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FgBUD14 is important for ascosporogenesis and involves both stage-specific alternative splicing and RNA editing during sexual reproduction

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Summary

In wheat head blight fungus *Fusarium graminearum*, A-to-I RNA editing occurs specifically during sexual reproduction. Among the genes with premature stop codons (PSCs) that require RNA editing to encode full-length proteins, *FgBUD14* also had alternative splicing events in perithecia. In this study, we characterized the functions of *FgBUD14* and its post-transcriptional modifications during sexual reproduction. The *Fgbud14* deletion mutant was slightly reduced in growth, conidiation and virulence. Although deletion of *FgBUD14* had no effect on perithecium morphology, the *Fgbud14* mutant was defective in crozier formation and ascus development. The FgBud14-GFP localized to the apex of ascogenous hyphae and croziers, which may be related to its functions during early sexual development. During vegetative growth and asexual reproduction, FgBud14-GFP localized to hyphal tips and both ends of conidia. Furthermore, mutations blocking the splicing of intron 2 that has the PSC site had no effect on the function of *FgBUD14* during sexual reproduction but caused a similar defect in growth with *Fgbud14* mutant. Expression of the non-editable *FgBUD14*$^{Intron2\text{-}TAA}$ mutant allele also failed to complement the *Fgbud14* mutant. Taken together, *FgBUD14* plays important roles in ascus development, and both alternative splicing and RNA editing occur specifically to its transcripts during sexual reproduction in *F. graminearum*.

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Introduction

Fusarium head blight (FHB) or scab is one of the most important diseases of wheat and barley worldwide. One major causal agent of FHB is *Fusarium graminearum*, a homothallic ascomycete (Bai and Shaner, 2004; Goswami and Kistler, 2004). Besides causing yield losses and reducing grain quality, *F. graminearum* produces deoxynivalenol (DON) and other mycotoxins (Audenaert *et al*., 2013; Park *et al*., 2016). Ascospores are the primary inoculum for FHB (Trail *et al*., 2002). *Fusarium graminearum* survives and overwinters in plant debris and forms melanized perithecia on the surface of plant tissues. Mature ascospores are forcibly discharged from perithecia, which is important for the dispersal of airborne ascospores (Maldonado-Ramirez *et al*., 2005; Trail *et al*., 2005). After landing on flowering wheat or barley heads, ascospores germinate and infect floral tissues that are predisposed to infection (Kazan *et al*., 2012). On diseased plant tissues, *F. graminearum* also produces asexual spores (conidia), which spread the infection to other parts of host plants later in the season.

As in *Neurospora crassa*, repeat-induced point mutation and meiotic silencing by unpaired DNA occur specifically during sexual development in *F. graminearum* (Cuomo *et al*., 2007; Son *et al*., 2011). Although it lacks adenosine deaminase acting on RNA orthologues, genome-wide A-to-I RNA editing, an important post-transcriptional modification that converts adenosine (A) to inosine (I) in RNA molecules via hydrolytic deamination (Bian *et al*., 2018), has been shown to occur specifically during sexual reproduction in *F. graminearum* (Liu *et al*., 2016). Stage-specific RNA editing also exists in *N. crassa*, *Sordaria macrospora* and other filamentous ascomycetes during sexual reproduction (Liu *et al*., 2017; Teichert *et al*., 2017). In *F. graminearum*, the *PUK1* (Perithecium Unique Kinase 1) gene, known to be important for sexual development, has two tandem premature stop codons (PSCs) and requires A-to-I RNA editing during sexual reproduction to encode the full-length functional protein (Liu *et al*., 2016). Among the other genes

with PSC in their coding regions that have been functionally characterized for the importance of A-to-I RNA editing, *FgAMA1* also plays critical roles in ascospore formation and release in *F. graminearum* (Cao *et al*., 2017; Hao *et al*., 2019). Its orthologue in the budding yeast *Saccharomyces cerevisiae* encodes a meiosis-specific APC activator. In *F. graminearum*, the *Fgama1* mutant produced oval, single-celled, binucleate ascospores instead of the four-celled, uninucleate ascospores produced by the wild-type PH-1 strain (Hao *et al*., 2019).

In the budding yeast, one of the Ama1-interacting genes is Bud14 that is involved in bud site selection (Tonikian *et al*., 2009). The Bud14 homologue functions as a cell end marker for polarized growth in the fission yeast and plays a role in the growth and asexual reproduction (conidiation) in filamentous ascomycetes *Aspergillus nidulans* and *Magnaporthe oryzae* (Higashitsuji *et al*., 2009; Patkar *et al*., 2010; Valinluck *et al*., 2014). However, the function of Bud14 orthologues in sexual reproduction has not been characterized in these ascomycetes. Interestingly, in *F. graminearum*, *FgBUD14* expression was upregulated in perithecia, and its transcripts contained one PSC editing event based on published RNA-seq data (Liu *et al*., 2016). In this study, we showed that FgBud14 localized to the tip of croziers and plays important roles in crozier formation, ascus development and ascospore genesis. We also confirmed that both alternative splicing and RNA editing occur in *FgBUD14* transcripts specifically during sexual development, and they synergistically regulate the function of *FgBUD14* during sexual reproduction in *F. graminearum*.

Results

Both alternative splicing and RNA editing occur in FgBUD14

The predicted gene FGSG_04118 of *F. graminearum* (named *FgBUD14* in this study) is orthologous to *BUD14* of *S. cerevisiae*. It has typical structural features that are conserved among the Bud14 orthologues in other fungi, including a conserved SH3 (Src-homology 3) domain (363–420 aa) and a Glc7 binding domain (Fig. S1). However, the overall homology between yeast Bud14 and its orthologues from filamentous ascomycetes is relatively low. FgBud14 shares only 37.5% identity in amino acid sequences with yeast Bud14.

Based on published RNA-seq data (Liu *et al*., 2016), the *FgBUD14* gene is upregulated during sexual reproduction in comparison with vegetative hyphae (Fig. 1A). The first two introns of its five introns, intron 1 and intron 2, had alternative splicing events in perithecia (Fig. 1B). Interestingly, intron 2 of *FgBUD14* has an in-frame PSC (UA^{1334}G) (Fig. 1B). Among the transcripts that retain intron 2 (approximately 45.8%), 19% of them had this premature stop codon edited to UG^{1334}G in perithecia at 8 days after sexual induction (dai) (Fig. 1C). Therefore, *FgBUD14* transcripts are subjected to both alternative splicing and A-to-I RNA editing specifically during sexual reproduction, which has not been reported for any other fungal genes.

Fgbud14 *mutant is defective in vegetative growth and conidiogenesis*

To determine the function of *FgBUD14*, we generated *Fgbud14* deletion mutants (Table 1) with the gene replacement approach (Fig. S2). In comparison with the wild-type strain PH-1, the *FgBud14* mutant had normal colony morphology (Fig. 2A) although its growth rate was reduced approximately by 16% (Table 2). Deletion of *FgBUD14* also resulted in a reduction in conidiation (Table 2) and affected conidium morphology. Mutant conidia were shorter and had fewer septa than those of the wild type (Fig. 2B). Although wild-type conidia normally had four or five septa, the majority of *Fgbud14* conidia (over 80%) had three or fewer septa (Fig. 2C).

Conidia of the *Fgbud14* mutant also were defective in germination. After incubation at 25°C for 6 h, the *Fgbud14* mutant had shorter germ tubes than the wild type (Fig. 2D). When observed at 12 h post-incubation (hpi), germlings of the *Fgbud14* mutant also showed reduced germ tube growth in comparison with PH-1 (Fig. 2D). In comparison with the wild type, hyphae of the *Fgbud14* mutant had a shorter inter-septal cell distance (Fig. 2E and F). These results indicated that the *Fgbud14* mutant was defective in vegetative growth, conidiogenesis and septation in *F. graminearum*.

FgBUD14 *plays a minor role in plant infection*

In infection assays with flowering wheat heads, the *Fgbud14* mutant still caused typical scab symptoms on the inoculated kernels and was able to spread to nearby spikelets (Fig. 2G). However, it was slightly reduced in virulence in comparison with PH-1. On average, the disease indices for PH-1 and the *Fgbud14* mutant were 10.6 and 7.7 respectively (Table 2). The *Fgbud14* mutant also was reduced in virulence in infection assays with corn silks (Fig. 2H), which may be related to its defects in growth rate. We also assayed the DON production in TBI medium and found that the *Fgbud14* mutant had no obvious defect in DON production in comparison with the wild type (Table 2). These results indicate that *FgBUD14* does not play a critical role in plant infection.

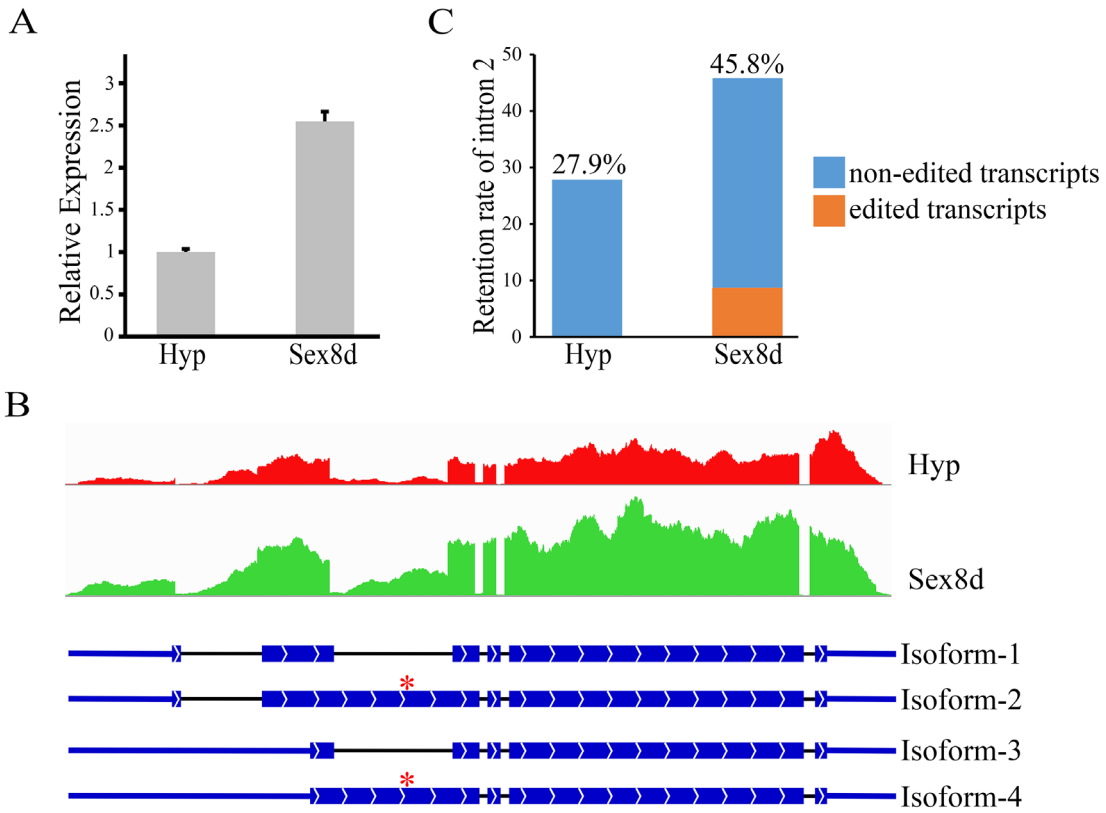

Fig. 1. The transcriptional expression pattern and RNA editing of *FgBUD14* in different stages.
A. The abundance of *FgBUD14* transcripts in hyphae collected from 24 h YEPD cultures (Hyp) and 8-dai perithecia (Sex8d). The relative expression level of *FgBUD14* in hyphae was arbitrarily set to 1.
B. Isoforms of *FgBUD14* transcripts derived from alternative splicing and A-to-I RNA editing. IGV-Sashimi plots showing the read coverage and transcript isoforms of *FgBUD14* in RNA-seq data of 24 h germlings harvested from YEPD (Hyp) and perithecia harvested at 8 dai (Sex8d). The asterisk symbol * marks the $TA^{1334}G$ stop codon in the second intron that is changed to TGG by RNA editing.
C. Bar graphs show the retention rate of intron 2 and the edited portion of *FgBUD14* transcripts. [Color figure can be viewed at wileyonlinelibrary.com]

FgBUD14 *is important for ascospore formation*

In comparison with the wild-type strain, the *Fgbud14* mutant produced melanized perithecia with normal morphology on mating plates (Fig. 3A), but its perithecial density was reduced approximately by 30% (Fig. S3A). However, unlike the wild type, ascospore cirrhi were not observed in the *Fgbud14* mutant at 8 dai or longer (Fig. 3A). The *Fgbud14* mutant also failed to forcibly release ascospores in ascospore discharge assays (Fig. 3B).

We then examined ascus development and ascospore formation inside perithecia. Mature asci of the wild type contained eight ascospores that had four compartments. On average, the number of asci per perithecium was reduced over 95% in the *Fgbud14* mutant in comparison with the wild type (Fig. S3B), suggesting a defect in ascus development. Among the few asci formed by the *Fgbud14* mutant at 7 dai, most of them lacked mature ascospores (Fig. 3C). Interestingly, some asci appeared to produce mature ascospores in 12-dai or 18-dai perithecia produced by the *Fgbud14* mutant. For the rare asci with ascospores in 12-dai perithecia, about 40% of them contained fewer than eight ascospores (Fig. 3D; Fig. S3C). These results indicated that the *FgBUD14* gene is dispensable for perithecium morphology but plays a critical role in ascus development and ascospore formation.

Deletion of FgBUD14 *significantly reduces crozier formation and ascus development*

To further characterize the defects of the *Fgbud14* mutant in ascus development, we examined the formations of croziers in perithecia at 4 dai. In perithecia formed by PH-1, croziers were observed (Fig. 4A). Under the same conditions, ascogenous hyphae were observed inside perithecia formed by the *Fgbud14* mutant. However, croziers were rarely observed (Fig. 4A).

Table 1. The wild-type and mutant strains of *F. graminearum* used in this study.

Strains	Brief description	Reference
PH-1	Wild type	Cuomo *et al*. (2007)
mat1-1 H1-GFP	Transformant of *mat1-1* mutant expressing H1-GFP	Lee *et al*. (2003)
M11	*Fgbud14* deletion mutant of PH-1	This study
M22	*Fgbud14* deletion mutant of PH-1	This study
C1	Transformant of M11 expressing *FgBUD14*-GFP	This study
C8	Transformant of M11 expressing *FgBUD14*-GFP	This study
N6	*Fgama1* deletion mutant of PH-1	Hao *et al*. (2019)
MN4	*Fgbud14 Fgama1* double mutant	This study
MN9	*Fgbud14 Fgama1* double mutant	This study
Ri1-19	Transformant of M11 expressing $FgBUD14^{Intron1}$	This study
Ri1-22	Transformant of M11 expressing $FgBUD14^{Intron1}$	This study
Ri2-5	Transformant of M11 expressing $FgBUD14^{Intron2}$	This study
Ri2-10	Transformant of M11 expressing $FgBUD14^{Intron2}$	This study
$Ri2^{TGG}$-5	Transformant of M11 expressing $FgBUD14^{Intron2\text{-}TGG}$	This study
$Ri2^{TGG}$-7	Transformant of M11 expressing $FgBUD14^{Intron2\text{-}TGG}$	This study
$Ri2^{TAA}$-9	Transformant of M11 expressing $FgBUD14^{Intron2\text{-}TAA}$	This study
$Ri2^{TAA}$-10	Transformant of M11 expressing $FgBUD14^{Intron2\text{-}TAA}$	This study

At 5 dai, young asci with developing ascospores were observed inside perithecia of PH-1. In the *Fgbud14* mutant, ascogenous hyphae rarely gave rise to elongated developing asci (Fig. 4B). When stained with DAPI, partial elongated asci formed by the *Fgbud14* mutant had eight nuclei (Fig. 4B). These results indicated that FgBud14 plays a critical role in the formations of croziers and ascus development during the early stages of sexual development.

The Fgbud14 *mutant is normal in ascosporogenesis in outcrossing*

Although it is a homothallic fungus, *F. graminearum* can be forced to outcross. When the *Fgbud14* mutant was crossed as the male with a *mat1-1* H1-GFP strain (Zheng *et al*., 2013), normal asci with eight ascospores were formed (Fig. 4C). Four of them had GFP signals in the nucleus, suggesting the segregation of H1-GFP in the progeny (Fig. 4C). Therefore, the wild-type *FgBUD14* allele is dominant in the diploid or dikaryotic stage and its presence in one parental strain is sufficient for normal ascus development and ascospore formation.

FgBud14-GFP localizes to the tips of conidia and hyphae

For complementation assays, we generated the *FgBUD14*-GFP fusion construct under the control of its native promoter and transformed it into the *Fgbud14* mutant. The resulting transformants were confirmed to contain the *FgBUD14*-GFP construct by PCR. The *Fgbud14*/*FgBUD14*-GFP transformant was normal in growth (Fig. 2A; Table 1), conidium morphology (Fig. 2B), conidiation (Table 1), plant infection (Fig. 2G and H; Table 1) and sexual reproduction (Fig. 3). These results indicated that the expression of *FgBUD14*-GFP completely rescued the defects of the *Fgbud14* mutant. Therefore, deletion of *FgBUD14* is directly responsible for the defects observed in the *Fgbud14* mutant.

When examined by epifluorescence microscopy, FgBud14-GFP signals were mainly localized to the tip in developing conidia (Fig. 5A) but observed at both ends in mature conidia (Fig. 5B). Because of the strong background fluorescence, no reliable FgBud14-GFP signal was observed at the septum of conidia. (Fig. 5B). During germination, FgBud14-GFP appeared to be enriched at the germ tube tip (Fig. 5C). Enrichment of FgBud14 at the septum also was observed in germ tubes (Fig. 5C), further indicating the involvement of FgBud14 in septation in *F. graminearum*. Therefore, FgBud14 may play a role in end marking and polarized growth as well as septum formation.

When treated with 10 μM latrunculin A (an actin polymerization inhibitor) for 20 min, the apical actin patch was no longer visible at hyphal tips (Fig. S4A). However, the FgBud14-GFP is still localized to the hyphal tip, although its GFP intensity was significantly reduced (Fig. S4B). Thus the actin cytoskeleton is not essential but contributes to the localization of FgBud14 to the hyphal tip.

FgBud14-GFP is localized to the apex of ascogenous hyphae and croziers

Because one of the major defects of the *Fgbud14* mutant was crozier formation and ascus development, we then examined the subcellular localization of FgBud14 during sexual reproduction. In developing perithecia at 4 dai, FgBud14-GFP was localized to the tips of ascogenous hyphae and croziers (Fig. 6A). To our knowledge, this is the first report on proteins localizing to the tips of croziers in filamentous ascomycetes. In some croziers, FgBud14-GFP signals appeared to be enriched at the contact region between crozier terminal cell and stalk cell (Fig. 6B). However, GFP fluorescence signals were not observed in young asci (Fig. 6C). One possible function of FgBud14 may be related to the hooking and crozier fusion during crozier formation and ascus development as shown in the diagram (Fig. 6D).

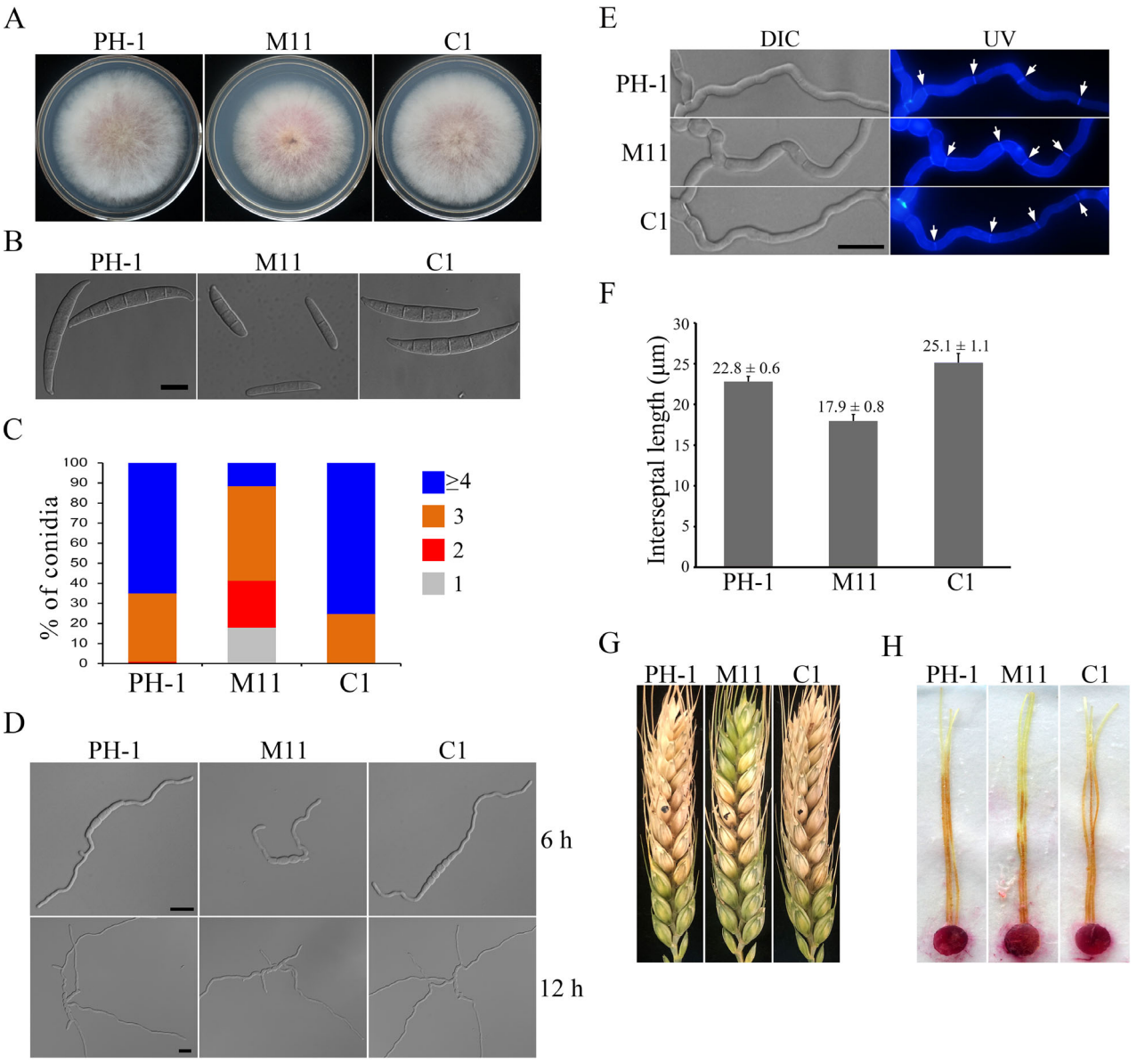

Fig. 2. Defects of the *Fgbud14* mutant in growth, conidiation, germination, septation and plant infection.
A. Three-day-old PDA cultures of the wild-type PH-1, *Fgbud14* mutant (M11) and *Fgbud14*/*FgBUD14* complemented transformant (C1).
B. Conidia of the same set of strains were examined by differential interference contrast (DIC) microscopy. Bar = 10 μm.
C. Percentage of conidia with different numbers of septa in PH-1, M11 and C1.
D. Germlings of the same set of strains were examined for defects in germination and germ tube growth by DIC microscopy after incubation in YEPD for 6 or 12 h. Bar = 20 μm.
E. 12-hpi germlings of the same set of strains were examined for septum formation by staining with CFW. Arrows point to septa. Bar = 20 μm.
F. The inter-septal distance of 12-hpi germlings of PH-1, M11 and C1.
G. Flowering wheat heads were drop-inoculated with conidia from PH-1, M11 and C1. Black dots mark the inoculated spikelets. Photographs were taken 14 dpi.
H. Corn silks were inoculated with culture blocks of labelled strains and examined at 5 dpi. [Color figure can be viewed at wileyonlinelibrary.com]

The FgBud14 may not interact with FgAma1 during sexual reproduction

To investigate the relationship between FgBud14 and FgAma1, we performed BiFC assay to detect their interaction. We generated the *FgBUD14*-NYFP and *FgAMA1*-CYFP fusion constructs under the control of their native promoters and co-transformed them into PH-1 strain. The resulting transformants were confirmed to contain both *FgBUD14*-NYFP and FgAMA1-CYFP constructs by PCR. In the resulting transformants, no reliable YFP signals were observed at the apex of ascogenous hyphae and croziers (Fig. S5A). Moreover,

Table 2. Defects of *Fgbud14* mutant in growth, conidiation, virulence and DON production.

Strains	Growth rate[a] (mm day^{-1})	Conidiation[b] (10^5 conidia/ml)	Disease index[c]	DON[d] (mg g^{-1})
PH-1	10.1 ± 0.02^{A}	13.8 ± 0.9^{A}	10.0 ± 1.6^{A}	248.7 ± 33.9^{A}
M11	8.4 ± 0.03^{B}	5.5 ± 0.5^{B}	7.7 ± 0.5^{B}	240.5 ± 13.3^{A}
C1	10.2 ± 0.09^{A}	14.0 ± 0.5^{A}	9.6 ± 1.6^{A}	NA

NA, not assayed.
Means ± SE were calculated from the results of three independent experiments. Data from three replicates were analysed with the protected Fisher least significant difference (LSD) test. The same letter indicates no significant difference and different letters indicate a statistically significant difference ($P = 0.05$).
[a]Growth rate was measured as the average daily expansion of colony radius after incubation on PDA plates for 3 days.
[b]Number of conidia per ml in 5-day-old CMC liquid cultures.
[c]Disease index was estimated by counting the number of diseased spikelets per head at 14 dpi. At least 10 wheat heads were examined for each strain.
[d]DON production was measured with TBI cultures inoculated for 7 days.

we also generated the *Fgbud14 Fgama1* double mutant and compared its sexual reproduction phenotype with *Fgbud14* and *Fgama1* single mutants. Unlike the *Fgbud14* mutant, the *Fgama1* mutant produced abundant asci with oval and single-celled ascospores (Fig. S5B) as previously reported (Hao *et al*., 2019). Interestingly, the *Fgbud14 Fgama1* double mutant showed a combined defect of both *Fgbud14* and *Fgama1* mutants in sexual reproduction (Fig. S5B). These results indicated that the FgBud14 may not interact with FgAma1 during sexual reproduction.

Expression of an FgBUD14 *allele with retained intron 2 complemented the defect of* Fgbud14 *mutant in sexual reproduction but not its growth defect*

Unlike intron 1, intron 2 is subjected to both alternative splicing and RNA editing. To determine the effects of retaining these two introns, we introduced site-specific mutations into the *FgBUD14* complementation construct by changing the 'gt-ag' splicing sites to 'ct-aa' to block intron splicing. The resulting *FgBUD14*^{Intron1} and *FgBUD14*^{Intron2} were transformed into the *Fgbud14* mutant. The resulting *Fgbud14*/*FgBUD14*^{Intron1} transformants, similar to the complemented transformant, were normal in vegetative growth and sexual reproduction (Fig. 7A). In contrast, the *Fgbud14*/*FgBUD14*^{Intron2} transformants, similar to the *Fgbud14* mutant, were defective in vegetative growth (Fig. 7A) but normal in ascospore formation and discharge (Fig. 7B). These results indicate that retaining the 47-bp intron 1 has no obvious effect on *FgBUD14* functions. However, the expression of *FgBUD14*^{Intron2} failed to rescue the growth defect of *Fgbud14* but complemented its defect in sexual reproduction. Moreover, we performed RT-PCR to confirm that intron 2 was totally retained in transcripts of *FgBUD14*^{Intron2} during sexual reproduction (Fig. S6A). We also assayed the PSC editing event in the transcripts of *FgBUD14*^{Intron2} in perithecia of the *Fgbud14*/*FgBUD14*^{Intron2} transformant. The sequencing peak map showed that there were two distinct signal peaks (A and G) at TA^{1334}G (Fig. S6B), indicating the occurrence of A-to-I editing within the 681-bp intron 2. In perithecia, the transcripts produced by *Fgbud14*/*FgBUD14*^{Intron2} transformants correspond to isoform-2 and isoform-4 of *FgBUD14* (Fig. 1B). Therefore, stage-specific A-to-I RNA editing of the premature stop codon in the retained intron 2 is essential for *FgBUD14*^{Intron2} transcripts to encode functional proteins.

Editing of TA^{1334}G in retained intron 2 to TGG is essential for the function of FgBUD14

To verify the importance of the editing of TA^{1334}G in the second intron (681-bp), we introduced the A1334G mutation or inserted an extra stop codon TAA after the TA^{1334}G to the *FgBUD14*^{Intron2} construct. The resulting *FgBUD14*$^{Intron2\text{-}TGG}$ and non-editable *FgBUD14*$^{Intron2\text{-}TAA}$ alleles were transformed into the *Fgbud14* mutant. Subsequently, we performed RT-PCR and confirmed by sequencing analyses that intron 2 was completely retained and not further edited during sexual reproduction in both *Fgbud14*/*FgBUD14*$^{Intron2\text{-}TGG}$ and *Fgbud14*/*FgBUD14*$^{Intron2\text{-}TAA}$ transformants (Fig. S6). The *Fgbud14*/*FgBUD14*$^{Intron2\text{-}TGG}$ transformants were normal in vegetative growth (Fig. 8A), ascus development and ascospore discharge (Fig. 8B and C), indicating that retaining the in-frame intron 2 with the TG^{1334}G mutation had no obvious effects on *FgBUD14* functions. In contrast, the *Fgbud14*/*FgBUD14*$^{Intron2\text{-}TAA}$ transformant expressing the non-editable allele was defective in growth rate and sexual reproduction (Fig. 8), indicating that the expression of non-editable *FgBUD14*$^{Intron2\text{-}TAA}$ allele failed to complement the defects of *Fgbud14* mutant. Therefore, stage-specific editing of TA^{1334}G to TGG in the retained intron 2 of *FgBUD14* is important for its function during sexual reproduction.

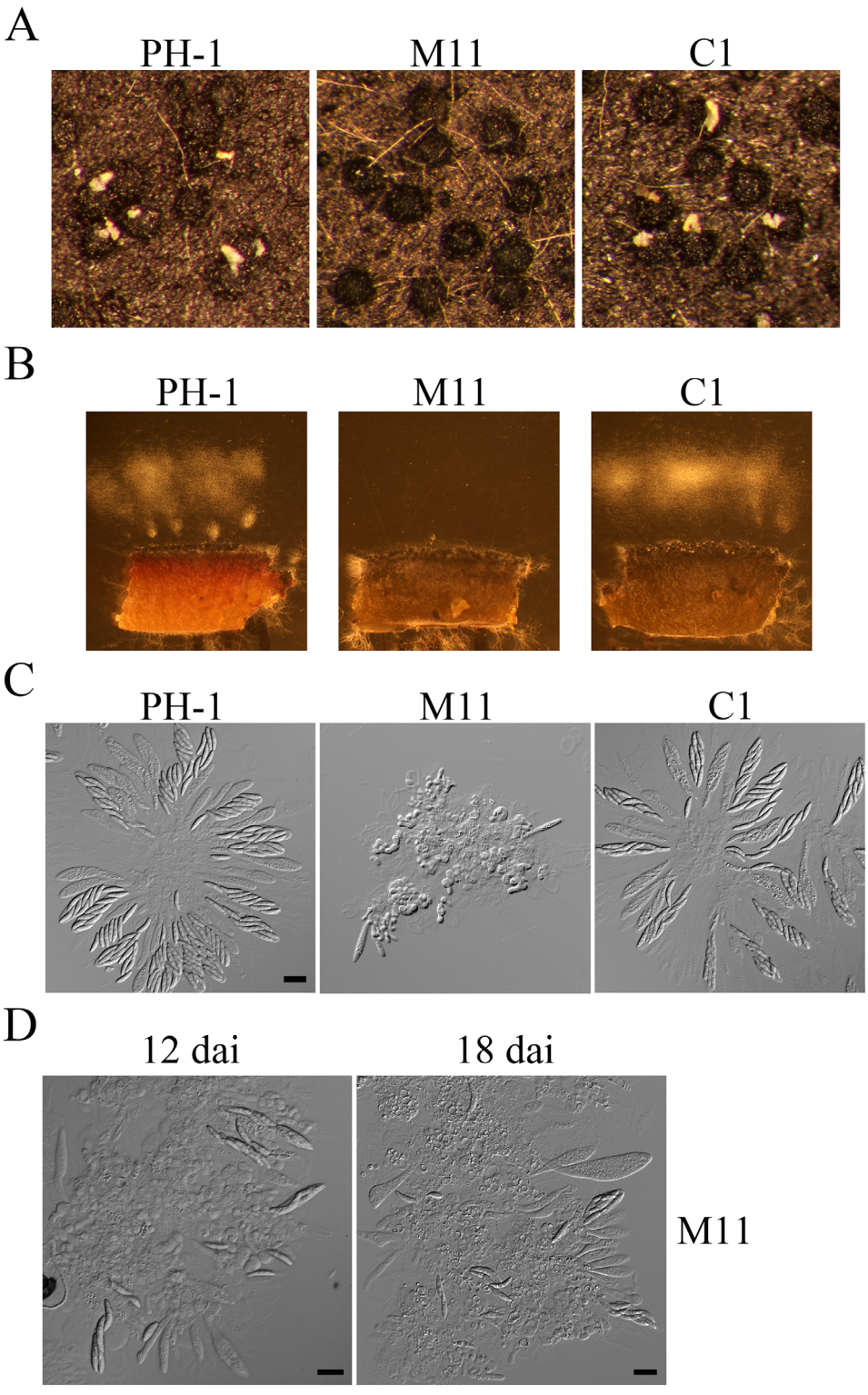

Fig. 3. Defects of the *Fgbud14* mutant in sexual reproduction.
A. Selfing cultures of the wild type (PH-1), *Fgbud14* mutant (M11) and *Fgbud14*/*FgBUD14* complemented transformant (C1) were examined for perithecium formation and ascospore cirrhi at 8 dai.
B. Perithecia of the same set of strains were assayed for ascospore discharge. Ascospores discharged from perithecia accumulated as whitish masses after incubation for 24 h. The *Fgbud14* mutant had no visible ascospore discharge.
C. Asci and ascospores formed by PH-1, mutant M11 and complemented transformant C1 were examined at 7 dai. Normal asci with eight ascospores were rarely observed in the *Fgbud14* mutant. Bar = 20 μm.
D. Asci and ascospores formed by the *Fgbud14* mutant M11 were examined at 12 and 18 dai. Bar = 20 μm. [Color figure can be viewed at wileyonlinelibrary.com]

Discussion

In the budding yeast, Bud14 is one of the proteins that interact with Ama1 and it is involved in bud-site selection and polarized growth. Although the amino acid sequences of Bud14 homologues are not well conserved, FgBud14 is the only Bud14 homologue in *F. graminearum* and has the

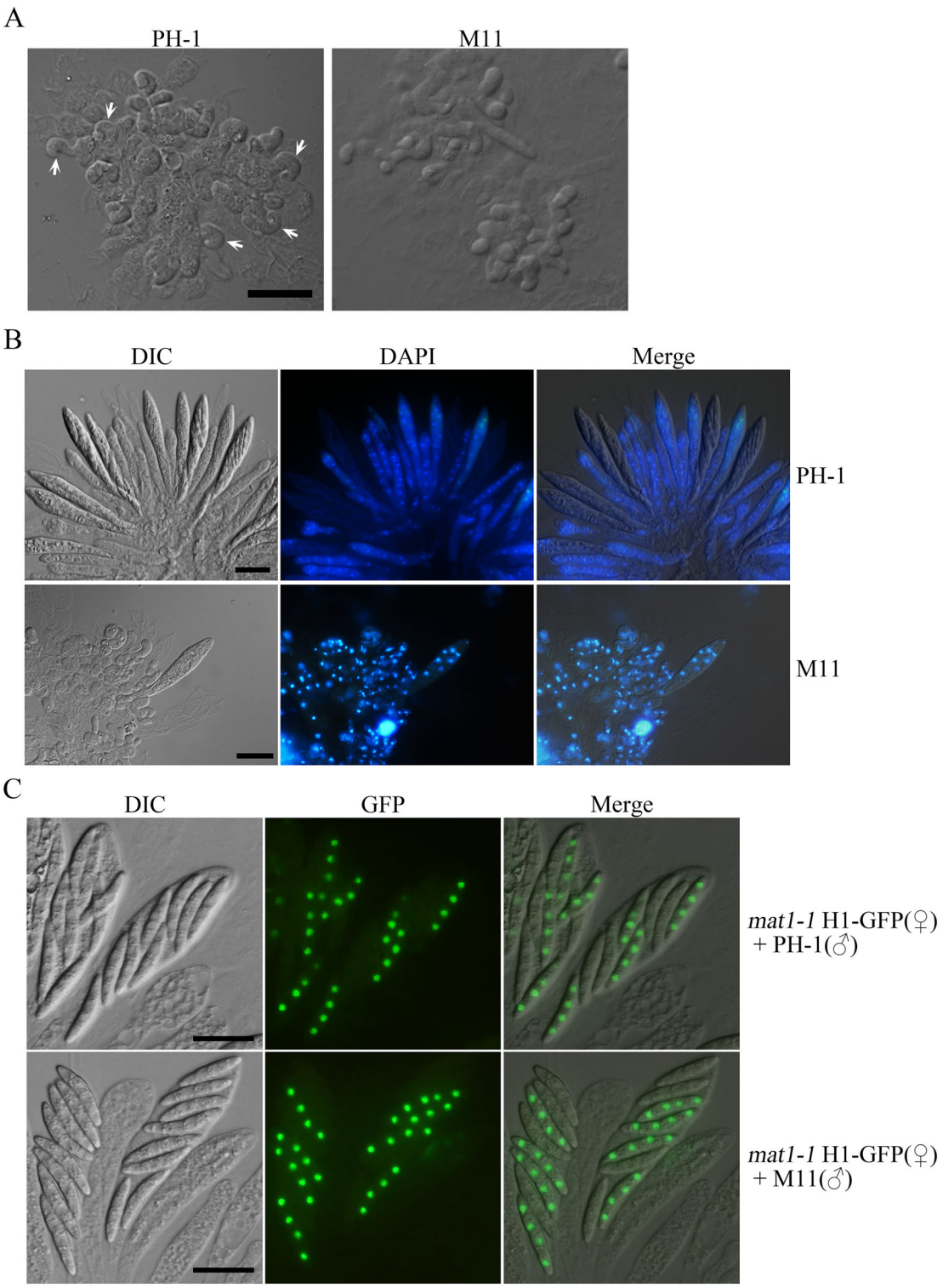

Fig. 4. FgBud14 is important for crozier formation and ascus development.
A. Perithecia from mating cultures of PH-1 and *Fgbud14* mutant (M11) were collected at 4 dai and examined for crozier and ascus development. Abundant croziers (marked with white arrows) were observed only in the wild type. Bar = 20 μm.
B. Developing asci and ascogenous tissues in perithecia of PH-1 and M11 at 5 dai were stained with DAPI and examined by DIC and epi-fluorescence microscopy. Fascicles of developing asci with eight nuclei were observed only in PH-1. Bar = 20 μm.
C. Asci and ascospores from perithecia from the out-crosses between the *mat1-1* H1-GFP strain as the female with PH-1 (upper row) or *Fgbud14* (lower row) at 7 dai were examined by DIC and epifluorescence microscopy. Eight ascospores in each ascus showed 1:1 segregation for GFP signals in the nucleus. Bar = 20 μm. [Color figure can be viewed at wileyonlinelibrary.com]

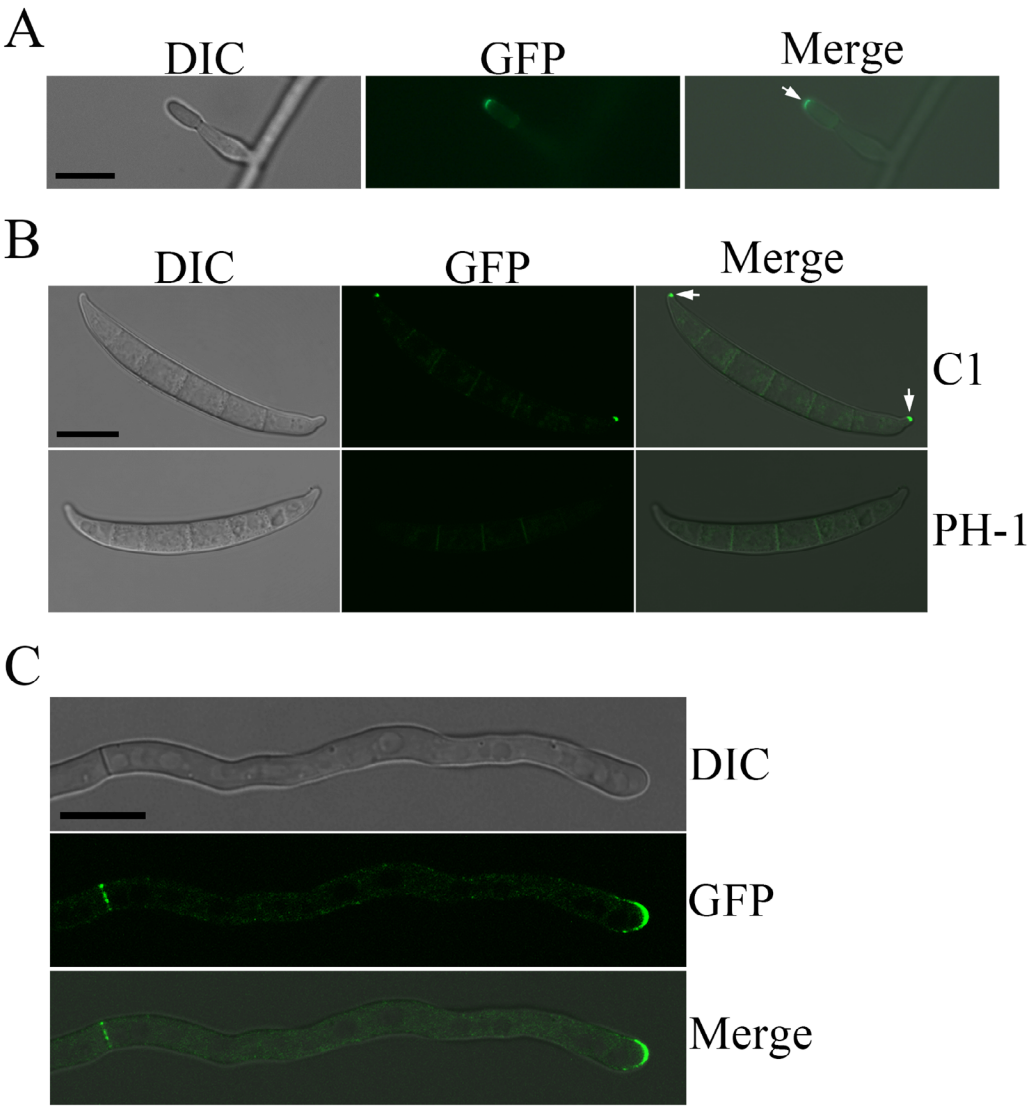

Fig. 5. Subcellular localization of FgBud14 during conidiogenesis and vegetative hyphal growth.
A. Developing conidia on conidiophores of the *Fgbud14*/*FgBUD14*-GFP complemented transformant. Arrow points to GFP signals observed at the tip of developing conidia by DIC and epifluorescence microscopy. Bar = 10 μm.
B. Mature conidia of the *Fgbud14*/*FgBUD14*-GFP complemented transformant. GFP signals were observed at both ends of mature conidia ((marked with arrows) by Olympus FV3000 confocal microscope. Bar = 10 μm.
C. Germ tubes (12 h) of *Fgbud14*/*FgBUD14*-GFP transformant were examined by Olympus FV3000 confocal microscope. GFP signals were observed at the tip and septa of germ tubes. Bar = 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]

conserved SH3 and Glc7-binding domains (Valinluck *et al*., 2014). However, we failed to detect the interaction between FgBud14 and FgAma1 in BiFC assays. The *Fgama1* mutant was normal in ascus development but produced deformed ascospores as reported (Hao *et al*., 2019). However, the *Fgbud14* mutant showed a different defect from the *Fgama1* mutant during sexual development. Therefore, the FgAma1 and FgBud14 may not be functionally related during sexual reproduction.

Similar to the *bud14* mutant in *S*. *cerevisiae* (Cullen and Sprague, 2002), the *Fgbud14* deletion mutant was reduced approximately by 16% in growth rate. The Bud14 orthologues also are known to play a role in normal vegetative growth in *A*. *nidulans*, *M*. *oryzae* and *Ustilago maydis* (Higashitsuji *et al*., 2009; Patkar *et al*., 2010; Valinluck *et al*., 2014). In *F*. *graminearum*, the *Fgbud14* mutant had a slightly shorter inter-septal distance in hyphae than that of the wild type. Similarly, an increased number of septa were observed in germ tubes of a Δ*teaC* mutant in *A*. *nidulans* (Higashitsuji *et al*., 2009). In *U*. *maydis*, the filaments of a *tea4* mutant formed under dimorphic switch-inducing conditions also

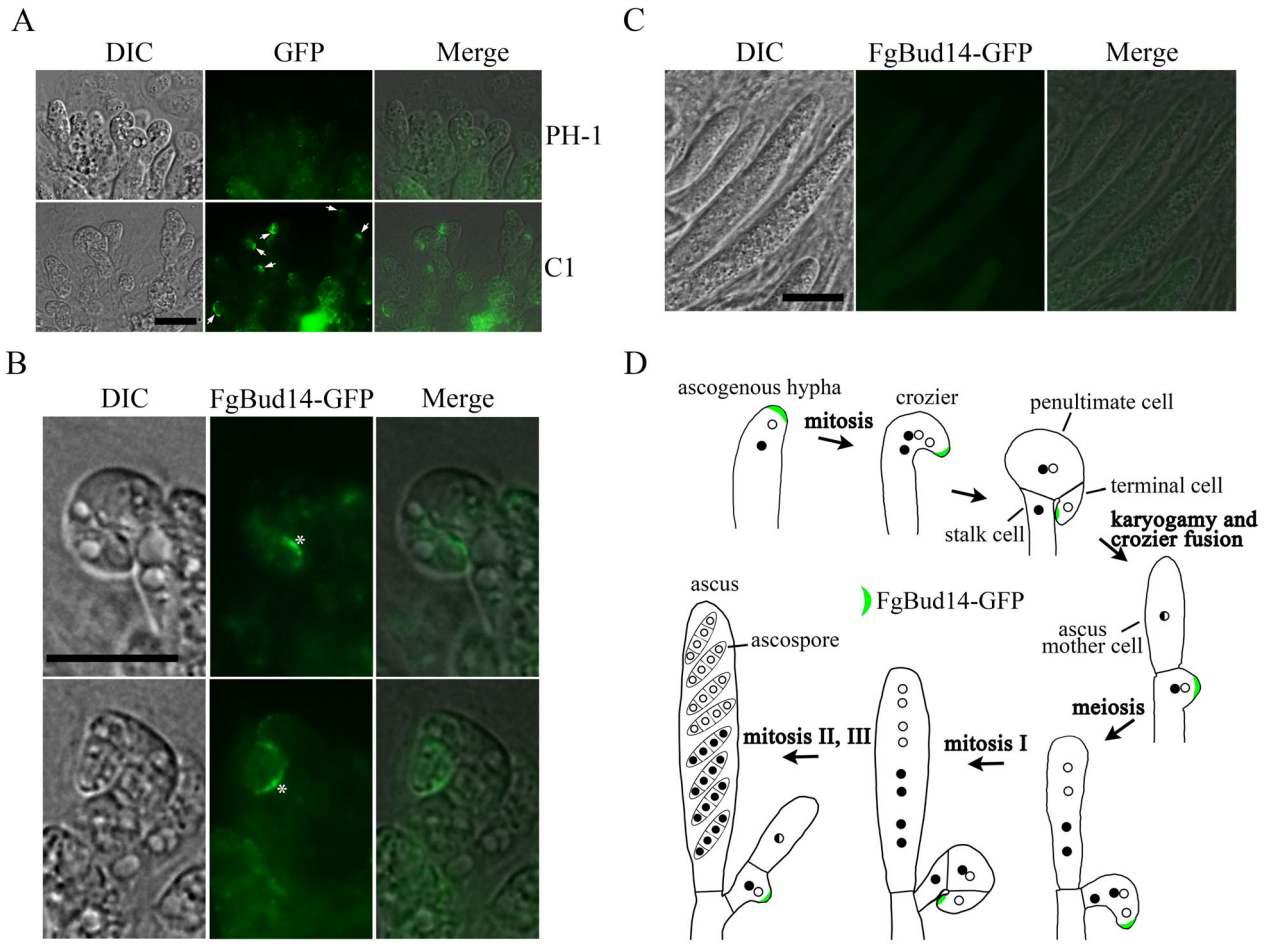

Fig. 6. Subcellular localization of FgBud14 during sexual development.
A. Ascogenous tissues from perithecia formed by the *Fgbud14*/*FgBUD14*-GFP transformant at 4 dai were examined by DIC and epifluorescence microscopy. GFP signals were localized to the apexes of ascogenous hyphae and croziers (marked with arrows).
B. GFP signals were also accumulated at the contact region (marked with asterisks) between crozier terminal cell and stalk cell, where the crozier fusion would occur. Bar = 10 μm.
C. GFP signals were not present at the tip of developing asci from the *Fgbud14*/*FgBUD14*-GFP transformant at 4.5 dai. Bar = 10 μm.
D. A model of dynamic localization of FgBud14 during ascospore genesis. [Color figure can be viewed at wileyonlinelibrary.com]

were increased in the number of septa (Valinluck *et al*., 2014). Therefore, the Bud14 homologues seem to play a conserved role in hyphal septation.

The *Fgbud14* mutant was reduced over 60% in conidiation and the majority of mutant conidia were shorter and had fewer septa than the wild-type conidia. In *M*. *oryzae*, the *Motea4* mutant also was significantly reduced in conidiation and formed two-celled conidia instead of three-celled pyriform conidia (Patkar *et al*., 2010). However, the Δ*teaC* mutant appeared to have normal conidium morphology, but *A*. *nidulans* forms unicellular conidia (Higashitsuji *et al*., 2009). It is possible that *BUD14* homologues are important only for conidiogenesis in fungi that produce multi-septate conidia, which may be related to the function of Bud14 proteins in septation.

BUD14 homologues have been characterized as a cell end marker for polarized growth in the budding and fission yeasts, and in the filamentous ascomycetes *A*. *nidulans* and *M*. *oryzae* (Higashitsuji *et al*., 2009; Patkar *et al*., 2010; Valinluck *et al*., 2014). In this study, we showed that FgBud14 localized to the tip of vegetative hyphae and conidia. Conidia of the *Fgbud14* mutant were normal in the initial germination from conidium compartments, and 12-hpi germlings of *Fgbud14* were normal in hyphal branching, but reduced in germ tube growth. In *A*. *nidulans*, conidia of Δ*teaC* mutant were defective in the emergence of the second germ tube and the number of branches increased in germlings of Δ*teaC* mutant (Higashitsuji *et al*., 2009). In the *Motea4* mutant of *M*. *oryzae*, one or more branched germ tubes were often formed from the end compartment of conidia, and only 9% of *Motea4* conidia formed abnormal appressoria and even fresh germ tubes emerged from the appressorium-like structures (Patkar *et al*., 2010). Therefore, it is likely that

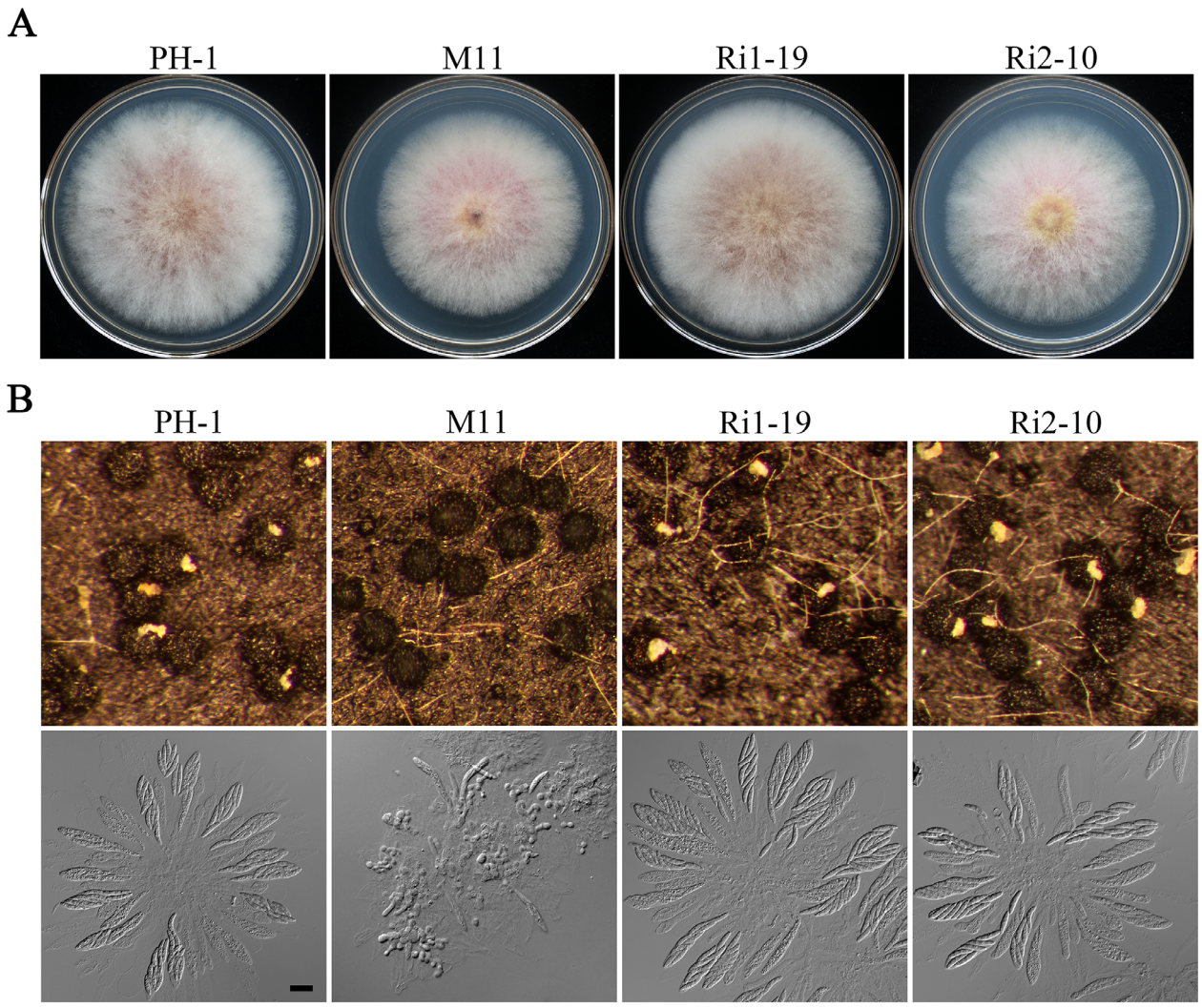

Fig. 7. Functional characterization of the first and second introns of the *FgBUD14* gene.
A. Three-day-old PDA cultures of the wild-type strain PH-1, *Fgbud14* mutant (M11), *Fgbud14*/*FgBUD14*^{Intron1} transformant (Ri1-19) with non-splicable intron 1 and *Fgbud14*/*FgBUD14*^{Intron2} transformant (Ri2-10) with non-splicable intron 2.
B. Mating cultures of the same set of strains were examined for perithecia with ascospore cirrhi (upper panels) at 8 dai and asci from crushed perithecia (lower panels) at 7 dai. Bar = 20 μm. [Color figure can be viewed at wileyonlinelibrary.com]

the role of *BUD14* homologues in conidium germination and establishing polarity is species-specific in filamentous fungi. In *Saccharomyces pombe*, Tea4 mediates the connection between Tea1 and the formin For3 that catalyses F-actin assembly (Evangelista *et al*., 2002; Martin *et al*., 2005; Higashitsuji *et al*., 2009). Tea1 and Tea4 localize to the cell end in a Mod5-dependent manner. The Tea4–formin interaction was also proved in *A. nidulans*, *M. oryzae* and *U. maydis* (Higashitsuji *et al*., 2009; Patkar *et al*., 2010; Valinluck *et al*., 2014). Both polarized growth and septation are actin-dependent processes, and actin usually accumulates at sites of germination, growing hyphal tips and sites of septa formation (Kono *et al*., 2005; Berepiki *et al*., 2011). Thus, FgBud14 likely has its conserved role in regulating the organization of actin cytoskeleton in *F. graminearum*.

In *F. graminearum*, deletion of *FgBUD14* only slightly reduced its virulence in infection assays with wheat heads and corn silks inoculated with conidia. However, deletion of *MoTEA4* affected appressorium formation and penetration, and the *Motea4* mutant was non-pathogenic in *M. oryzae* (Patkar *et al*., 2010). In *U. maydis*, the *TEA4* orthologue also is essential for the fusion between cells of compatible mating types, which is required for the yeast-to-hypha transition and plant infection (Valinluck *et al*., 2014). The different roles of *BUD14* homologues in plant infection among these fungal pathogens may be related to their differences in infection strategies.

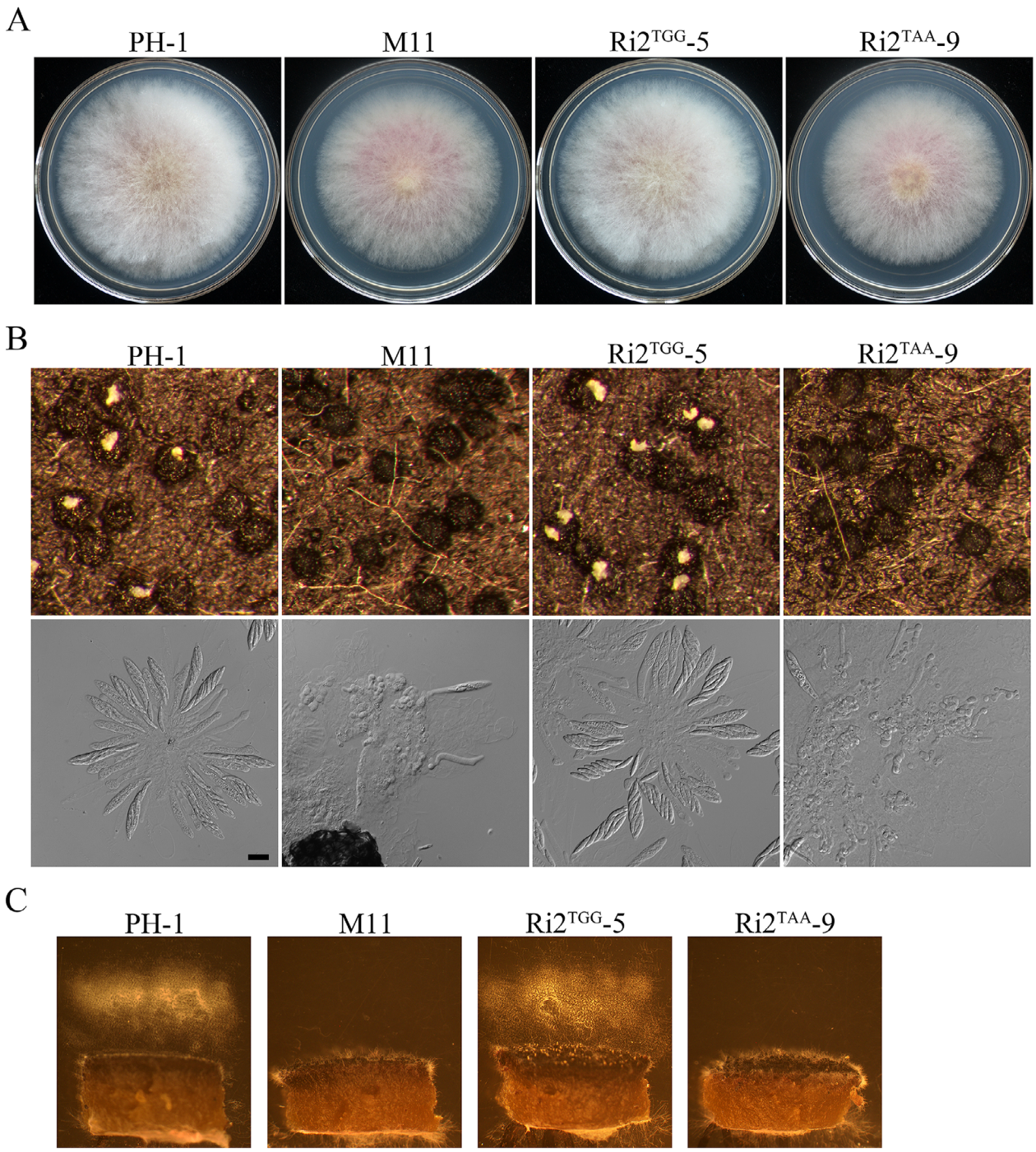

Fig. 8. Functional characterization of the RNA editing in the second intron.
A. Three-day-old PDA cultures of the wild-type PH-1, *Fgbud14* mutant (M11), *Fgbud14*/*FgBUD14*$^{Intron2\text{-}TGG}$ (Ri2^{TGG}-5) and *Fgbud14*/*FgBUD14*$^{Intron2\text{-}TAA}$ (Ri2^{TAA}-9) transformants.
B. Mating cultures of the same set of strains were examined for perithecia with ascospore cirrhi (upper panels) at 8 dai and asci from crushed perithecia (lower panels) at 7 dai. Bar = 20 μm.
C. Perithecia of the same set of strains were assayed for ascospore discharge. Ascospores discharged from perithecia accumulated as whitish masses after incubation for 24 h. [Color figure can be viewed at wileyonlinelibrary.com]

Infection of floral tissues by *F. graminearum* does not involve a mating-associated dimorphic transition or heavily melanized appressoria.

Although Bud14 orthologues have been well characterized for their functions during vegetative growth and asexual reproduction in yeasts and other ascomycetes,

their functions in sexual reproduction are not clear. We found that FgBud14, like FgAma1, is dispensable for perithecium formation. However, FgBud14 is important for crozier formation, ascus development, and ascosporogenesis. In *F. graminearum*, FgBud14 may be involved in the tip bending of ascogenous hyphae to form croziers and may also play a role in the crozier fusion event leading to the formation of elongated developing asci in ascogenous tissues (Bouhouche *et al*., 2004; Pöggeler *et al*., 2018). In *U. maydis*, a basidiomycete, the *TEA4* orthologue is essential for the fusion between yeast cells of compatible mating types, which lead to hyphal growth and plant infection (Valinluck *et al*., 2014). Besides its role in the early stages of sexual reproduction, FgBud14 is also important for ascospore formation although the underlying mechanism is not clear. It is possible that FgBud14 is involved in the proper elongation of developing asci and formation of mature asci, because the *Fgbud14* mutant often produced deformed asci with fewer than eight ascospores in mature asci of *F. graminearum*.

Unlike *FgAMA1* that is specifically expressed during sexual reproduction, *FgBUD14* is constitutively expressed, although its expression level is elevated in perithecia. Interestingly, *FgBUD14* transcripts are subjected to both alternative splicing and RNA editing during sexual reproduction. Because of a PSC in the second intron, A-to-I RNA editing is essential for the transcripts that retain intron 2 to encode functional FgBud4 proteins. During sexual development, this stop codon can be removed by alternative splicing or be corrected by A-to-I RNA editing to encode full-length functional proteins. Interestingly, its orthologue in *N. crassa* (NCU00006) also has the second intron that is subjected to both alternative splicing and RNA editing based on published RNA-seq data (Liu *et al*., 2017). To our knowledge, *FgBUD14* and NCU00006 are the first two genes that are found to have both stage-specific alternative splicing and RNA editing during sexual reproduction in fungi. Alternative splicing and RNA editing would ensure the greatest protein diversity of FgBud14, which may ensure the normal sexual process under harsh environmental conditions.

Materials and methods

Strains and culture conditions

The wild-type strain PH-1 (Cuomo *et al*., 2007) and transformants derived from it in this study were routinely cultured on potato dextrose agar (PDA) plates at 25°C (Ren *et al*., 2019). Colony morphology and growth rate were assayed with 3-day-old PDA cultures as described (Li *et al*., 2015). Conidiation and conidium morphology were assayed with 5-day-old CMC cultures (Wang *et al*., 2012). Freshly harvested conidia were incubated in liquid YEPD medium for 6 and 12 h and examined for defects in conidium germination and germ tube growth. For selfing, aerial hyphae of 7-day-old carrot agar cultures were pressed down with sterile 0.1% Tween 20 (Kim *et al*., 2015). For outcrossing, 7-day-old carrot agar cultures of *mat1-1* H1-GFP were pressed down with conidium suspensions (10^5 conidia ml^{-1}) of the PH-1 or *Fgbud14* mutant (Zheng *et al*., 2013). Mating cultures were incubated under a black light lamp at 25°C for 7–18 days and examined for perithecium formation, ascosporogenesis and ascospore discharge as described (Wang *et al*., 2011; Zheng *et al*., 2013). Protoplast preparation and PEG-mediated transformation were performed as previously described (Hou *et al*., 2002). For selection of transformants, hygromycin B (CalBiochem, La Jolla, CA, USA) and G418 (Sigma-Aldrich, St Louis, MO, USA) were added to the final concentration at 300 and 400 μg ml^{-1} respectively (Wang *et al*., 2018).

Generation of Fgbud14 *and* Fgbud14 Fgama1 *mutants*

The *FgBUD14* (FGSG_04118) gene replacement construct was generated with the split-marker approach as described (Catlett *et al*., 2003; Li *et al*., 2011). A 0.9-kb upstream and a 1.0-kb downstream fragment of *FgBUD14* were amplified with the primer pairs 1F/2R and 3F/4R respectively. The resulting PCR products were fused to *NEO* (Neomycin-resistance gene) fragments amplified with the primer pairs NEO-F/EO-R and EO-F/NEO-R from pFL7 plasmid (Zhou *et al*., 2010) by overlapping PCR and co-transformed into protoplasts of PH-1. Transformants resistant to G418 were screened with primers 5F/6R that amplified a 0.7-kb *FgBUD14* fragment. The *Fgbud14* deletion mutants were confirmed by PCR with both upstream and downstream anchor primers as described (Wang *et al*., 2012). For generating the *Fgbud14 Fgama1* double mutants, the *FgAMA1* gene replacement construct generated with the hygromycin resistance gene (*hph*) was transformed into the *Fgbud14* mutant M11. The putative *Fgbud14 Fgama1* mutants were identified by PCR. All primers used for PCR are listed in Table S1.

Complementation of the Fgbud14 *mutant*

For complementation assays, the gap repair method (Bruno *et al*., 2004) was used to generate the *FgBUD14*-GFP construct. The entire *FgBUD14* gene with its promoter region was amplified with primers HB-F and HB-R and co-transformed with XhoI-digested pDL2 (carrying the hygromycin resistance marker) into yeast strain XK1-25 as described (Zhou *et al*., 2011). The

FgBUD14-GFP fusion construct recovered from Trp + yeast transformants was transformed into the *Fgbud14* mutant. The *Fgbud14*/*FgBUD14*-GFP transformants resistant to both hygromycin and G418 were analysed by PCR and further confirmed by examination for GFP signals with an Olympus BX53 epifluorescence microscope (Olympus, Tokyo, Japan) or an Olympus FV3000 confocal microscope (Olympus).

Plant infection and DON production

For infection assays with flowering wheat heads of cultivar Xiaoyan 22, conidia were harvested from 5-day-old CMC cultures and re-suspended to 2×10^5 conidia ml^{-1} in sterile distilled water. For each head, a center spikelet was drop-inoculated with 10 μl of conidium suspensions as described (Gale *et al*., 2002). Wheat spikelets with typical FHB symptoms were examined at 14 days post-inoculation (dpi). The disease index was estimated as the number of diseased spikelets per head from three independent replicates, with at least 10 wheat heads inoculated in each replicate (Jonkers *et al*., 2012). Corn silks of cultivar Pioneer 2375 were infected with culture blocks and examined as described (Hou *et al*., 2002).

For DON production assays, conidia harvested from 5-day-old CMC cultures were re-suspended in TBI medium to a final concentration of 10^4 conidia ml^{-1}. 2 ml of conidial suspension was transferred to the well of a 24-well plate and incubated for 7 days at 25°C without shaking in the dark. Subsequently, 400 μl liquid culture was mixed with 1.6 ml methanol, and then cleaned up with a C18 SPE cartridge. 800 μl of filtrate was transferred to a new 2 ml tube and vacuum-evaporated to dryness at 25°C. The residue was dissolved in 50 μl complex solvent of TMSI:TMCS (100:1 vol./vol.) by vortexing vigorously for 10 min. The resulting solution was mixed with 800 μl isooctane and 800 μl ultrapure water successively. The mixture was allowed to stand at room temperature for 20 min for separation of liquid phases. The upper organic phase was analysed by a capillary gas chromatography–mass spectrometry (GCMS-QP2010 Shimadzu, Kyoto, Japan) with an Rxi-5MS column (30 m long; 0.25 mm internal diameter; 0.25 μm film thick) and nitrogen was used as the carrier gas at a constant flow rate of 1 ml min^{-1}. The operating conditions were as follows: injector temperature: 260°C; interface temperature: 280°C; oven temperature: 150°C (1 min), slope 30°C min^{-1} and 280°C (15 min); ion source: 250°C. The *m*/*z* fragment ions monitored for quantification of trichothecenes were 193 and 295 for DON, 235 and 377 for 3-ADON and 235 and 392 for 15-ADON. The calculation of trichothecene concentration was based on the average area counts of the fragment ions of each standard toxin. The retention time for each toxin was 6.375 min for DON, 6.890 min for 3-ADON and 6.960 min for 15-ADON. The values are reported in μg g^{-1} dried mycelia, and the assays were repeated three times for each strain. Because the wild-type PH-1 strain tends to produce predominantly 15-ADON in TBI culture (Alexander *et al*., 2011), we just showed the concentrations of 15-ADON of tested strains.

Generation of different mutant alleles of FgBUD14

To generate the *FgBUD14*^{Intron1} construct by yeast gap repair, three fragments of *FgBUD14* were amplified with primer pairs FR-1F/BPU-1R, BPU-1F/BP1-R and BP1-F/HB-R respectively and co-transformed with *Xho*I-digested pDL2 into yeast strain XK1-25 (Bruno *et al*., 2004; Zhou *et al*., 2011). The resulting *FgBUD14*^{Intron1} construct rescued from Trp + yeast transformants was verified by sequencing for the 'gt-ag' to 'ct-aa' mutation at the splicing sites of intron 1. Similar approaches were used to generate the *FgBUD14*^{Intron2}, *FgBUD14*$^{Intron2\text{-}TGG}$ and *FgBUD14*$^{Intron2\text{-}TAA}$ alleles with PCR primers listed in Table S1. All the mutant alleles of *FgBUD14* were verified by sequencing and transformed into the *Fgbud14* mutant M11 for complementation assays.

Staining for the nucleus and cell wall

To assay defects in septation, conidia and hyphae of the wild-type and *Fgbud14* mutant strains were stained with Calcofluor white (CFW) as described (Li *et al*., 2015). To assay nuclear division during ascus development, perithecia were harvested from mating plates at 4–6 dai, gently crushed, and stained with 20 μg ml^{-1} 4,6-Diamidino-2-phenylindole for 5 min. Samples were examined for CFW and DAPI staining signals with an Olympus BX53 epifluorescence microscope.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Alignment of FgBud14 with its orthologues from other fungi.

Sequences of FgBud14 and its homologues from *Saccharomyces cerevisiae* (Bud14), *Magnaporthe oryzae* (MGG_06439T0), *Neurospora crassa* (NCU00006), *Aspergillus nidulans* (AN1099), and *Sclerotinia sclerotiorum* (SS1G_03195) were aligned with MEGA software. Identical residues are highlighted in black and conserved residues in grey. The conserved SH3 and Glc7-binding domains are marked with blue overline and red box, respectively.

Fig. S2. PCR assays for confirmation of the deletion of *FgBUD14*.

A. The *FgBUD14* locus and gene replacement construct. The *FgBUD14* and *NEO* genes are marked with empty and black arrows, respectively. Primer pairs 1F/2R and 3F/4R were used to amplify the upstream and downstream flanking sequences of *FgBUD14*. Primers pairs 5F/6R, G850/G852, 7F/G856R, and G855F/8R were used for mutant screen and verification. B. Verification of the labelled *Fgbud14* deletion mutants by PCR with primer pairs 5F/6R (L1), G850/G852 (L2), 7F/G856R (L3), G855F/8R (L4). DNA isolated from the wild-type strain PH-1 was used as the control. Whereas mutant strains M11 and M22 had only the expected bands derived from homologous recombination events in the flanking sequences, mutant M6 had an additional band when amplified with primers 7F and G856R.

Fig. S3. Quantitative analysis of *Fgbud14* defects in sexual reproduction.

A. 7-dai selfing cultures of PH-1, *Fgbud14* mutant (M11) and complemented transformant (C1) were counted to calculate the number of perithecia per cm^2.

B. The average number of wild-type and *Fgbud14* asci per perithecium at 7 dai. C. The percentage of *Fgbud14* asci with indicated number of ascospores per ascus in perithecia at 12 dai.

Fig. S4. The effect of Latrunculin A on FgBud14 localization.

The localizations of LifeAct-GFP (A) and FgBud14-GFP (B) in transformants PH-1/LifeAct-GFP and *Fgbud14*/*FgBUD14*-GFP treated with or without Latrunculin A (Lat A). These transformants were grown on SYM agar for 1 day and treated with 10% DMSO (-LatA) or 10 μM Lat A (+LatA) for 20 min, and then observed under Olympus FV3000 confocal microscope.

Fig. S5. The relationship between FgBud14 and FgAma1.

A. Ascogenous tissues from perithecia formed by PH-1 expressing both *FgBUD14*-NYFP and *FgAMA1*-CYFP at 4 dai were examined by DIC and epifluorescence microscopy. No reliable YFP signals were detected at the apexes of ascogenous hyphae and croziers, suggesting that the FgBud14 may not interact with FgAma1 during sexual development. Bar = 10 μm. B. The morphology of asci and ascospores in deletion mutants of *Fgbud14* (M11), *Fgama1* (N6) and *Fgbud14 Fgama1* (MN4) at 12 dai. Bar = 20 μm.

Fig. S6. Detecting the intron 2 retention and RNA editing of *FgBUD14* transcripts in different transformants.

A. The intron 2 retentions were verified by RT-PCR with primers (marked with arrows) in 8-dai perithecia of transformants Ri2-10 (*Fgbud14*/*FgBUD14*^{Intron2}), Ri2^{TGG}-5

(*Fgbud14*/*FgBUD14*$^{Intron2\text{-}TGG}$) and Ri2^{TAA}-9 (*Fgbud14*/*FgBUD14*$^{Intron2\text{-}TAA}$). B. RNA editings were examined by direct sequencing of PCR products in 8-dai perithecia of the same set of strains. The blue boxes point the loci where RNA editing may occur. The two peaks (A and G) marked by the red arrow indicated that the A-to-I RNA editing occurred in transformants Ri2-10.

Table S1. PCR primers used in this study.