogen^{TM}) before examination for invasive hyphas with an Olympus BX-53 microscope or Nikon A1 confocal microscope after removal of epiphytical hyphas by Q-tips as described previously (Jiang *et al*., 2019). At least three independent replicates were examined, with five samples in each replicate.

Quantitative reverse transcription (qRT)-PCR analysis

RNA was isolated from germlings collected from 24-h YEPD cultures and perithecia harvested at 4 dpf with the TRIzol reagent and used for cDNA synthesized with the Fermentas First cDNA synthesis kit (Thermo Fisher Scientific). The relative expression levels of target genes were assayed by qRT-PCR with primers listed in Table S1 with the *GzUBH* gene (Kim & Yun, 2011) as the internal control (Livak & Schmittgen, 2001). Means and SD were calculated from three biological replicates.

Feeding assays with plant extracts

Spikelet and rachis tissues of cultivar XiaoYan 22 were collected separately (100 g), ground in liquid nitrogen, and resuspended in

100 ml of double-distilled water. After filtration with a 0.45-μm Millipore filter, each filtrate was concentrated to 10 ml. For assaying their effects on growth, spikelet or rachis extracts were added to minimal medium (MM) (Ingraham *et al.*, 1995) plates at the 1:10 (V/V) ratio. For assaying their effects on conidiation and perithecium formation, 5 ml and 1 ml of spikelet or rachis extracts were added to 50 ml CMC and 10 ml of CA medium, respectively.

Assays for the intracellular purine concentrations

hyphas were harvested from 24-h YEPD cultures and perithecia were collected from 4-dpf mating cultures. Spikelet and rachis tissues of cultivar XiaoYan 22 were collected separately. All samples were lyophilized at −40°C for 16 h, ground with liquid nitrogen, and transferred to a centrifuge tube. For every 1 mg of lyophilized samples, 200 μl of 6% (w/v) trichloroacetic acid (TCA) was added. After mixing thoroughly, samples were centrifuged at 1377 ***g*** for 15 min at 4°C. The supernatants were washed four times with 5 × volumes of anhydrous ether to remove TCA before being analyzed by LC-MS as described (Liu *et al.*, 2016b).

RNA-seq analysis

Perithecia of the *acd1* mutant were collected from 6-dpf mating cultures with two biological replicates. Total RNA of each replicate was extracted with the Qiagen RNeasy Micro kit, and poly (A)+ mRNA was enriched with immobilized oligo (dT) as described previously (Liu *et al.*, 2017). Strand-specific RNA-seq libraries were prepared with the NEBNext® Ultra™ Directional RNA Library Prep Kit and sequenced by Illumina HiSeq-2500 with a 2 × 150-bp paired-end read mode at the Novogene Bioinformatics Institute (Beijing, China). For each replicate, ≥ 20 Mb of paired end reads were generated.

The published RNA-seq data of PH-1 (Liu *et al.*, 2016a; Kim *et al.*, 2018; Jiang *et al.*, 2019) were downloaded from NCBI SRA database under accession numbers SRS1044644, SRS1044675, SRS1044677, SRS4360510-SRS4360512, SRS2827555, SRS28 27558 and SRS2827560. The RNA-seq reads of each sample were mapped to the PH-1 genome via HISAT2 (Kim *et al.*, 2015). The number of reads aligned to each gene was calculated by featureCounts (Liao *et al.*, 2014). Gene expression counts were normalized using the Transcripts Per Kilobase Million (TPM) method (Wagner *et al.*, 2012). A-to-I mRNA editing sites were identified as described (Liu *et al.*, 2017).

Results

Characterizing genes encoding putative adenosine/cytidine/guanine deaminase

The *F. graminearum* genome has 18 genes predicted to encode proteins with the putative adenosine/cytidine/guanine deaminase domains (named *ACD* genes; Table S2). Based on published RNA-seq data (Liu *et al.*, 2016a; Jiang *et al.*, 2019), none of them were specifically expressed during sexual reproduction (Fig. S1). However, *ACD6*, *ACD10*, *ACD11* and *ACD15* were upregulated in perithecia compared to vegetative hyphas.

We generated deletion mutants for all of these *ACD* genes except *ACD17* and *ACD18* that are orthologous to yeast *TAD2* and *TAD3*, respectively (Table 1). The *ACD17* and *ACD18* genes likely are essential for viability in *F. graminearum* because we failed to identify knockout mutants after repeated attempts, and their yeast orthologs are essential genes (Gerber & Keller, 1999). For the *ACD* genes with viable mutants, only the *acd1* and *acd16* mutants had detectable phenotypes. All of the other *acd* mutants were normal in growth, sexual reproduction and plant infection (Fig. S2).

ACD1 is important for sexual/asexual reproduction and pathogenesis

ACD1 is orthologous to the *AMD1* AMP deaminase gene (Sollitti *et al.*, 1993) responsible for the hydrolytic cleavage of AMP to IMP and the yeast *amd1* mutant grows normally but has defects in sporulation (Walther *et al.*, 2014). In *F. graminearum*, the *acd1* deletion mutant was not affected in growth but significantly reduced in conidiation (Fig. 1a; Table 2). In CMC cultures, it rarely formed clusters of phialides (Fig. 1b). The *acd1* mutant formed small perithecia lacking asci/ascospores (Fig. 1c) but sporadic normal-sized perithecia were occasionally observed at the edge of mating cultures. It caused only limited symptoms on the inoculated wheat spikelet and corn silks (Fig. 2a,b; Table 2). The *acd1*/*ACD1* and *acd1*/*ACD1*-GFP transformants both were normal as the WT in all phenotypes (Figs 1, 2a,b; Table 2). GFP signals were observed in the cytoplasm in the *acd1*/*ACD1*-GFP transformant (Fig. S3), which is similar to the localization of Amd1-GFP (Huh *et al.*, 2003). These results indicate that although *ACD1* is dispensable for vegetative growth, it is important for conidiation, ascosporogenesis and pathogenesis. Therefore, Acd1 may play a stage-specific role in purine homeostasis during reproduction and infectious growth in *F. graminearum*.

Because *ACD1* is important for ascosporogenesis, we performed RNA-seq analysis to determine whether it is related to A-to-I mRNA editing. We identified a total of 2889 editing sites in 6-dpf perithecia of the *acd1* mutant (Table S3). Based on published RNA-seq data (Kim *et al.*, 2018), developing perithecia of the WT at the same developmental stage (no asci/ascospores) had 2319 editing sites. Thus, *ACD1* is unlikely responsible for A-to-I mRNA editing in *F. graminearum*.

The *acd1* mutant is defective in infectious growth in rachis tissues

Similar to the WT, the *acd1* mutant formed abundant dome-shaped infection cushions on wheat lemma at 2 dpi (Fig. 2c). The extent of invasive hyphal growth in infected lemma tissues also was similar between the WT and *acd1* mutant (Fig. 2d). However, at 5 dpi, invasive hyphas of the *acd1* mutant were observed only rarely in the rachis tissues (Fig. 2e). Under the

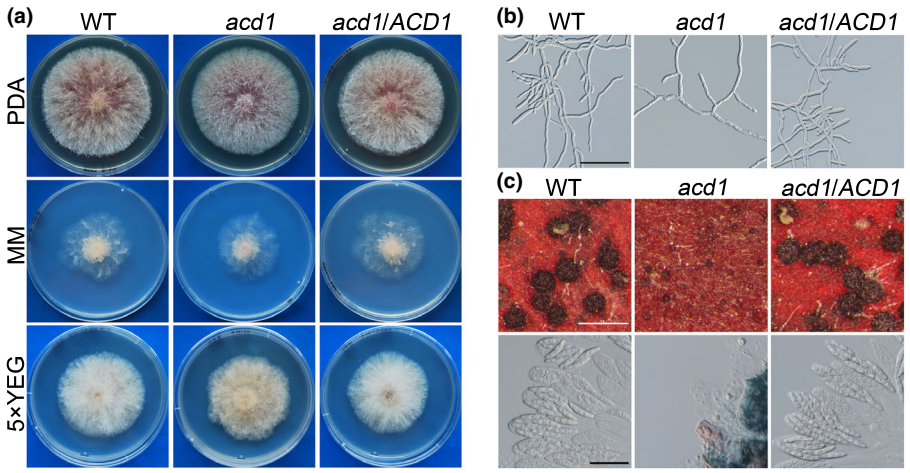

Fig. 1 Defects of the *acd1* mutant of *Fusarium graminearum* in growth, conidiation and sexual reproduction (*ACD*, genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain). (a) Three-day-old cultures of the wild-type stain PH-1 (WT), *acd1* mutant and *acd1/ACD1* complemented transformant grown on potato dextrose agar (PDA), minimal medium (MM) and yeast-extract-glucose (5 × YEG) plates. (b) Phialides produced by PH-1, *acd1* mutant and *acd1/ACD1* transformant in 24-h carboxymethylcellulose (CMC) cultures. Bar, 50 μm. (c) Mating cultures of PH-1, *acd1* mutant and *acd1/ACD1* transformant were examined for formation of perithecia (upper row) and asci/ascospores at 8 d post-fertilization (dpf). Bars: (white) 1 mm; (black) 20 μm.

Table 2 Growth rate, conidiation, and virulence of the *acd1* mutant and complemented transformant of *Fusarium graminearum*.

Strain	Growth rate (mm d^{-1})[a]			Conidiation (10^5 spores ml^{-1})[b]	Disease index[c]
	PDA	MM	5 × YEG		
PH-1 (wt)	10.8 ± 0.1^{A}	7.3 ± 0.1^{A}	9.1 ± 0.2^{A}	18.2 ± 5.8^{A}	13.0 ± 1.6^{A}
ACD1-1 (*acd1*)	10.1 ± 0.1^{A}	7.1 ± 0.1^{A}	9.0 ± 0.2^{A}	3.2 ± 1.3^{B}	0.7 ± 0.5^{B}
AMC1 (*acd1/ACD1*)	11.3 ± 0.1^{A}	7.2 ± 0.2^{A}	8.9 ± 0.3^{A}	20.2 ± 2.9^{A}	12.5 ± 1.8^{A}
AMG1 (*acd1/ACD1*-GFP)	11.0 ± 0.1^{A}	7.1 ± 0.1^{A}	8.9 ± 0.2^{A}	19.2 ± 3.0^{A}	12.8 ± 1.5^{A}

Mean and SD were calculated with results from at least three biological replicates. Data were evaluated by one-way ANOVA followed by Fisher's protected least significant difference test. Different letters mark significant differences ($P = 0.05$). *ACD*, genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain.

[a]Average daily extension in colony radius on potato dextrose agar (PDA), minimal medium (MM) and yeast-extract-glucose (5 × YEG) plates.

[b] Conidiation in 5-d-old carboxymethylcellulose (CMC) cultures.

[c]The average number of diseased spikelets per inoculated wheat head at 14 d post-inoculation (dpi).

same conditions, invasive hyphas of the WT spread from the inoculated spikelets and grew extensively in the rachis tissues (Fig. 2e). These results indicate that *ACD1* is not essential for the initial penetration and colonization but important for the spread and growth of invasive hyphas in the wheat rachis.

ACD1 is important for the differentiation and spread of invasive hyphas

The *acd1* mutant also was defective in infection of wheat coleoptiles and caused only limited necrosis at the inoculation sites (Fig. 3a). Like the WT, the *acd1* mutant invaded coleoptile tissues through the wounding sites and formed compact invasive hyphas longitudinally along coleoptiles at 36 hpi. However, deletion of *ACD1* reduced infectious growth, and invasive hyphas that grew across coleoptiles were rarely observed in the *acd1*-inoculated samples (Fig. 3b). Even at 72 hpi, the *acd1* mutant was significantly reduced in infectious growth.

In inoculated wheat lemma, the *acd1* mutant penetrated cuticles and formed coralloid invasive hyphas (Fig. 3c) that were morphologically distinct from vegetative hyphas (Rittenour & Harris, 2010; Bormann *et al.*, 2014). However, in comparison with PH-1, the *acd1* mutant was significantly reduced in the growth of coralloid hyphas (Fig. 3c), confirming the importance of *ACD1* for the differentiation and spreading of invasive hyphas in plant tissues.

Exogenous IMP or GMP rescues the defect of *acd1* in sexual reproduction and conidiation

Because Amd1 catalyzes the conversion of AMP to IMP and regulates the homeostasis of purine nucleotides in yeast (Saint-Marc *et al.*, 2009), we assayed the effects of exogenous AMP, IMP and GMP on the *acd1* mutant. Addition of 1 mM IMP or GMP rescued its defect in sexual reproduction (Fig. 4a). Interestingly, treatment with AMP not only failed to rescue, but also enhanced

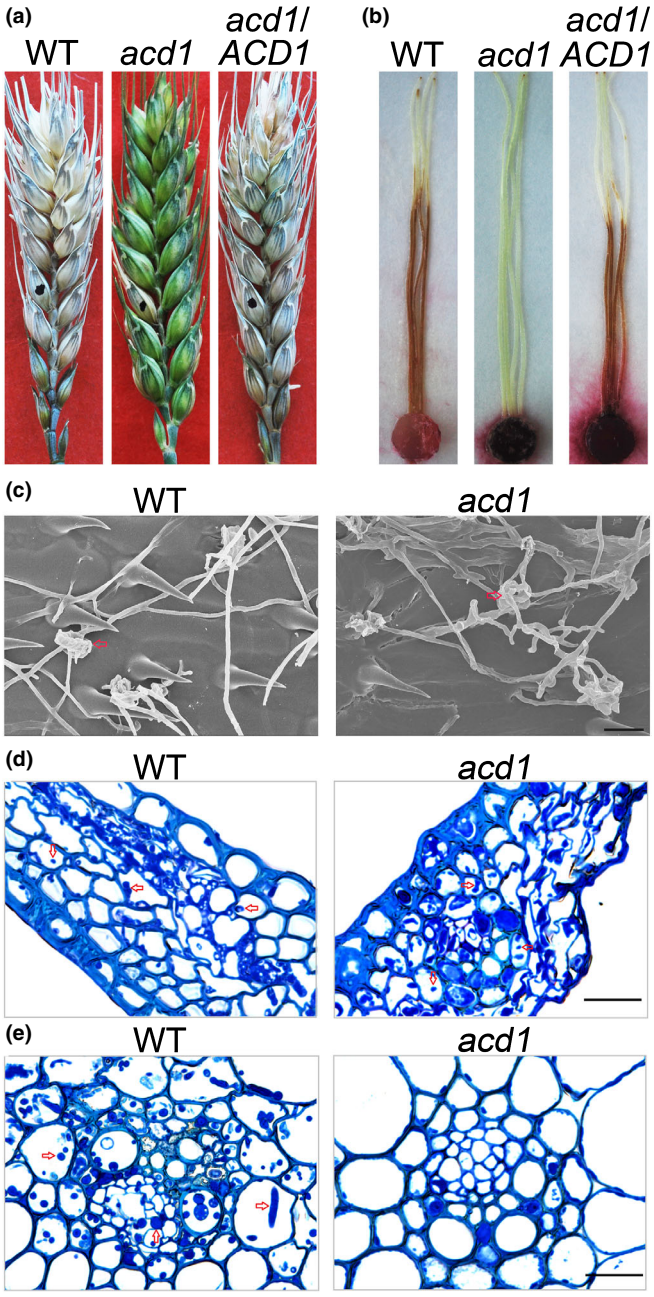

Fig. 2 Defects of *acd1* mutant of *Fusarium graminearum* in plant infection (*ACD*, genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain). (a) Wheat heads inoculated with conidia from the wild-type strain PH-1 (WT), *acd1* mutant and *acd1/ACD1* complemented transformant were photographed at 14 d post-inoculation (dpi). Black dots mark the inoculated spikelets. (b) Corn silks were inoculated with culture blocks of the same set of strains and examined at 5 dpi. (c) Infection cushions (marked with red arrows) formed on lemma inoculated with the marked strains were examined by scanning electron microscope (SEM) at 48 h post-inoculation (hpi). (d) Lemma from spikelets inoculated with WT and *acd1* mutant were examined for infectious growth at 48 hpi. (e) Thick sections of rachis tissues directly below and above the inoculated spikelet were examined for infectious growth at 5 dpi. For (d) and (e), red arrows point to invasive hyphas. Bars, 20 μm.

the defect of *acd1* in sexual reproduction in a concentration-dependent manner (Fig. 4b). Similarly, its defect in conidiation was rescued by exogenous IMP or GMP but enhanced by AMP (Fig. 4c). These results suggest that *ACD1* is important for the homeostasis of AMP/IMP/GMP and deletion of *ACD1* likely results in an imbalance of the intracellular purine nucleotide concentration, which may negatively impact sexual reproduction and conidiation.

We also assayed the effects of exogenous IMP and GMP on the defect of the *acd1* mutant in plant infection. Addition of these compounds to *acd1* conidium suspensions had no obvious effects on its virulence on wheat coleoptiles (Fig. 4d). It is possible that IMP or GMP in conidium suspensions is not sufficient to support infectious growth or is not available to invasive hyphas growing inside plant cells.

Deletion of *ACD1* affects intracellular concentrations of IMP and GMP but not AMP

We then measured the intracellular concentrations of AMP, IMP and GMP by LC-MS. In hyphas collected from 24-h YEPD cultures, the intracellular concentrations of IMP and GMP were significantly reduced in the *acd1* mutant in comparison with the WT although they had a similar concentration of AMP (Table 3). The *acd1* mutant also had lower intracellular concentrations of IMP and GMP than the WT in perithecia collected at 4 dpf (Table 3). However, in the presence of exogenous IMP or GMP, the intracellular IMP and GMP concentrations in both *acd1* hyphas and perithecia returned to the WT concentration (Table 3). These results confirmed that deletion of *ACD1* resulted in a shortage of IMP and GMP during sexual reproduction and vegetative growth.

Expression of yeast *AMD1* partially rescues the defect of the *acd1* mutant in perithecium formation

Similar to the yeast *amd1* mutant (Saint-Marc *et al.*, 2009), the *F. graminearum acd1* mutant showed a growth defect in the presence of adenine (Fig. S4). The domain sequences and active sites of AMP deaminase are well conserved in Acd1 (Fig. S5). To determine whether Acd1 can function as AMP deaminase, we expressed *ACD1* in the yeast *amd1* mutant. The *amd1/ACD1* transformant was normal in growth in the presence of adenine (Fig. S4), suggesting that *ACD1* can functionally replace *AMD1* in yeast. We then replaced the coding region of *ACD1* with that of *AMD1* in *F. graminearum*. In comparison with the *acd1* mutant, the *acd1/AMD1* transformant had similar defects in conidiation and plant infection but produced more normal-sized perithecia (Fig. S6). Approximately 25% of the perithecia formed by the *acd1/AMD1* transformant were normal in size, indicating that expression of yeast *AMD1* can partially rescue the defect of *acd1* mutant in sexual reproduction.

Genes involved in purine *de novo* biosynthesis are downregulated during sexual reproduction and plant infection

One possible explanation for the *acd1* mutant with stage-specific defects in ascosporogenesis and pathogenesis is that *de novo* IMP/GMP synthesis is downregulated in these stages, which makes

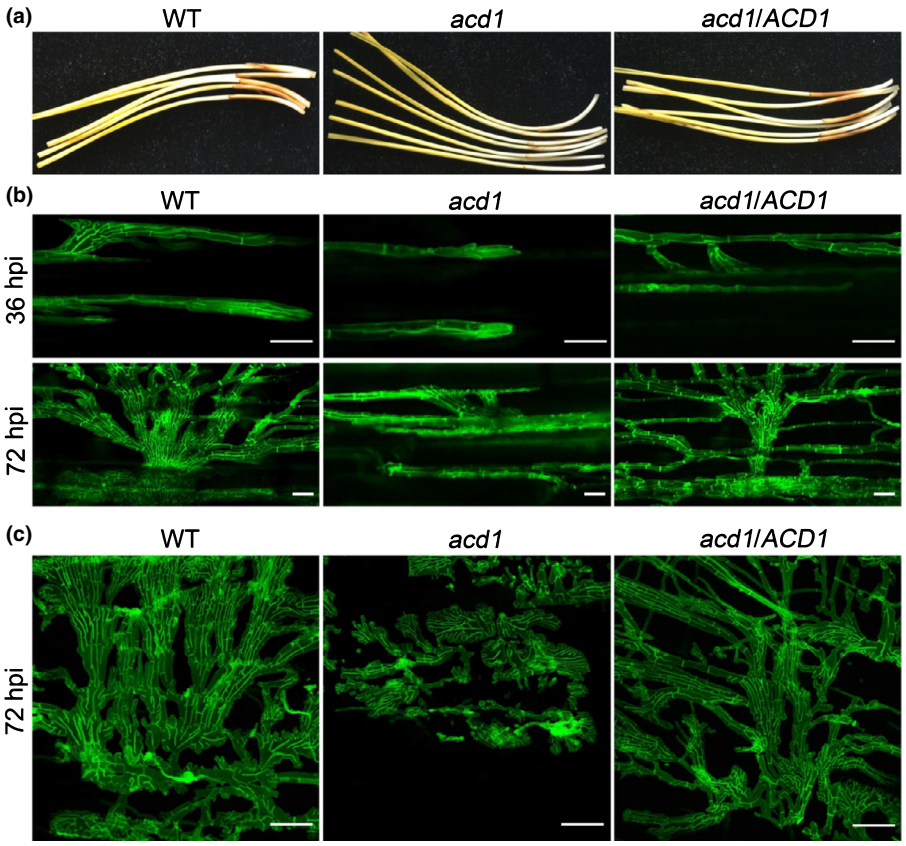

Fig. 3 Defects of the *acd1* mutant of *Fusarium graminearum* in infectious growth in wheat coleoptile cells and lemma tissues (*ACD*, genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain). (a) Wheat coleoptiles inoculated with PH-1 (WT), *acd1* mutant and *acd1/ACD1* transformant were photographed at 7 d post-inoculation (dpi). (b) Infected coleoptiles were examined for invasive hyphas stained with Alexa Fluor 488 at 36 and 72 h post-inoculation (hpi). Bars, 20 μm. (c) Flat, coralloid invasive hyphas growing under the cuticle in infected lemma tissues were examined after staining with Alexa Fluor 488 at 72 hpi. Bars, 20 μm.

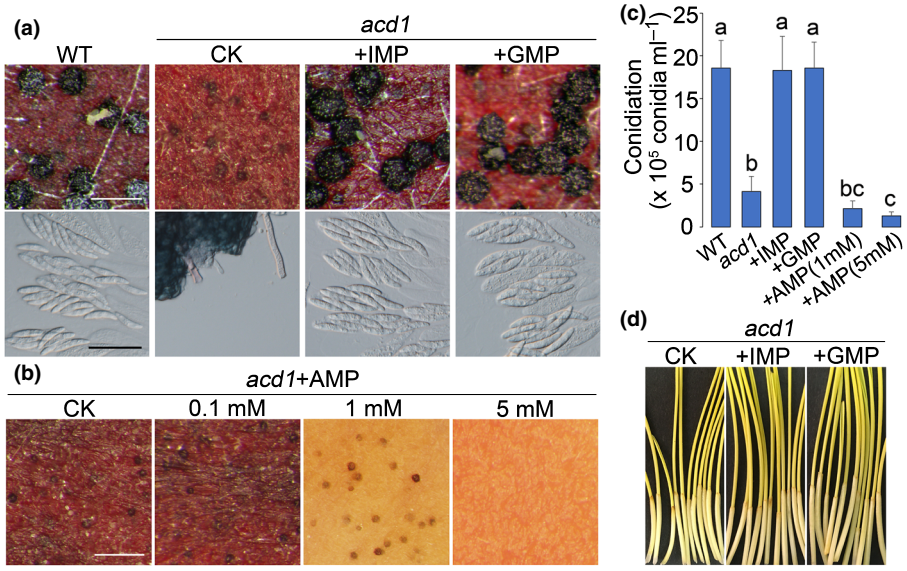

Fig. 4 Effects of exogenous inosine, adenosine and guanosine monophosphate (IMP, AMP and GMP) on sexual/asexual reproduction and virulence of the *acd1* mutant of *Fusarium graminearum* (*ACD*, genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain). (a) Mating cultures of the *acd1* mutant on regular carrot agar (CK) and carrot agar supplemented with 1 mM IMP or GMP were examined for perithecium formation (upper row) and ascus or ascospore development at 8 d post-fertilization (dpf). WT, mating culture of PH-1 as the control. Bars: (white) 1 mm; (black) 20 μm. (b) Mating cultures of the *acd1* mutant on carrot agar supplemented with different concentrations of AMP as indicated were examined for perithecium formation at 8 dpf. Bar, 1 mm. (c) Conidiation in 5-d-old carboxymethylcellulose (CMC) cultures of WT and the *acd1* mutant with or without addition of 1 mM of IMP, GMP or AMP, or 5mM of AMP. Mean and SD were calculated with data from five biological replicates. Different letters show significant difference based on one-way ANOVA analysis followed by Fisher's protected least significant difference test ($P = 0.05$). (d) Wheat coleoptiles inoculated with conidium suspensions of the *acd1* mutant with 1 mM IMP or GMP were photographed at 7 d post-inoculation (dpi).

Table 3 Intracellular inosine, adenosine and guanosine monophosphate (IMP, AMP and GMP) concentrations in the wild-type (WT) and *acd1* mutant of *Fusarium graminearum*.

Samples	Strain	Intracellular concentration (μg g^{-1} DW)*		
		AMP	IMP	GMP
Hyphas	PH-1 (WT)	210.41 ± 6.26^{A}	82.56 ± 5.45^{A}	111.92 ± 8.72^{A}
	ACD1-1 (*acd1*)	204.08 ± 9.39^{A}	52.33 ± 7.99^{B}	83.06 ± 11.58^{B}
	ACD1-1 + IMP	201.86 ± 12.62^{A}	80.01 ± 3.06^{A}	107.15 ± 6.76^{A}
	ACD1-1 + GMP	204.02 ± 5.05^{A}	83.17 ± 3.78^{A}	104.81 ± 10.80^{A}
Perithecia	PH-1 (WT)	17.83 ± 1.76^{A}	2.80 ± 0.72^{A}	4.88 ± 0.75^{A}
	ACD1-1 (*acd1*)	16.96 ± 1.60^{A}	0.94 ± 0.46^{B}	2.13 ± 0.56^{B}
	ACD1-1 + IMP	18.52 ± 1.42^{A}	2.44 ± 0.28^{A}	4.93 ± 0.64^{A}
	ACD1-1 + GMP	17.55 ± 1.00^{A}	2.61 ± 0.63^{A}	4.74 ± 0.39^{A}

Final concentration of IMP and GMP used is 1 mM. Mean and SD were calculated with results from at least three replicates. Data were evaluated by one-way ANOVA followed by Fisher's protected least significant difference test. Different letters mark significant differences ($P = 0.05$). *ACD*, genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain.
*The intracellular concentration of IMP, AMP or GMP was measured as μg g^{-1} of lyophilized hyphas harvested from 24-h yeast-extract-peptone-dextrose (YEPD) cultures and perithecia collected at 4 d post-fertilization (dpf).

salvage synthesis of IMP by AMP deaminase essential. To test this hypothesis, we examined the expression profiles of genes involved in purine *de novo* synthesis with published RNA-seq data (Liu *et al.*, 2016a; Jiang *et al.*, 2019). In comparison with hyphas, the expression levels of almost all of the genes related to purine *de novo* biosynthesis were decreased in perithecia and/or infected wheat heads (Fig. S7). The expression of *ACD16* was upregulated but this upregulation was minor (less than two-fold) during infection compared to vegetative hyphas. Among the genes selected for qRT-PCR assays, six of them, including *FgADE1*, *FgIMD1* and *FgGUA1* were downregulated more than two-fold in perithecia compared to vegetative hyphas (Fig. S7). These results indicate that IMP/GMP *de novo* biosynthesis is indeed downregulated in ascogenous tissues and invasive hyphas. It is possible that the downregulation of the *de novo* purine biosynthesis, which is more energy-consuming than the purine salvage pathway, is required for coping with the high-energy demand needed for ascosporogenesis and infectious growth in *F. graminearum*.

The *acd16* mutant has pleiotropic defects

The *acd16* deletion mutant had severe growth defects on PDA, MM and CA plates and rarely produced conidia in CMC culture (Fig. 5a; Table 4). It failed to form perithecia (Fig. 5a) and cause disease symptoms on inoculated spikelets and corn silks (Fig. 5b, c). At 2 dpi, the WT formed abundant dome-shaped infection cushions on wheat lemma. Infection cushions were observed rarely although a few runner hyphas were seen on the surface of *acd16*-inoculated lemma (Fig. 5d). In addition, invasive hyphas were observed frequently in lemma tissues in PH-1-inoculated but rarely in *acd16*-inoculated samples (Fig. 5e). The *acd16*/*ACD16* transformants were normal as the WT in all phenotypes (Fig. 5; Table 4). Therefore, *ACD16* is important for vegetative growth, sexual/asexual reproduction and pathogenesis.

ACD16 is orthologous to yeast *ADE16* and *ADE17*. To determine whether they are functionally interchangeable, we generated transformants of *F. graminearum* in which the coding region of *ACD16* was replaced with *ADE16* or *ADE17*. The *acd16*/*ADE16* and *acd16*/*ADE17* transformants grew faster than *acd16* but were still defective in sexual reproduction and plant infection (Fig. S6), indicating that expression of *ADE16* and *ADE17* individually can only partially replace the function of *ACD16* in *F. graminearum*.

Addition of adenine and histidine partially rescues the defects of *acd16* mutant

Ade16 and Ade17 catalyze the last two steps of the *de novo* IMP biosynthesis pathway (Tibbetts & Appling, 2000). The yeast *ade16 ade17* double mutant is auxotrophic for adenine and histidine. Because the domain sequences and active sites of Ade16/Ade17 are well-conserved in Acd16 (Fig. S5), it is likely that Acd16 has a similar function in *de novo* IMP biosynthesis in *F. graminearum*. The *acd16* mutant also was auxotrophic for adenine and histidine. Although addition of 1 mM histidine or adenine alone had no or only a minor effect, addition of both rescued the defects of *acd16* in growth (Fig. 6a) and conidiation (Fig. 6b), indicating that Acd16 is involved in *de novo* IMP biosynthesis. Interestingly, on CA plates with 1 mM each of adenine and histidine, the *acd16* mutant still failed to produce perithecia (Fig. 6c). Because exogenous adenine and histidine rescued the defects of *acd16* in growth and conidiation but not perithecium formation, sexual reproduction in *F. graminearum* may require a functional *de novo* purine synthesis pathway.

We also assayed the effects of exogenous histidine and adenine on plant infection with wheat coleoptiles and heads. Based on results presented in Fig. 6(d), addition of 1 mM adenine and 1 mM histidine to conidium suspensions of *acd16* had no obvious effects on its virulence. Therefore, a functional *de novo* purine synthesis pathway also may be required for plant infection in *F. graminearum* although it remains possible that addition of these compounds to conidia is not sufficient to support extensive infectious growth inside plant tissues.

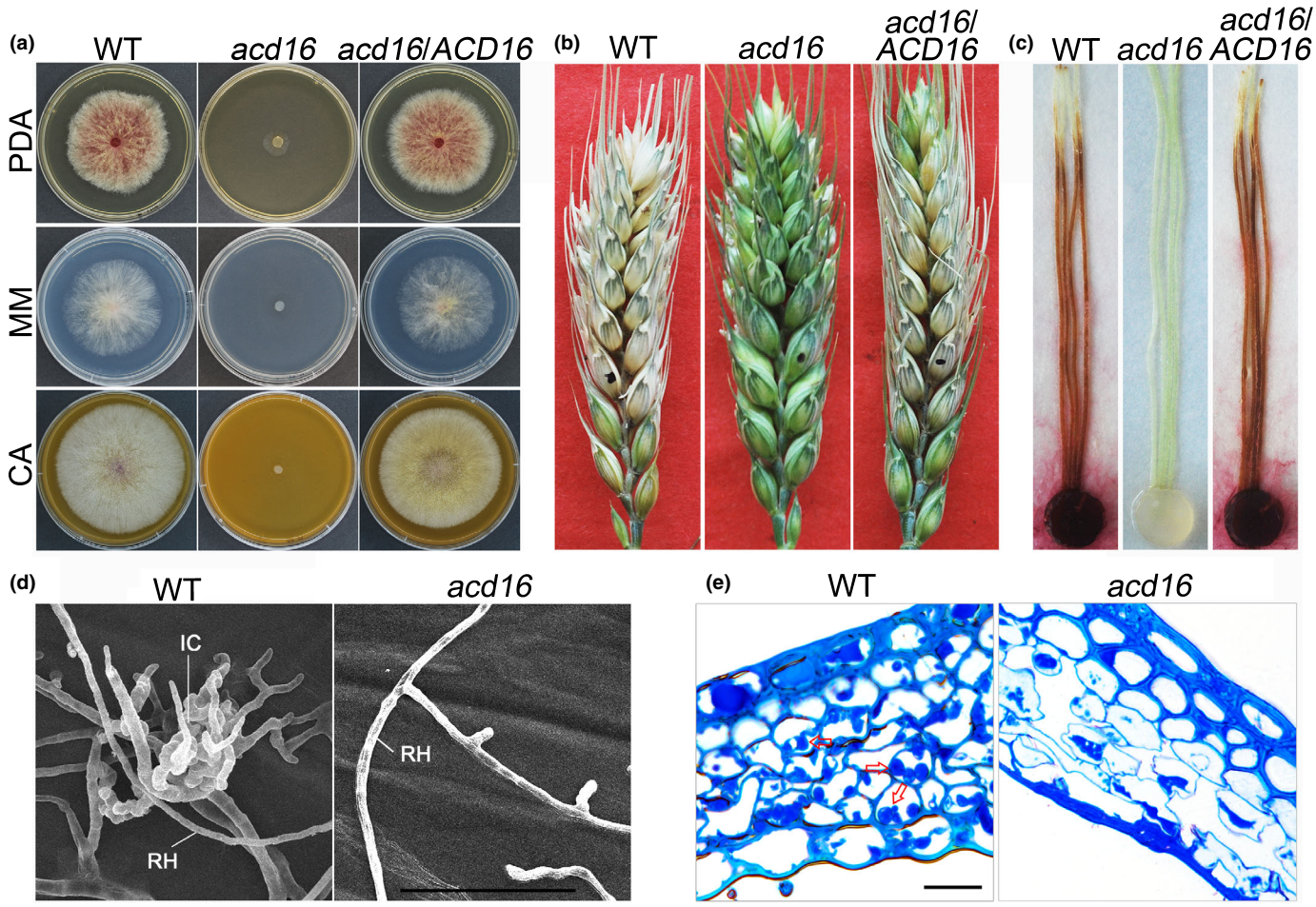

Fig. 5 Defects of the *acd16* mutant of *Fusarium graminearum* in growth and plant infection (*ACD*, genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain). (a) Three-day-old potato dextrose agar (PDA), minimal medium (MM) and carrot agar (CA) cultures of PH-1 (WT), *acd16* deletion mutant and *acd16/ACD16* transformant. (b) Wheat heads inoculated with the indicated strains were examined for head blight symptoms at 14 d post-inoculation (dpi). Black dots mark the inoculated spikelets. (c) Corn silks inoculated with the marked strains were photographed at 5 dpi. (d) Infection cushion (IC) and epiphytic runner hypha (RH) formed by the WT and *acd16* mutant on wheat lemma at 48 h post-inoculation (hpi) were examined by scanning electron microscope (SEM). Bar, 20 μm. (e) Thick sections of infected wheat heads were examined for invasive hyphas (red arrows) in lemma tissues at 48 hpi. Bar, 20 μm.

Table 4 Growth rate, conidiation and virulence of the *acd16* mutant and complemented transformant of *Fusarium graminearum*.

Strain	Growth rate (mm d^{-1})[a]			Conidiation (10^5 spores ml^{-1})[b]	Disease index[c]
	PDA	MM	CA		
PH-1 (wild-type)	9.9 ± 0.2^{A}	7.3 ± 0.2^{A}	11.8 ± 0.1^{A}	13.5 ± 0.9^{A}	11.3 ± 1.0^{A}
ACD16-1 (*acd16*)	2.4 ± 0.1^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}
ADC1 (*acd16/ACD16*)	10.0 ± 0.2^{A}	7.5 ± 0.2^{A}	11.8 ± 0.1^{A}	14.3 ± 1.4^{A}	12.0 ± 1.3^{A}

Mean and SD were calculated with results from at least three biological replicates. Data were evaluated by one-way ANOVA followed by Fisher's protected least significant difference test. Different letters mark significant differences ($P = 0.05$). *ACD*, genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain.
[a]Average daily extension in colony radius on potato dextrose agar (PDA), minimal medium (MM) and carrot agar (CA) plates.
[b] Conidiation in 5-d-old carboxymethylcellulose (CMC) cultures.
[c]The average number of diseased spikelets per inoculated wheat head at 14 d post-inoculation (dpi).

Purines in wheat head tissues may be insufficient for the *acd1* and *acd16* mutants

Besides *de novo* and salvage synthesis of purines in cells, fungi also can acquire purines from extracellular sources, such as plant tissues. The reduced virulence of the *acd1* and *acd16* mutants may be due to the lack of sufficient purines in infected plant tissues. To test this hypothesis, we prepared extracts from wheat rachis and spikelet tissues. Addition of these extracts to the cultures failed to rescue the defects of the *acd1* and *acd16* mutants

(Fig. S8). When assayed for the concentration of purines, we failed to detect adenine in either rachis or spikelet tissues but guanine was detected in spikelets at a very low concentration (1 μg g^{-1} DW). AMP, IMP and GMP were detected in both tissues but their concentrations were all < 12 μg g^{-1} DW (Fig. S8). In cultures, addition of 10 μg ml^{-1} each of IMP, GMP and guanine failed to rescue the defects of *acd1* in conidiation and sexual reproduction (Fig. S8). Likewise, addition of 10 μg ml^{-1} each of AMP, IMP, GMP and guanine together with 1 mM histidine also failed to rescue the growth defect of *acd16* (Fig. S8). These results suggest that the low concentrations of these compounds in wheat spikelet and rachis tissues may be insufficient to support infectious growth of the *acd1* and *acd16* mutants.

Deletion of *FgADE12* results in severe defects in growth, reproduction, and pathogenesis

In yeast, *ADE12* encodes the adenylosuccinate synthase (AdSS) involved in the *de novo* synthesis of AMP from IMP (Lipps & Krauss, 1999). *FgADE12* (FGSG_05187) is the ortholog of *ADE12* in *F. graminearum* and has conserved domain sequences and active sites of AdSS (Fig. S5). The *Fgade12* deletion mutant was severely reduced in vegetative growth and failed to cause disease symptoms on the inoculated wheat spikelets (Fig. 7a–c). At 2 dpi, complex infection cushions and invasive hyphas were rarely observed in lemma inoculated with *Fgade12* (Fig. 7d–e). In addition, the *Fgade12* mutant was reduced in conidiation and blocked in perithecium formation (Fig. 7f; Table S4). All of the defects of *Fgade12* were rescued in the *Fgade12*|*FgADE12* transformants (Fig. 7; Table S4), indicating that *FgADE12* is important for vegetative growth, sexual/asexual reproduction and pathogenesis.

We then assayed the effects of exogenous AMP on the *Fgade12* mutant. Addition of 1 mM AMP to MM plates significantly increased its growth rate (Fig. 7b) and rescued its defects in sexual reproduction and conidiation (Fig. 7f; Table S5). The defects of *Fgade12* mutant also were rescued by the addition of 1 mM adenine (Figs 7b, 8f; Table S5). These observations indicate that *FgADE12* is indeed involved in *de novo* AMP biosynthesis in *F. graminearum*.

Deletion of *FgIMD1* affects growth, sexual reproduction and pathogenesis

Although *S. cerevisiae* has three IMP dehydrogenase (IMPDH) genes for *de novo* GMP biosynthesis (McPhillips *et al.*, 2004), *F. graminearum* has only one that was named *FgIMD1* (FGSG_00861). The domain sequences and active sites of IMPDH are well conserved in FgImd1 (Fig. S5). The *Fgimd1* deletion mutant was significantly reduced in vegetative growth and conidiation (Fig. 8a,b; Table S6). On CA plates, like the *acd1* mutant, it produced relatively small perithecia that lacked asci and ascospores (Fig. 8c). In infection assays with wheat heads, the *Fgimd1* mutant caused only limited symptoms on the inoculated spikelet (Fig. 8d). Like the *acd1* mutant, abundant infection cushions and invasive hyphas were observed in the *Fgimd1*-infected wheat lemma tissues at 2 dpi (Fig. 8e–f), but invasive hyphas were observed rarely in the rachis tissues at 5 dpi (Fig. 8g). The *Fgimd1*|*FgIMD1* transformant was normal as the WT in all phenotypes (Fig. 8; Table S6), indicating the

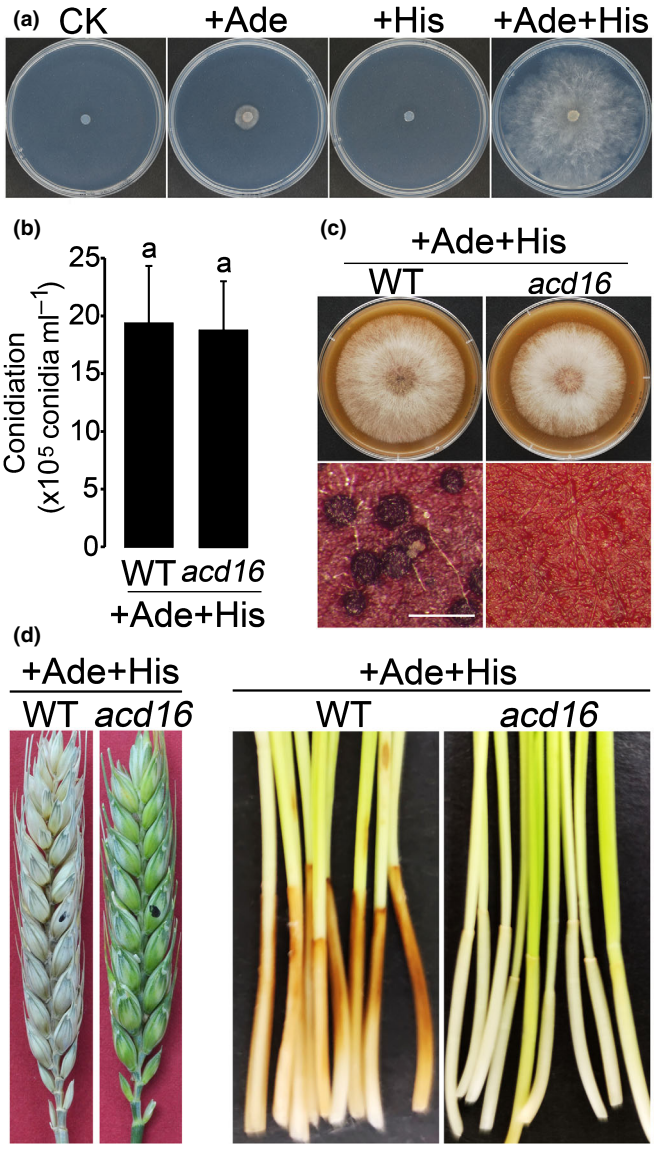

Fig. 6 Assays for the effects of adenine and histidine supplements on the *acd16* mutant of *Fusarium graminearum* (*ACD*, genes encoding proteins with a putative adenosine/cytidine/guanine deaminase domain). (a) Five-day-old minimal medium (MM) cultures of the *acd16* mutant with 1 mM adenine (+Ade) and/or 1 mM histidine (+His). CK, no adenine or histidine added. (b) Conidiation in 5-d-old carboxymethylcellulose (CMC) cultures of PH-1 (WT) and the *acd16* mutant with addition of 1 mM adenine and 1 mM histidine (+Ade + His). Mean and SD were calculated with data from five biological replicates. Same letter indicates no significant difference based on one-way ANOVA analysis followed by Fisher's protected least significant difference test ($P = 0.05$). (c) Cultures of the WT and *acd16* mutant grew on carrot agar supplemented with 1 mM adenine and 1 mM histidine (+Ade + His) were photographed after incubation for 5 d (upper row) and examined for perithecia formation at 8 d post-fertilization (dpf) (lower row). Bar, 1 mm. (d) Flowering wheat heads and wheat coleoptiles inoculated with conidium suspensions of the WT and *acd16* mutant with 1 mM adenine and 1 mM histidine (+Ade + His) were photographed at 14 d post-inoculation (dpi) and 7 dpi, respectively.

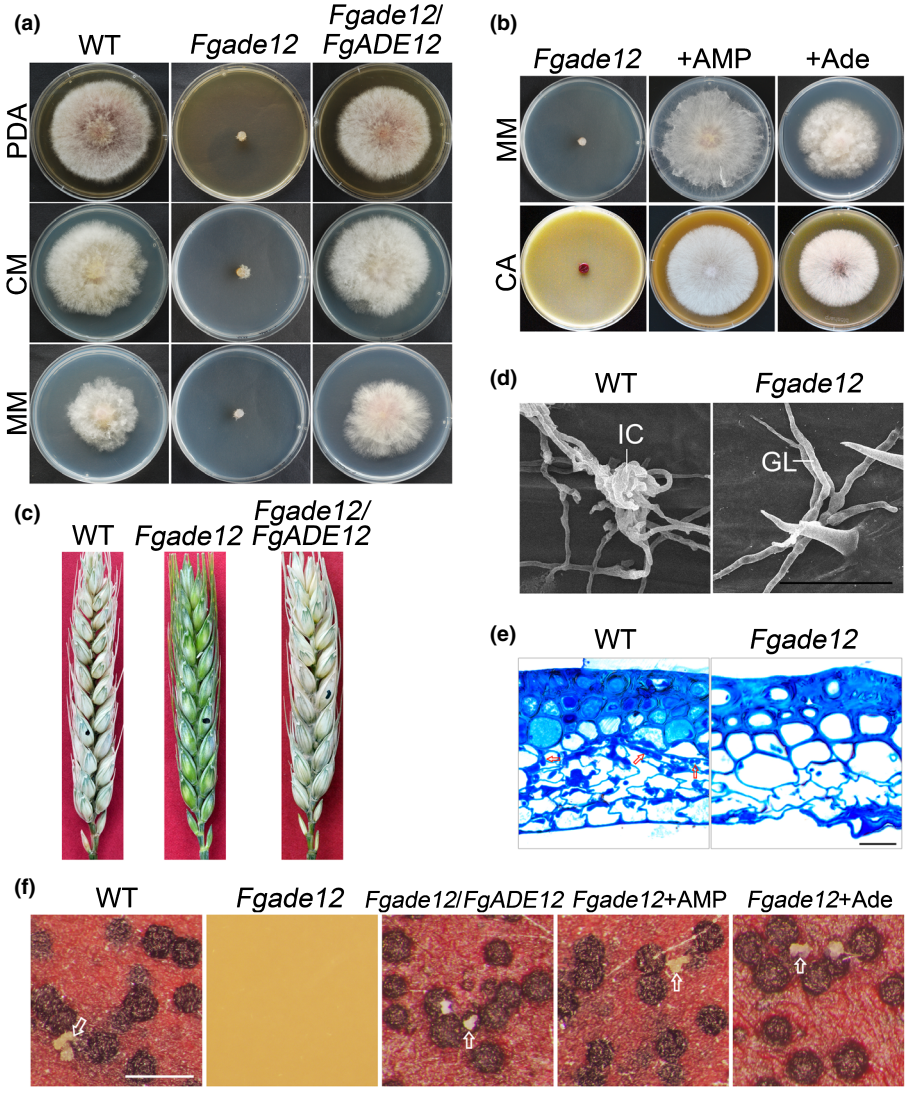

Fig. 7 Defects of the *Fgade12* mutant of *Fusarium graminearum* in growth, sexual reproduction and plant infection (*FgADE12*, ortholog of yeast *ADE12*). (a) Three-day-old cultures of PH-1 (WT), *Fgade12* mutant and *Fgade12/FgADE12* transformant grown on potato dextrose agar (PDA), complete medium (CM) and minimal medium (MM) plates. (b) The *Fgade12* mutant grew on MM (5-d-old) and carrot agar (CA) (3-d-old) cultures with or without 1 mM AMP or adenine (Ade). (c) Wheat heads inoculated with WT, *Fgade12* mutant and *Fgade12/FgADE12* transformant were photographed at 14 d post-inoculation (dpi). Black spots mark the inoculated spikelets. (d) Infection cushion (IC) and germlings (GL) formed by the WT and *Fgade12* mutant on wheat lemma at 48 h post-inoculation (hpi) were examined by scanning electron microscope (SEM). Bar, 20 μm. (e) Thick sections of infected wheat heads were examined for invasive hyphas (red arrows) in the lemma tissues at 48 hpi. Bar, 20 μm. (f) Perithecia formed by WT, *Fgade12/FgADE12* transformant and the *Fgade12* mutant on CA with or without 1 mM AMP or adenine (Ade) at 8 d post-fertilization (dpf). Arrows point to ascospore cirrhi. Bar, 1 mm.

importance of *FgIMD1* in vegetative growth, sexual/asexual development and plant infection.

Addition of 1 mM GMP significantly increased the growth rate of the *Fgimd1* mutant on MM plates and rescued its defect in conidiation but not sexual reproduction (Fig. 8b,c; Table S7). Similarly, the defects of *Fgimd1* in vegetative growth but not sexual reproduction were rescued by supplementing with 1 mM guanine (Fig. 8b,c; Table S7). These observations indicate that *FgIMD1* is indeed involved in *de novo* GMP biosynthesis, which may be essential for sexual reproduction in *F. graminearum*.

Many of the putative purine transporter genes are upregulated in mutants defective in purine synthesis

Exogenous purines can be transported across the plasma membrane to be utilized by the salvage pathway. Based on homology searches, we identified seven genes that encode putative purine transporters in *F. graminearum*. Among these genes, three (FGSG_13426, FGSG_07858, FGSG_12980) are homologous to yeast *FCY2* and *FUN26* (Weber *et al.*, 1990; Boswell-Casteel *et al.*, 2014). The other four (FGSG_11617, FGSG_03523, FGSG_12024, FGSG_07495) are homologous to purine transporter genes *AzgA*, *UapA* and *UapC* of *Aspergillus nidulans* (Cecchetto *et al.*, 2004).

Based on published RNA-seq data, FGSG_07858, FGSG_07495, FGSG_11617 and FGSG_12980 were downregulated during sexual reproduction and plant infection compared to vegetative hyphas or conidia (Fig. S9). FGSG_13426 was expressed only during vegetative growth and pathogenesis, whereas FGSG_03523 was expressed only in perithecia and conidia. FGSG_12024 was specifically expressed during sexual reproduction. When assayed for their expression in vegetative hyphas, FGSG_13426, FGSG_07858 and FGSG_07495 were upregulated over two-fold in the *acd1*, *Fgade12* and *Fgimd1* mutants compared to PH-1. The other four putative purine transporter genes were upregulated over two-fold in at least one of these three mutants (Fig. S9). These results suggest that defects in purine synthesis likely result in the upregulation of purine transporter genes.

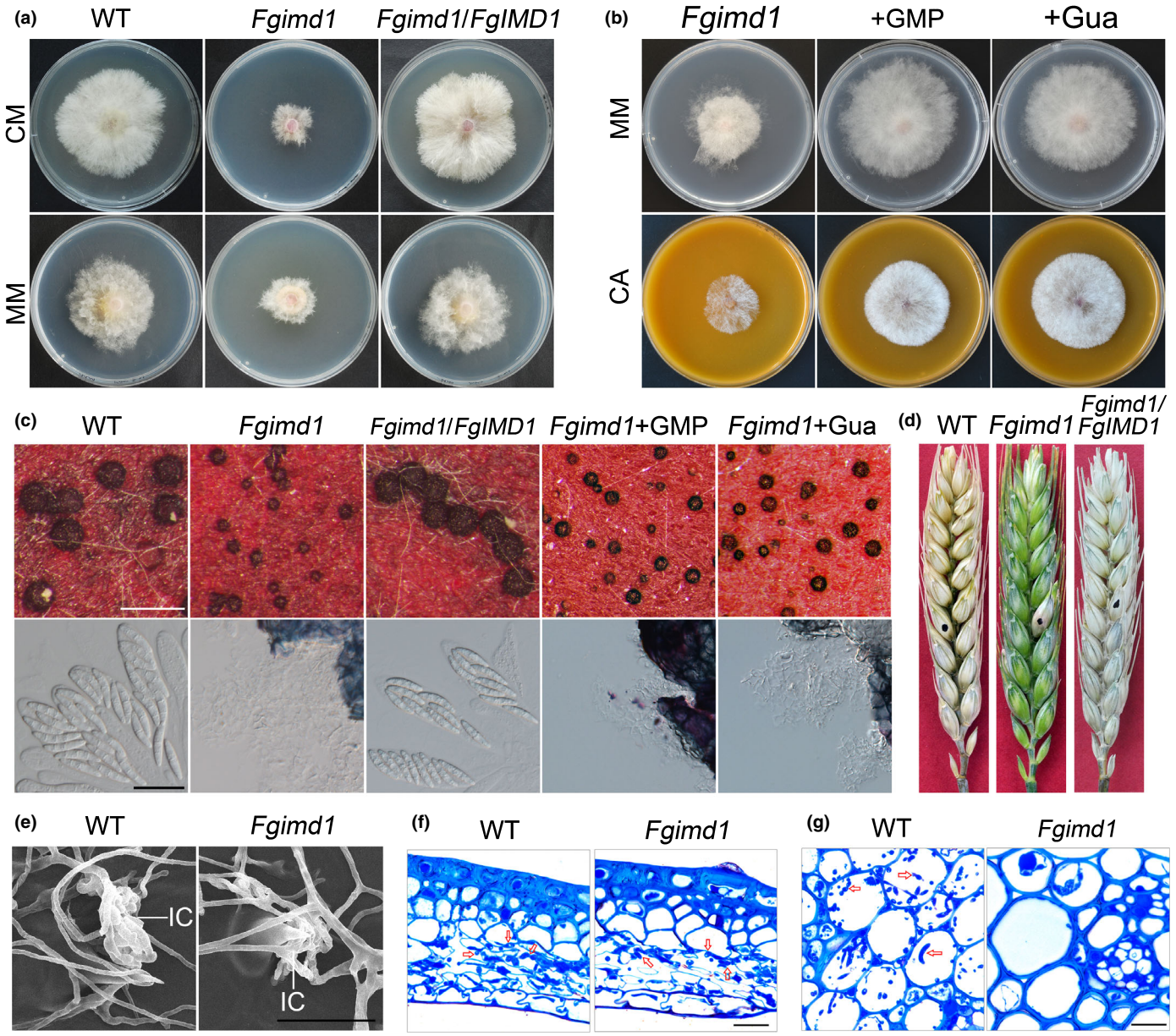

Fig. 8 Defects of the *Fgimd1* mutant of *Fusarium graminearum* in growth, sexual reproduction and plant infection (*FgIMD1*, ortholog of yeast *IMD1/2/3/4*). (a) Three-day-old minimal medium (MM) and complete medium (CM) cultures of PH-1 (WT), *Fgimd1* mutant and *Fgimd1/FgIMD1* complemented transformant. (b) *Fgimd1* mutant grown on MM (5-d-old) and carrot agar (CA) (3-d-old) cultures with or without 1 mM GMP or guanine (Gua). (c) Perithecia formed by WT, *Fgimd1/FgIMD1* transformant and the *Fgimd1* mutant with or without 1 mM GMP or guanine (Gua), on carrot agar at 8 d post-fertilization (dpf) (upper row) were examined for the formation of asci and ascospores (bottom row). Ten-day-old carrot agar cultures were used for self-fertilization of the *Fgimd1* mutant due to its growth defect. Bars: (white) 1 mm; (black) 20 μm. (d) Wheat heads inoculated with WT, *Fgimd1* mutant and *Fgimd1/FgIMD1* transformant were photographed at 14 d post-inoculation (dpi). (e) Infection cushion (IC) formed by the WT and *Fgimd1* mutant on wheat lemma at 48 h post-inoculation (hpi) were examined by scanning electron microscope (SEM). Bar, 20 μm. (f) Thick sections of infected wheat heads were examined for invasive hyphas (red arrows) in the lemma tissues at 48 hpi. Bar, 20 μm. (g) Thick sections of rachis tissues directly below and above the inoculated spikelet were examined for invasive hyphas (red arrows) at 5 dpi. Bar, 20 μm.

Purine salvage synthesis is dispensable for growth, sexual reproduction and virulence

According to yeast orthologs, salvage biosynthesis of purine nucleotides from the purines obtained from extracellular sources in *F. graminearum* likely is catalyzed by FgHpt1, FgApt1, FgAah1 and FgGud1 (Fig. 9). However, mutants with *ACD3* (*FgAAH1*), *ACD14* (*FgGUD1*), *FgHPT1* (FGSG_08275) and *FgAPT1* (FGSG_00722) deleted had no obvious defects in vegetative growth, sexual reproduction and plant infection (Figs S2, S10). These results indicated that Acd3, Acd14, FgHpt1 and FgApt1 are not important in *F. graminearum*, when the *de novo* purine synthesis pathway is functional.

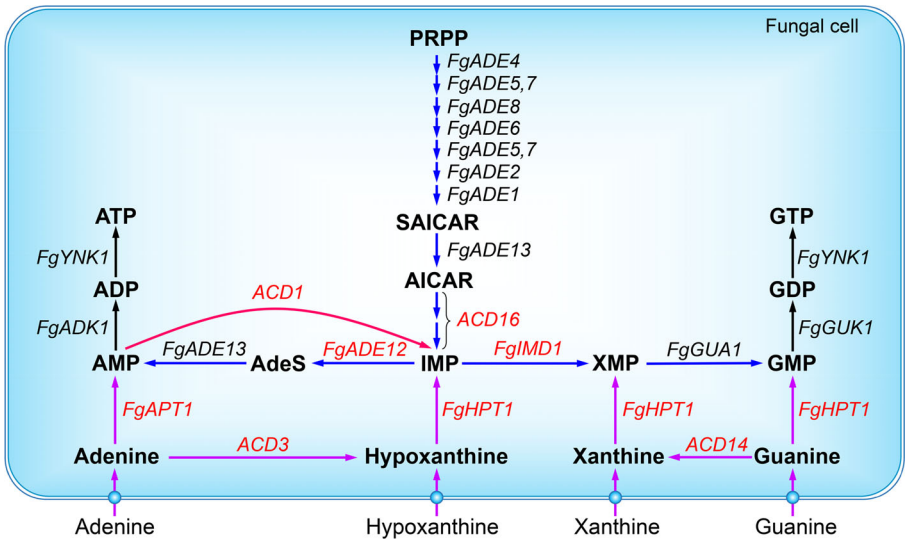

Fig. 9 Schematic representation of purine *de novo* and salvage biosynthesis pathways in *Fusarium graminearum*. Blue arrows represent the steps of purine *de novo* biosynthesis pathway. Purple arrows represent the steps of purine salvage biosynthesis pathway. Red arrow indicates the crucial intersection for purine salvage biosynthesis and catabolism. Gene names are italicized. The genes functionally characterized in this study are shown in red. The following abbreviations are used: AICAR, 5′-phosphoribosyl-5-amino-4-imidazole carboxamide; AdeS, adenylosuccinate; PRPP, 5-phosphoribosyl-1-pyrophosphate; SAICAR, 5′-phosphoribosyl-4-(*N*-succinocarboxamide)-5-amino-imidazole. Gene ID for each gene: *FgADE4* (FGSG_05278), *FgADE5,7* (FGSG_02506), *FgADE8* (FGSG_08429), *FgADE6* (FGSG_09440), *FgADE2* (FGSG_10669), *FgADE1* (FGSG_09453), *FgADE13* (FGSG_09185), *ACD16* (FGSG_00969), *FgADE12* (FGSG_05187), *FgIMD1* (FGSG_00861), *FgGUA1* (FGSG_10358), *FgAPT1* (FGSG_00722), *FgHPT1* (FGSG_08275), *ACD3* (FGSG_01567), *ACD14* (FGSG_05323), *FgYNK1*(FGSG_05972), *FgGUK1*(FGSG_05956) and *FgADK1* (FGSG_10737).

We then assayed the response of these mutants to adenine, adenine analog 8-azaadenine and hypoxanthine/guanine analog 8-azaguanine. Same as the yeast *aah1* mutant (Saint-Marc *et al.*, 2009), the *acd3* mutant showed a slightly reduced resistance to adenine (Fig. S4). Likewise, the *Fgapt1* and *Fghpt1* mutants were resistant to 8-azaadenine and 8-azaguanine, respectively (Fig. S10), as the yeast cognate mutants *apt1* and *hpt1* (Woods *et al.*, 1983; Sahota *et al.*, 1987). We also expressed *ACD3* in the yeast *aah1* mutant and found that its growth defect in response to adenine was rescued in the *aah1/ACD3* transformant (Fig. S4), suggesting that *F. graminearum ACD3* can functionally replace *AAH1*. Therefore, similar to their yeast orthologs, Acd3, FgHpt1 and FgApt1 are likely involved in purine salvage biosynthesis.

Discussion

The biosynthesis and catabolism of purine nucleotides play important roles in growth and development in fungi (Ljungdahl & Daignan-Fornier, 2012). In yeast, *ADE16* and *ADE17* are two paralogous genes (encoding the enzymes that catalyze the last two steps of *de novo* synthesis of inosine monophosphate (IMP) from phosphoribosyl pyrophosphate (PRPP) (Tibbetts & Appling, 2000). Deletion of both *ADE16* and *ADE17* results in the accumulation of 5′-phosphoribosyl-4-carboxamide-5-aminoimidazole (AICAR) that interferes with histidine and methionine biosynthesis and other cellular processes (Rebora *et al.*, 2005). In *Fusarium graminearum*, deletion of *ACD16* (gene encoding protein with a putative adenosine/cytidine/guanine deaminase domain), the only ortholog of *ADE16* and *ADE17*, resulted in a severe growth defect. The *acd16* mutant, like the yeast *ade16 ade17* double mutant, was auxotrophic for adenine and histidine. These results suggest that *ACD16* is indeed involved in *de novo* IMP biosynthesis in *F. graminearum* (Fig. 9). Furthermore, the *acd16* mutant was blocked in conidiation, perithecium formation and plant infection, suggesting that *de novo* synthesis of IMP is not only critical for growth and development, but also important for plant infection. In *Candida albicans*, mutants disrupted of the *ADE8* gene encoding an enzyme that catalyzes the third step of *de novo* IMP synthesis also had attenuated virulence (Jiang *et al.*, 2010). In *Magnaporthe oryzae*, deletion of *ADE1* that encodes the enzyme catalyzing the seventh step of *de novo* IMP synthesis caused infectious growth defects *in planta* (Fernandez *et al.*, 2013). Interestingly, addition of adenine and histidine rescued the defects of the *acd16* mutant in growth and conidiation but not sexual reproduction. Therefore, the requirement for Acd16 and a functional *de novo* purine synthesis pathway must differ between sexual and asexual stages in *F. graminearum*.

After its synthesis, IMP can be used for *de novo* synthesis of adenosine and guanosine monophosphate (AMP and GMP) (Fig. 9). In yeast, conversion of IMP to AMP is catalyzed by the Ade12 adenylosuccinate synthase and Ade13 adenylosuccinate lyase (Walther *et al.*, 2010). The last also is involved in *de novo* IMP synthesis. IMP dehydrogenase (IMPDH) is a rate-limiting enzyme that catalyzes the first committed step of GMP *de novo* synthesis in *Saccharomyces cerevisiae* (Hyle *et al.*, 2003). Our results showed that the *Fgade12* and *Fgimd1* deletion mutants had pleiotropic defects, suggesting the importance of both AMP and GMP *de novo* synthesis in *F. graminearum*. In *Cryptococcus neoformans* and *M. oryzae*, disruption of the IMPDH gene also resulted in defects in growth and virulence (Morrow *et al.*, 2012;

Yang *et al.*, 2019). It is likely that the important roles of IMPDH and *de novo* purine synthesis in growth, reproduction and pathogenesis are conserved in other fungal pathogens. In *in vitro* assays, *C. neoformans* is sensitive to MPA, an IMPDH inhibitor (Morrow *et al.*, 2012), suggesting its potential as an antifungal target. Notably, addition of AMP or adenine rescued the defects of *Fgade12* in both vegetative growth and sexual reproduction, whereas addition of GMP or guanine rescued the defects of *Fgimd1* in vegetative growth but not sexual reproduction. These results suggest that the requirement for *de novo* GMP synthesis rather than AMP synthesis is distinct in the sexual stage in *F. graminearum*.

Purine catabolism is an important pathway to balance nitrogen metabolism. In *S. cerevisiae*, the hydrolytic cleavage of AMP to IMP, the only pathway of AMP catabolism (Fig. 9), is catalyzed by the Amd1 AMP deaminase. The yeast *amd1* mutant grows normally but has defects in sporulation due to the disturbance of purine homeostasis (Walther *et al.*, 2014). Similarly, the *acd1* mutant was normal in hyphal growth but blocked in ascosporogenesis, suggesting that AMP catabolism is dispensable for vegetative growth but essential for sexual reproduction in *F. graminearum*. Expression of *ACD1* complemented the defect of yeast *amd1* mutant and *AMD1* also could partially complement the defects of *acd1* in perithecium formation. In *Arabidopsis*, the Fac1 AMP deaminase is essential for the zygote to embryo transition (Xu *et al.*, 2005). Therefore, Amd1 orthologs may have a conserved function during sexual reproduction.

Our results also showed that Acd1 plays a critical role in pathogenesis. To our knowledge, stage-specific functions of AMP catabolism during plant infection have not been reported in other fungi. Interestingly, the *acd1* mutant was normal in the formation of infection cushions and initial penetration but defective in infectious growth. Therefore, ascogenous hyphas in perithecia and invasive hyphas in plant tissues may differ from vegetative hyphas in cultures in the regulation of purine metabolism, making them more sensitive to disturbance in purine homeostasis caused by the deletion of *AMD1*. Consistent with this hypothesis, the expression of almost all of the genes involved in *de novo* purine synthesis was downregulated in perithecia and infected wheat heads in comparison with vegetative hyphas. Although the *acd1* mutant had stage-specific defects in ascosporogenesis and pathogenesis, *ACD1* is expressed in vegetative hyphas. The *acd1* mutant showed a growth defect in the presence of adenine, suggesting that it is indeed functional during vegetative growth in *F. graminearum*. The *acd1* mutant had similar defects with the *Fgimd1* mutant in ascosporogenesis and pathogenesis, suggesting that GMP depletion may be responsible for its defects. Because the *acd1* mutant was normal in vegetative growth, the effect of *ACD1* deletion on GMP depletion may be not as severe in vegetative hyphas as in developing perithecia and invasive hyphas.

Like other organisms, fungi also have both *de novo* and salvage pathways of purine synthesis. In *S. cerevisiae*, *APT1, AAH1, HPT1, XPT1* and *GUD1* are involved in the salvage synthesis of AMP, IMP and GMP from exogenous purines (Ljungdahl & Daignan-Fornier, 2012). Whereas *XPT1* and *HPT1* are paralogous genes catalyzing different reactions in *S. cerevisiae*, *F. graminearum* has only one orthologous gene (named *FgHPT1*) (Fig. 9). Similar to the yeast cognate mutants, the *acd3* (*Fgaah1*) mutant had a slightly increased sensitivity to adenine, whereas the *Fgapt1* and *Fghpt1* mutants were resistant to 8-azaadenine and 8-azaguanine, respectively. Addition of adenine rescued the *Fgade12* mutant but was suppressive to the growth of the *acd1* mutant. Also, addition of exogenous guanine rescued the *Fgimd1* mutant. These results indicate that the purine salvage biosynthesis pathway is functional in *F. graminearum*. Nevertheless, the *acd3*, *Fgapt1*, *acd14* (*Fggud1*) and *Fghpt1* mutants had no detectable phenotypes, suggesting that salvage synthesis of AMP/GMP is not important when *de novo* purine synthesis is functional. Consistent with these results, many putative purine transporter genes were upregulated in the *acd1*, *Fgade12* and *Fgimd1* mutants. In *C. neoformans*, *HPT1* and GTP salvage synthesis also are dispensable for growth and virulence (Morrow *et al.*, 2012).

In *F. graminearum*, A-to-I mRNA editing specifically occurs during sexual reproduction and is essential for ascosporogenesis (Liu *et al.*, 2016a; Cao *et al.*, 2017; Hao *et al.*, 2019). Therefore, 14 of the 18 *ACD* genes dispensable for sexual reproduction are not involved in RNA editing. Whereas genome-wide RNA editing events were identified in the *acd1* mutant by RNA-seq, similar analysis could not be done with the *acd16* mutant due to its defect in perithecium formation. Nevertheless, our data showed that the distinct function of *ACD16* in sexual reproduction is likely related to *de novo* GMP synthesis. Therefore, only the two ADAT orthologs, *ACD17* (*FgTAD2*) and *ACD18* (*FgTAD3*), remain to be candidate deaminase genes responsible for mRNA editing in *F. graminearum*. Unfortunately, these two genes may be essential because we failed to identify deletion mutants. In bacteria, the tadA ADAT is responsible for A-to-I editing (Bar-Yaacov *et al.*, 2017). In fungi, editing preferentially targets adenosine in the hairpin loop of folded mRNAs, a structure similar to the anticodon loop of tRNA targeted by ADATs (Wang *et al.*, 2016; Bian *et al.*, 2019). Therefore, A-to-I mRNA editing in *F. graminearum* is likely catalyzed by Acd17 and Acd18. It will be important to generate and characterize site-specific mutations that have no effect on their ADAT functions but affect mRNA editing during sexual reproduction.

Overall, our data showed that genes involved in *de novo* IMP, AMP and GMP synthesis are important for vegetative growth, sexual/asexual reproduction and pathogenesis in *F. graminearum*. Interestingly, the requirement for *de novo* GMP synthesis but not AMP synthesis is distinct for the sexual stage compared to the asexual stage. More importantly, our results revealed that the conversion of AMP to IMP catalyzed by Acd1 is dispensable for vegetative growth but essential for ascosporogenesis and infectious growth, suggesting that AMP catabolism and Acd1 have stage-specific functions during sexual reproduction and plant infection in *F. graminearum*. This stage-specific regulation of purine metabolism has not been reported but may be common to other plant pathogenic fungi.

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Author contributions

HL and JRX planned and designed the research; MS, ZB, QL, YC, WW, YD, LC and CH performed the experiments; MS, HL and JRX analyzed the data; and MS, HL and JRX wrote the manuscript.

ORCID

Huiquan Liu https://orcid.org/0000-0002-4723-845X
Jin-Rong Xu https://orcid.org/0000-0001-5999-5004

Data availability

RNA-seq data generated in this study were deposited at the NCBI SRA database under accession nos. SRR12677793 and SRR12677794.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Expression levels (TPM) of the *ACD* genes in *F. graminearum* based on RNA-seq data of conidia (Coni), hyphas harvested from 24-h YEPD cultures (Hy24h), perithecia collected at 8 dpf (Sex8d), and infected wheat heads sampled at 3 dpi (Inf3d).

Fig. S2 The 14 *ACD* deletion mutants of *F. graminearum* with normal growth, fertility and virulence.

Fig. S3 Subcellular localization of the Acd1-GFP fusion protein in *F. graminearum*.

Fig. S4 Effects of exogenous adenine on the phenotypes of yeast *amd1* and *aah1* mutants and *F. graminearum acd1* and *acd3* mutants.

Fig. S5 Multiple sequence alignments of Acd1, Acd16, FgAde12 and FgImd1 orthologs in different fungi.

Fig. S6 Phenotypes of the *acd1*|*AMD1*, *acd16*|*ADE16*, and *acd16*|*ADE17* transformants of *F. graminearum* in growth, conidiation, sexual reproduction and plant infection.

Fig. S7 Expression of genes involved in purine *de novo* biosynthesis in *F. graminearum*.

Fig. S8 Effects of wheat extracts and exogenous purines on the phenotypes of *acd1* and *acd16* mutants of *F. graminearum*.

Fig. S9 Expression of putative purine transporter genes in *F. graminearum*.

Fig. S10 Phenotypes of *Fghpt1* and *Fgapt1* deletion mutants of *F. graminearum*.

Table S1 Primers used in this study.

Table S2 The *ACD* genes identified in *F. graminearum*.

Table S3 RNA variant sites identified in RNA-Seq data of WT and *acd1* mutant of *F. graminearum*.

Table S4 Growth rate, conidiation and virulence of the *Fgade12* mutant and complemented transformant of *F. graminearum*.

Table S5 Effects of exogenous AMP and adenine on the phenotypes of the *Fgade12* mutant of *F. graminearum*.

Table S6 Growth rate, conidiation and virulence of the *Fgimd1* mutant and complemented transformant of *F. graminearum*.

Table S7 Effects of exogenous GMP and guanine on the phenotypes of the *Fgimd1* mutant of *F. graminearum*.

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