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Sexual specific functions of Tub1 beta-tubulins require stage-specific RNA processing and expression in *Fusarium graminearum*

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Summary

The wheat head blight fungus *Fusarium graminearum* has two highly similar beta-tubulin genes with overlapping functions during vegetative growth but only *TUB1* is important for sexual reproduction. To better understand their functional divergence during ascosporogenesis, in this study we characterized the sequence elements important for stage-specific functions of *TUB1*. Deletion of *TUB1* blocked the late but not initial stages of perithecium formation. Perithecia formed by *tub1* mutant had limited ascogenous hyphae and failed to develop asci. Silencing of *TUB1* by MSUD also resulted in defects in ascospore formation. Interestingly, the 3′-UTR of *TUB1* was dispensable for growth but essential for its function during sexual reproduction. RIP mutations that specifically affected Tub1 functions during sexual reproduction also were identified in two ascospore progeny. Furthermore, site-directed mutagenesis showed that whereas the non-editable mutations at three A-to-I RNA editing sites had no effects, the N347D (not T362D or I368V) edited mutation affected ascospore development. In addition, the F167Y, but not E198K or F200Y, mutation in *TUB1* conferred tolerance to carbendazim and caused a minor defect in sexual reproduction. Taken together, our data indicate *TUB1* plays an essential role in ascosporogenesis and sexual-specific functions of *TUB1* require stage-specific RNA processing and Tub1 expression.

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Introduction

Wheat head blight (WHB) caused by the homothallic ascomycete *Fusarium graminearum* is one of the most destructive diseases of wheat and barley (Bai and Shaner, 2004; Goswami and Kistler, 2004). Outbreaks of WHB often cause severe yield losses and contaminations of infested grains with deoxynivalenol (DON), zearalenone and other mycotoxic compounds (Desjardins, 2003). Although conidia also are infectious, ascospores are the primary inoculums in *F. graminearum*, which overwinters and forms perithecia on plant debris (Osborne and Stein, 2007; Geng *et al*., 2014). Ascospores are forcibly released from perithecia for dispersal and infection of floral tissues involves the differentiation of compound appressoria or penetrating hyphae (Jansen *et al*., 2005; Boenisch and Schäfer, 2011). After penetration, *F. graminearum* likely has a transient period of biotrophic phase and forms morphologically irregular invasive hyphae (Rittenour and Harris, 2010; Zhang *et al*., 2012). Eventually, infectious hyphae of *F. graminearum* become necrotrophic and spread from diseased kernels to other spikelets on the same heads via the rachis and causes the typical head blight symptoms (Wanjiru *et al*., 2002; Brown *et al*., 2010).

As a homothallic ascomycete with the linked *MAT1-1* and *MAT1-2* loci, *F. graminearum* is proficient at the production of perithecia and ascospores by selfing. However, it also can be forced to outcross (Bowden and Leslie, 1992, 1999; Hou *et al*., 2002; Lee *et al*., 2003). During sexual reproduction, dikaryotic ascogenous hyphae growing inside developing perithecia give rise to croziers, which are then developed into asci after karyogamy. In developing asci, meiosis is followed by one round of post-meiotic mitosis before the formation of eight ascospores (Bennett and Turgeon, 2016). After two additional rounds of mitosis and cytokinesis, mature ascospores are four-celled. To date, three genetic and epigenetic phenomena, repeat induced point mutation (RIP), meiotic silencing by unpaired DNA (MSUD) and A-to-I RNA editing, have been reported to specifically occur during sexual reproduction in *F. graminearum* (Cuomo *et al*., 2007; Son *et al*., 2011; Liu

et al., 2016). RIP and MSUD were first discovered in *Neurospora crassa* (Aramayo and Selker, 2013; Gladyshev and Kleckner, 2017; Hammond, 2017). Whereas RIP occurs pre-meiotically and leads to C-to-T point mutations in repeated sequences, MSUD likely occurs after karyogamy to silence the expression of unpaired DNA. Like MSUD, A-to-I RNA editing does not change genomic DNA sequences but may increase the proteome complexity during sexual reproduction in filamentous fungi (Liu *et al*., 2016, 2017; Teichert *et al*., 2017).

Beside these stage-specific genetic and epigenetic phenomena, functional characterization of the two highly similar Cdc2 and β-tubulin paralogs in *F. graminearum* have showed that sexual reproduction differs from vegetative growth in cell cycle regulation and microtubule organizations (Zhao *et al*., 2014; Liu *et al*., 2015). Whereas Cdc2A and Cdc2B have redundant functions during vegetative growth, only Cdc2A is important for ascosporogenesis (Liu *et al*., 2015). The *cdc2B* mutant has no obvious phenotypes but deletion of both *CDC2A* and *CDC2B* is lethal. Similar to the two Cdc2 paralogs, whereas most fungi have only one, *F. graminearum* has two beta-tubulin genes. The *tub1* mutant is only slightly reduced in growth rate but deletion of *TUB2* results in a severe growth defects (Chen *et al*., 2009; Qiu *et al*., 2011, 2012; Zhao *et al*., 2014). Interestingly, whereas *TUB2* is dispensable for sexual reproduction, the *tub1* deletion mutant is blocked in ascus development and ascospore formation. Deletion of both *TUB1* and *TUB2* is lethal in *F. graminearum*, further indicating their overlapping functions in hyphal growth. Phylogenetic analysis showed that *TUB1* is the ancestral copy that is under strong purifying selection. *TUB2* is likely derived from a duplication event and it is under divergent evolution (Zhao *et al*., 2014).

Based on published RNA-seq and microarray data (Qi *et al*., 2006; Liu *et al*., 2016), both *TUB1* and *TUB2* are constitutively expressed in hyphae and perithecia in *F. graminearum.* Besides their highly conserved amino acid sequences, Tub1 and Tub2 also have similar tertiary structures (Zhao *et al*., 2014). Therefore, it is not clear what confers functional difference between *TUB1* and *TUB2* during sexual reproduction and why Tub1 beta-tubulins have the stage-specific function during ascosporogenesis. Interestingly, various mutations conferring resistance to carbendazim fungicides targeting at fungal beta-tubulins have been identified in the *TUB2* gene but not in the *TUB1* gene in field isolates (Chen *et al*., 2009; Begaye *et al*., 2011; Zhu *et al*., 2018). Like in other fungi, resistant mutations in *TUB2* of *F. graminearum* often occur at amino acid residues F167, E198 and F200 that form a carbendazim-binding pocket in beta-tubulins (Qiu *et al*., 2011). These three residues are well-conserved between Tub1, Tub2 and other fungal beta-tubulins. However, the effects of mutations at F167, E198 and F200 in *TUB1* on resistance against benzimidazole fungicides and sexual reproduction have not been examined in *F. graminearum.*

To better understand its stage-specific function in *F. graminearum*, in this study we characterized the sequence elements and modifications of *TUB1* important for sexual reproduction. *TUB1* was dispensable for the initial stages of perithecium formation but essential for ascus and ascospore development. The *tub1* mutant displayed female-specific defects in out-crossing and silencing of *TUB1* by MSUD also lead to defects in ascosporogenesis. The 3′-UTR of *TUB1* was dispensable for vegetative growth but essential for its functions during sexual reproduction. RIP mutations that specifically affect the functions of Tub1 proteins during ascosporogenesis were identified in two ascospore progeny. Furthermore, we functionally characterized three A-to-I RNA editing sites and three conserved sites related to benzimidazole resistance in *TUB1* by site-directed mutagenesis. Taken together, our data indicate that *TUB1* plays an essential role in ascosporogenesis in a female-specific manner and the sexual-specific functions of *TUB1* require stage-specific RNA processing and Tub1 expression.

Results

Ascus development but not ascogenous hyphal growth is completely blocked in the tub1 *mutant*

In *F. graminearum,* whereas *TUB2* is more important for vegetative growth, *TUB1* plays a more critical role in sexual reproduction. In selfing, the *tub1* mutant formed perithecia but failed to produce ascospores (Zhao *et al*., 2014). To further characterize its defects in sexual reproduction, we measured the size of perithecia at 7 days-post-fertilization (dpf). Perithecia formed by the *tub1* mutant had a diameter of 0.93 ± 0.30 mm, which was significantly smaller than that of the wild-type perithecia (2.03 ± 0.23 mm) (Table 2; Fig. 1A). Furthermore, perithecia produced by *tub1* mutant failed to form ascospore cirrhi. When thick-sections of perithecia were examined, mature asci with ascospores were observed in perithecia formed by PH-1. Under the same conditions, only limited growth of ascogenous hyphae was observed in *tub1* mutant perithecia (Fig. 1A). Although ostioles were visible, the hymenium layer of asci was not observed in perithecia formed by the *tub1* mutant. These results further indicate that *TUB1* is essential for ascus development although it is dispensable for perithecium formation in *F. graminearum.*

TUB1 *has female-specific functions for perithecium maturation and ascus development*

To determine whether *TUB1* has male- or female-specific functions during sexual reproduction, the *tub1* mutant

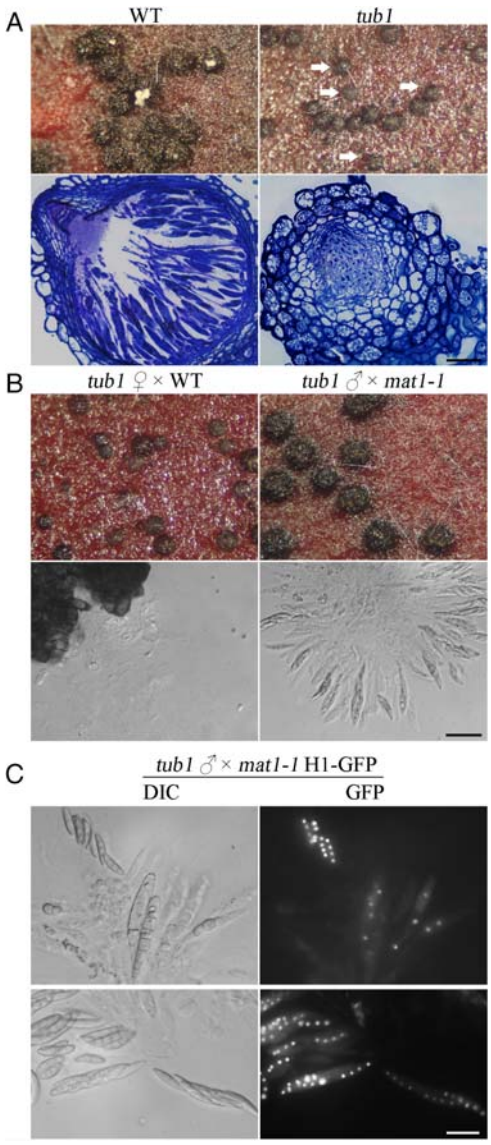

Fig. 1. Defects of the *tub1* mutant in ascosporogenesis. **A.** Perithecia produced by the wild type PH-1 and *tub1* mutant on selfing plates. When examined under a dissect microscope (upper panels), the *tub1* mutant produced smaller perithecia without ascospore cirrhi (marked with white arrows). Thick sections of perithecia (lower panels) showed the absence of hymenium layer in the *tub1* mutant. Bar = 20 μm. **B.** Perithecia formed by the *tub1* (♀) × PH-1 (♂) and *tub1* (♂) × *mat1-1* H1-GFP (♀) crosses were sampled at 7-days post-fertilization (pdf) and examined for ascus development and ascospore formation. Bar = 40 μm. **C**. Asci and ascospores from the *tub1* (♂) × *mat1-1* H1-GFP (♀) cross. Majority of normal asci with ascospores showed 1:1 segregation for GFP signals. However, some asci had defects in ascospore formation. Bar = 20 μm. [Color figure can be viewed at wileyonlinelibrary.com]

was crossed with the wild-type PH-1 or *mat1-1* (H1-GFP) deletion mutant (Lee *et al*., 2003; Cuomo *et al*., 2007). In the cross between *tub1* mutant as the female and conidia of PH-1 as the male, like selfing of *tub1* mutant, only small perithecia without ascus development were observed (Fig. 1B). However, normal-size perithecia with asci and ascospores were formed when the *tub1* mutant was crossed as the male with the *mat1-1* (H1-GFP) mutant (Fig. 1B). Four ascospores with and four ascospores without H1-GFP signals were observed in most asci (Fig. 1C). These results indicate that deletion of *TUB1* had no effect on male fertility and the formation of protoperithecia but blocked ascus development in *F. graminearum.*

Interestingly, although asci and ascospores were observed, approximately 67% of the asci from the *mat1-1* (female) × *tub1* (male) cross were defective in ascospore formation or produced ascospores with morphological defects (Fig. 1C). Meiotic silencing by unpaired DNA (MSUD) is known to occur in *F. graminearum* (Son *et al*., 2011). It is likely that MSUD of *TUB1* resulted in defects in ascospore formation in some of the asci in the *mat1-1* × *tub1* cross. Therefore, besides ascus development, *TUB1* may be also important for ascospore morphogenesis and maturation in *F. graminearum.*

TUB1-*GFP complements the defects of* tub1 *in vegetative growth but not sexual reproduction*

For complementation assays, we first transformed the *TUB1*-GFP construct generated in a previous study (Luo *et al*., 2014) into the *tub1* mutant. This GFP fusion construct contained a 1.58-kb promoter region of *TUB1* but lacked the 3′-UTR sequence. The resulting transformant TG3 (Table 1) had similar growth rate and colony morphology with PH-1 (Fig. 2A), indicating that the expression of Tub1-GFP fusion rescued the defect of *tub1* in growth. In comparison with *TUB1*-GFP transformants of PH-1, Tub1-GFP fusion proteins had the same expression and localization patterns in the *tub1*/*TUB1*-GFP transformant (Fig. 2B). Therefore, fusion with GFP and lack of 3′-end sequence had no obvious effect on *TUB1* expression and functions during vegetative growth.

Interestingly, perithecia produced by the *tub1*/*TUB1*-GFP transformants were blocked in ascosporogenesis. Similar to the *tub1* mutant, the *tub1*/*TUB1*-GFP transformants failed to form asci and ascospores (Fig. 2C). Because the *tub1* mutant generated in the previous study (Zhao *et al*., 2014) was deleted of only the ORF region (−6 205 300 to −6 207 039 of chromosome 4), repeat-induced pointed (RIP) mutations may occur to the promoter region of *TUB1* and affect the expression of ectopically integrated *TUB1*-GFP in the *tub1*/*TUB1*-GFP transformants during sexual reproduction. To avoid this problem, we generated a *tub1* deletion mutant TD4 (Table 1) in which the entire *TUB1* gene (−6 205 119 to −6 208 663 of chromosome 4), including the 1677 bp promoter region, was deleted (Supporting Information Fig. S1A). Transformants of the *tub1* mutant TD4 expressing the *TUB1*-GFP fusion had normal growth rate but were still defective in ascus development and ascospore

Table 1. Strains of *Fusarium graminearum* used in this study.

Strain	Genotype description	Reference
PH-1	Wild type	(Cuomo *et al*., 2007)
TD28	*tub1* deletion mutant of PH-1 (Δ −6205300 to −6207039)	(Zhao *et al*., 2014)
TD4	*tub1* deletion mutant of PH-1 (Δ −6205119 to −6208663)	This study
Δmat1-1::H1::GFP	*mat1-1-1* mutant expressing H1-GFP	(Lee *et al*., 2003)
T1P10	Transformant of PH-1 expressing *TUB1*-GFP	(Luo *et al*., 2014)
TG3	Transformant of TD28 expressing *TUB1*-GFP	This study
T150-6	Transformant of *tub1* mutant TD4 expressing *TUB1*-150	This study
T200-4	Transformant of *tub1* mutant TD4 expressing *TUB1*-200	This study
T665-2	Transformant of *tub1* mutant TD4 expressing *TUB1*-665	This study
347E6	Transformant of mutant TD4 expressing $TUB1^{N347D}$-665	This study
347NE1	Transformant of mutant TD4 expressing $TUB1^{*N347}$-665	This study
362E1	Transformant of mutant TD4 expressing $TUB1^{T362A}$-665	This study
362NE3	Transformant of mutant TD4 expressing $TUB1^{*T362}$-665	This study
368E5	Transformant of mutant TD4 expressing $TUB1^{I368V}$-665	This study
368NE3	Transformant of mutant TD4 expressing $TUB1^{*I368}$-665	This study
R21	Ascospore progeny of T1P10 selfing	This study
R27	Ascospore progeny of T1P10 selfing	This study
F167-3	*tub1*/$TUB1^{F167Y}$-665 transformant	This study
E198-4	*tub1*/$TUB1^{E198K}$-665 transformant	This study
F200-4	*tub1*/$TUB1^{F200Y}$-665 transformant	This study
Syn11	Transformant of mutant TD4 expressing $TUB1^{Syn}$-665	This study
Syn13	Transformant of mutant TD4 expressing $TUB1^{Syn}$-665	This study

Table 2. Growth rate and perithecium size of wild type, *tub1* and restored strain.

Strains	Growth rate[a] (cm day^{-1})	Diameter of perithecia[b] (mm)
PH-1 (WT)	$1.01 \pm 0.05^{A*}$	2.03 ± 0.23^{A}
TD4 (*tub1*)	0.81 ± 0.02^{B}	0.93 ± 0.30^{C}
Syn11 (*tub1*/$TUB1^{Syn}$-665)	1.08 ± 0.08^{A}	1.34 ± 0.30^{B}
T665-2 (*tub1*/*TUB1*-665)	1.01 ± 0.02^{A}	2.16 ± 0.14^{A}

*Mean and standard deviation were calculated from four independent replicates. Data were analyzed with the protected Fisher's least significant difference (LSD) test. Different letters marked statistically significant differences ($p = 0.05$).
[a]Daily extension in colony radius on CM plates.
[b]The diameter of perithecia were measured 7-dpf.

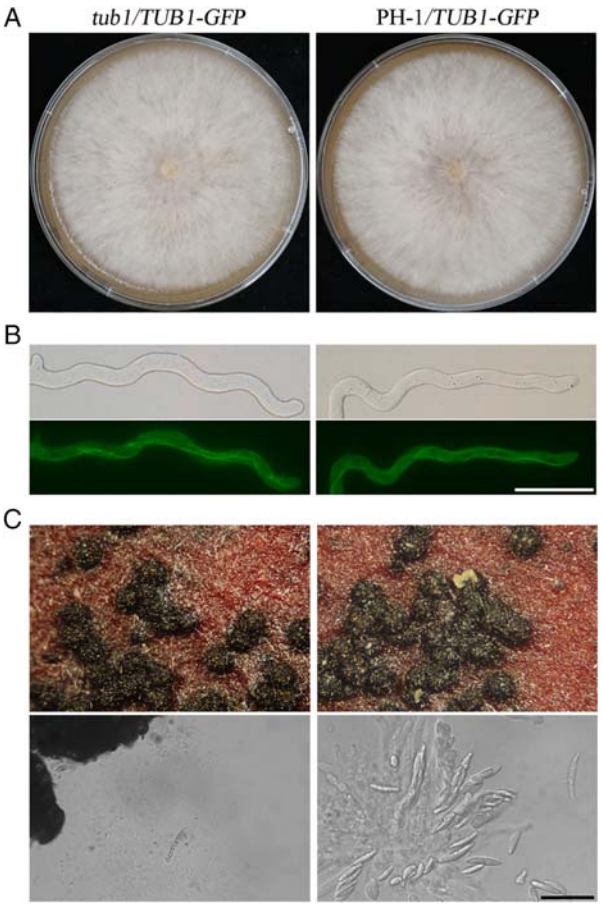

Fig. 2. Expression of *TUB1*-GFP failed to rescue the defects of *tub1* in ascosporogenesis. **A.** Three-day-old V8 cultures of the *tub1*/*TUB1*-GFP (TG3) and PH-1/*TUB1*-GFP (T1P10) transformants. **B.** GFP signals in the microtubules in the hyphae of *TUB1* transformants. Bar = 20 μm. **C.** Perithecia produced by the *tub1*/*TUB1*-GFP and PH-1/*TUB1*-GFP transformants were examined for ascospore cirrhi (upper panels) and asci (lower panels). Bar = 40 μm. [Color figure can be viewed at wileyonlinelibrary.com]

formation. Therefore, although it was functional during vegetative growth, *TUB1*-GFP failed to complement the defect of *tub1* in sexual reproduction.

The 3′-UTR sequence of TUB1 *is important for its function during sexual reproduction*

Because the *TUB1*-GFP fusion lacks the 3′-end sequences, we generated a *TUB1* complementation construct (named *TUB1*-665) with 1.58-kb upstream promoter and 665-bp downstream 3′-end (3′-UTR + terminator) sequences (Fig. 3A) and transformed it into the *tub1* deletion mutant TD4. The resulting *tub1*/*TUB1*-665 transformants were normal in growth rate (Fig. 3B) and ascospore formation in selfing (Fig. 3C; Table 2), suggesting that the 3′-end sequence of *TUB1* is important for its function during sexual reproduction. Interestingly, when the *TUB1*-665 transformant was crossed as the male with

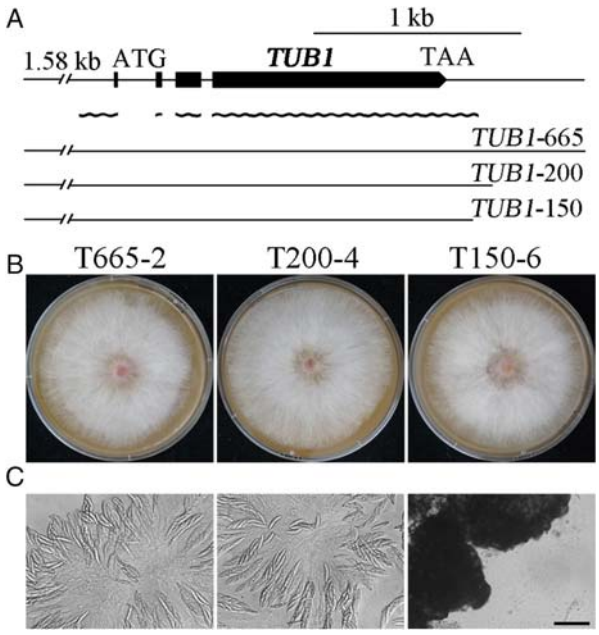

Fig. 3. Complementation of *tub1* mutant with *TUB1* constructs containing different 3′-end sequences. **A.** Diagrams of the *TUB1* gene and different complementation constructs. **B.** Colonies of wild type PH-1, *tub1* mutant (TD4), and transformants of the *tub1* mutant expressing the *TUB1*-665 (T665-2), *TUB1*-150 (T150-6) and *TUB1*-200 (T200-4) constructs. **C.** Asci from the mating plates of marked transformants. Expression of *TUB1*-665 and *TUB1*-200 but not *TUB1*-150 complemented the defects of *tub1* in ascosporogenesis. Bar = 40 μm. [Color figure can be viewed at wileyonlinelibrary.com]

the *mat1-1* mutant, although normal perithecia were formed, some perithecia were defective in ascosporogenesis and produced ascospores with abnormal morphology, which is similar to the *tub1* (male) × *mat1-1* (female) cross. Because *TUB1*-665 was integrated ectopically, these results further indicated that MSUD of unpaired *TUB1* sequences may cause defects in ascosporogenesis.

Based on ESTs in GenBank (XM_011329885) (Cuomo *et al*., 2007) and RNA-seq data, the length of 3′-UTR in *TUB1* transcripts is estimated to be 170-bp (Fig. 3A) and the predicted polyadenylation signal (AATAAA) is at 144–149 bp downstream from the stop codon. To determine the minimal 3′-end sequence required for *TUB1* function in ascosporogenesis, we then generated the *TUB1*-150 and *TUB1*-200 constructs that had the same 1.58-kb promoter but 150- and 200-bp 3′-end sequences, respectively (Fig. 3A), and transformed them into the *tub1* mutant TD4. At least three transformants were generated for each construct and confirmed by PCR analysis. All the *TUB1*-150 and *TUB1*-200 transformants were normal in vegetative growth (Fig. 3B). Whereas the *tub1*/*TUB1*-150 transformants were defective in sexual reproduction, the *tub1*/*TUB1*-200 transformants were normal in ascus development and ascospore formation (Fig. 3C). Because the 3′-UTR of *TUB1* is 170-bp in length, complementation of *tub1* mutant in ascosporogenesis by *TUB1*-200 but not *TUB1*-150 indicated the importance of its 3′UTR during sexual reproduction.

RIP mutations specifically affect TUB1 *functions during sexual reproduction*

As the control for the *tub1*/*TUB1*-GFP transformant, we also set up selfing plates of the transformant of PH-1 expressing the same *TUB1*-GFP construct ectopically in the genome that was generated in a previous study (Luo *et al*., 2014). Although perithecia with ascospore cirrhi were observed, significant variations in ascosporogenesis were observed in the PH-1/*TUB1*-GFP transformant. Besides normal asci with eight 4-celled ascospores, asci with defects in ascus development or ascospore morphology were often observed. In repeated mating tests, approximately 10% of the ascospores had abnormal morphology (Fig. 4A). Because RIP is known to occur efficiently to duplicated sequences in *F. graminearum* (Cuomo *et al*., 2007), defects of PH-1/*TUB1*-GFP transformants in ascosporogenesis may be caused by RIP mutations of both the endogenous and ectopically integrated *TUB1* alleles.

To confirm this observation, ascospore progeny were isolated from 32 ascospores with morphological defects. Two progeny, R21 and R27 (Table 1), were normal in vegetative growth and colony morphology (Fig. 4B) but had severe defects in sexual reproduction (Fig. 4C). Although normal perithecia were formed, ascus and ascospore development were significantly reduced in progeny R21 and rarely observed in R27 (Fig. 4C). These results indicated that RIP mutations in progeny R21 and R27 specifically affected the function of Tub1 proteins during sexual reproduction.

To identify RIP mutations, the endogenous *TUB1* fragment was amplified with primers Tub1-S1/F and Tub1-155/R (Supporting Information Table S1). Primer Tub1-155/R was in the 3′-end sequence of *TUB1* not present in the ectopically integrated *TUB1*-GFP construct, which was not functional during sexual reproduction due to the lack of 3′-UTR. Multiple RIP mutations were identified in progeny R21 and R27 (Table 3). Modelling after the published 3D structure of β1-tubulins (Zhao *et al*., 2014) showed that the T23, G34, V119, V180 and D197 residues are present on the surface (Fig. 4D). These residues may be involved in interacting with alpha-tubulins or other proteins.

Synonymous mutations in the C-terminal end region of TUB1 *affected sexual reproduction*

The importance of the 3′-UTR may be related to its role in the secondary structure of *TUB1* transcripts. To test

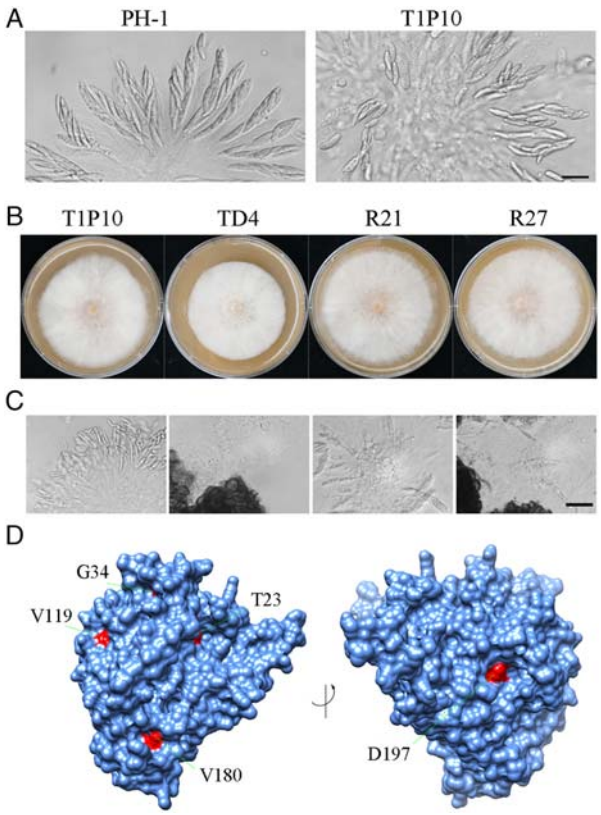

Fig. 4. Repeat-induced point mutations of *TUB1*. **A.** Asci and ascospores of PH-1 and transformant of PH-1 expressing the *TUB1*-GFP construct (T1P10). Ascospores with morphological defects were observed in transformant T1P10. Bar = 20 μm. **B**. Three-day-old V8 agar cultures of transformant T1P10 (*TUB1*/*TUB1*-GFP), *tub1* mutant TD4, and RIP progeny R21 and R27. **C.** Asci and ascospores of transformant T1P10 and RIP progeny R21 and R27. Progeny R27 had the most severe defects in ascosporogenesis and it rarely formed ascospores. Bar = 40 μm. **D.** The locations of RIP mutation sites T23, G34, V119, V180 and D197 on the predicted 3D structure of Tub1. [Color figure can be viewed at wileyonlinelibrary.com]

this hypothesis, we predicted the secondary structure of the *TUB1* transcripts (100-bp upstream and 200-bp downstream from the stop codon; *TUB1*$^{1526\text{-}1829}$) with the Vienna RNA Websuite (Gruber *et al*., 2008). Pairing between the 3′-end region of *TUB1* ORF and its 3′-UTR was observed (Fig. 5A). To determine the functions of base pairing in the predicted stem C1 (Fig. 5A), we introduced the C^{1609}TG^{1611}CCT^{1614}GAG^{1617}GGC^{1620}GAG^{1623} to T^{1609}TA^{1611}CCG^{1614}GAA^{1617}GGT^{1620}GAA^{1623} synonymous mutations corresponding to amino acid residues L441 to E445 (at 4-bp upstream from the stop codon) to the *TUB1*-665 construct to generate the *TUB1*^{Syn} allele. When the *TUB1*^{Syn} allele was transformed into the *tub1* mutant, the resulting transformants (Table 1) had normal growth but were still defective in sexual reproduction. Perithecia formed by *tub1*/*TUB1*^{Syn} transformants had the average diameter of 1.34 ± 0.30 mm, which was significantly larger than that of *tub1* mutant perithecia (Table 2). However, similar to the *tub1* mutant, the *tub1*/*TUB1*^{Syn} transformants was blocked in ascus development or

Table 3. Mutations identified in the *TUB1* gene in two RIP progeny.

Progeny	Nucleotide change	Amino acid change	Phenotype
R21	C^{184}T*, G^{340}A, C^{348}T, C^{372}T, C^{396}T, C^{693}T, C^{942}T	G34S, Y36Y, L44L, N52N, I135I, S218S	Reduced in ascus formation, rare ascospores.
R27	C^{309}T, G^{643}A, G^{729}A, G^{826}A, G^{877}A, G^{890}A	T23I, V119I, M147I, V180I, D197N, C201Y, A428A	Rare asci, no ascospores.

*The C184T mutation in the first intron has no effect on the Tub1 protein sequence.

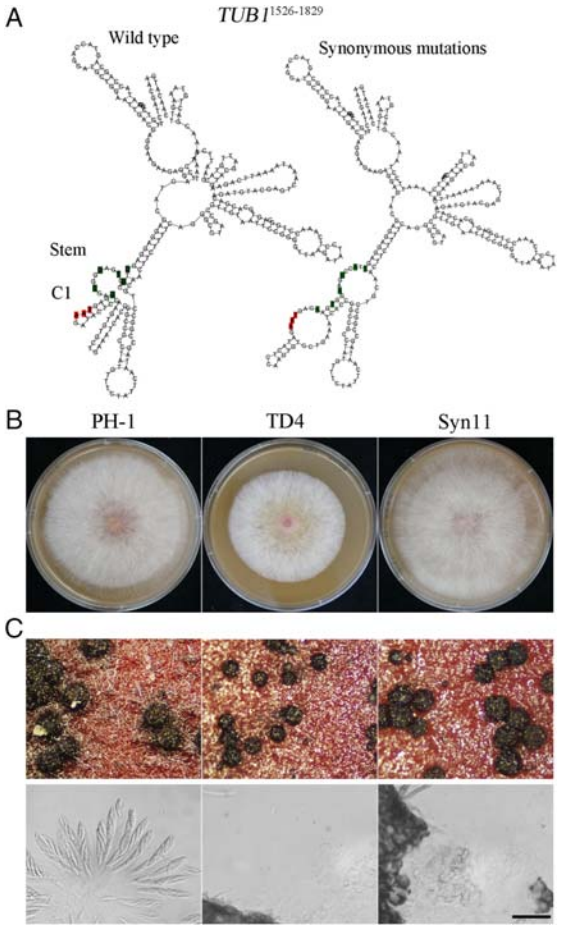

Fig. 5. Effects of synonymous mutations at the end of *TUB1* ORF on its structure and functions. **A.** Modelling of the secondary structure of *TUB1* transcripts (1526–1829 bp) with or without the synonymous mutations (C1609T, G1611A, T1614G, G1617A, C1620T and G1623A). Three bases of the stop codon are shaded in red. Six bases shaded in green are the mutation sites. **B.** Three-day-old V8 cultures of PH-1, *tub1* mutant (TD4) and *tub1*/*TUB1*^{Syn} transformant (Syn11). **C**. Perithecia of the same set of strains were examined for ascospore cirrhi (upper row) and ascospore (bottom row) formation. Bar = 40 μm. [Color figure can be viewed at wileyonlinelibrary.com]

ascospore formation (Fig. 5C), indicating that these synonymous mutations changed the secondary structure of *TUB1* transcripts may affect the expression of Tub1 proteins and functions during sexual reproduction.

The N347D mutation affects Tub1 function in ascosporogenesis

TUB1 transcripts had three A-to-I RNA editing sites during sexual reproduction (Liu *et al*., 2016). These three editing events resulting in the N347D, T362A and I368V had the editing levels of 41.1%, 62.1% and 36.7%, respectively, in perithecia harvested at 8 dpf. All these three edited sites are in the C-terminal region of Tub1 proteins. Modelling after the conserved domains of α-tubulin (cd02186) and β-tubulin (cd02187) in CDD of NCBI (Zhao *et al*., 2014) showed that N347 of Tub1 is at the interface with α-tubulin (Fig. 6A). To determine whether these editing events are related to the function of Tub1 during sexual reproduction, we first introduced the CCT^{1326}AAC to CCG^{1326}AAC mutation into the complementation construct *TUB1*-665 to generate the non-editable *TUB1*^{*N347} allele. We also generated the edited *TUB1*^{N347D} allele by introducing the A^{1327}AC (Asn) to G^{1327}AC (Asp) mutation to *TUB1*-665. Both alleles were transformed into the *tub1* mutant TD4 (Table 1). To our surprise, expressing the non-editable *TUB1*^{*N347} allele, but not the edited *TUB1*^{N347D} allele, fully complement the defect of *tub1* in sexual reproduction. Whereas the *tub1*/*TUB1*^{*N347} transformants were normal, the *tub1*/*TUB1*^{N347D} transformants were defective in ascosporogenesis (Fig. 6B). Because the editing level at N347 was only 41.1%, the failure of *TUB1*^{N347D} to fully complement *tub1* mutant indicated that the native Tub1 protein without editing at N347 is important for normal ascus formation.

The same strategy was used to generate the *TUB1*^{T362A} and *TUB1*^{I368V} edited alleles by introducing the A^{1372}CT (Thr) to G^{1372}CT (Ala) and A^{1390}TT (Ile) to G^{1390}TT (Val) mutations, respectively, into the *TUB1*-665 construct. The non-editable *TUB1* alleles of *TUB1*^{*T362} and *TUB1*^{*I368} was generated by introducing the CTT^{1371}ACT to CTG^{1371}ACT and TTT^{1389}ATT to TTC^{1389}ATT mutations to *TUB1*-665. These constructs were transformed into the *tub1* mutant. All the resulting transformants expressing these mutant alleles of *TUB1* were normal in growth rate and sexual reproduction (Fig. 6B), indicating that the T362A and I368V mutations or editing had no significant effects on Tub1 functions in *F. graminearum.*

The F167Y mutation in TUB1 *confers carbendazim resistance but causes defects in ascosporogenesis*

Mutations in beta-tubulins are known to confer resistance to carbendazim fungicides (Ma and Michailides, 2005). In *F. graminearum*, the F167Y, E198K and F200Y

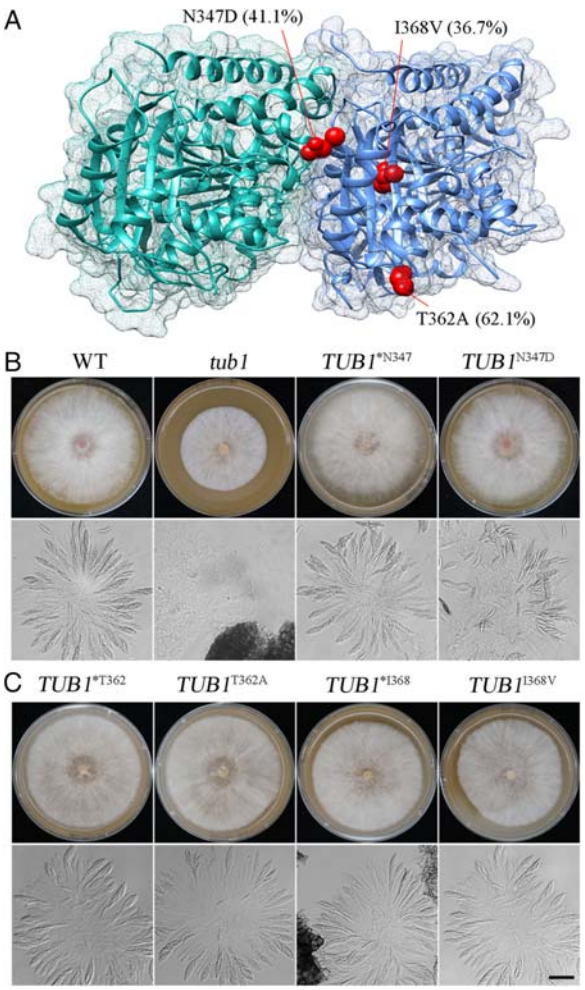

Fig. 6. Site-directed mutagenesis of the three RNA editing sites in *TUB1.* **A.** The locations of three A-to-I RNA editing sites (editing levels in the bracket) in Tub1 based on the model of the α- and β-tubulin dimer. **B.** Colonies and asci of transformants of wild type PH-1, *tub1* mutant and transformants of *tub1* expressing the marked mutant *TUB1* alleles. Bar = 40 μm. [Color figure can be viewed at wileyonlinelibrary.com]

mutations in *TUB2* have been identified in resistant field isolates and confirmed to be responsible for carbendazim resistance by site-directed mutations (Liu *et al*., 2010a, b; Qiu *et al*., 2012). Although Tub1 and Tub2 are highly similar in amino acid sequences, mutations in *TUB2* alone are sufficient to confer fungicide resistance and mutations in *TUB1* had not been reported.

Whereas mutations in both Tub1 and Tub2 may be more resistant against carbendazim fungicides, one possibility is that mutations in *TUB1* may have detrimental effect on sexual reproduction. To test this hypothesis, we introduced the mutations in *TUB2* that are known to confer carbendazim resistance into *TUB1*-665, including the F167Y (TTC to TAT), E198K (GAA to AAA) and F200Y (TTT to TAC) mutations. Expression of the *TUB1*^{E198K} or *TUB1*^{F200Y} allele fully complemented the *tub1* mutant in

growth (Fig. 7A) and sexual reproduction, indicating that these mutations had no effect on Tub1 function. However, although they were normal in growth, some of the asci formed by the *tub1*/*TUB1*^{F167Y} transformants were defective in ascus development or produced ascospores with morphological defects (Fig. 7B). Nevertheless, no obvious defects in ascospores discharge and ascospore germination were observed in the *tub1*/*TUB1*^{F167Y} transformants (Supporting Information Fig. S2).

Similar to the previous report (Qiu *et al*., 2012), the *tub1* mutant was slightly increased in carbendazim resistance on V8 plates with 1.0 μg ml^{-1} carbendazim in comparison with the wild-type strain PH-1 (Fig. 7A). The *tub1*/*TUB1*^{E198K} and *tub1*/*TUB1*^{F200Y} transformants were similar to the *tub1* mutant in sensitivities to carbendazim. When incubated on V8 with 1.4 μg ml^{-1} carbendazim, significant hyphal growth was only observed in the *tub1*/*TUB1*^{F167Y} transformants (Fig. 7A). Even in the presence of 5 μg ml^{-1} carbendazim, the *tub1*/*TUB1*^{F167Y} transformants had limited hyphal growth (Fig. 7C). These results indicated that the F167Y mutation in *TUB1*, but not E198K or F200Y, conferred resistance against carbendazim. However, the F167Y mutation had a minor cost effect on the function of Tub1 proteins during ascosporogenesis.

Discussion

Like in other eukaryotic organisms, microtubules formed by the polymerization of the α- and β-tubulins play important roles in cellular trafficking, nuclear division and hyphal growth in fungi. Although majority of ascomycetes, including the budding and fission yeast and model filamentous fungus *Neurospora crassa*, have only one β-tubulin gene, *F. graminearum* has two β-tubulin genes that differ in functions (Orbach *et al*., 1986; Yanagida, 1987; Reijo *et al*., 1994; Zhao *et al*., 2014). Earlier studies showed *TUB2* is dispensable for sexual reproduction but the *tub1* mutant is blocked in ascosporogenesis although Tub2 is more important for hyphal growth (Zhao *et al*., 2014). In this study, we showed that there was only limited hyphal growth inside the cavity of *tub1* perithecia and the *tub1* deletion mutant displayed a female-specific defect in outcrossing. Unlike many genes that is essential for female fertility, such as *MGV1*, and *CID1* (Hou *et al*., 2002; Zhou *et al*., 2010), *TUB1* is not essential for protoperithecium development. In fact, although smaller than those of the wild type, melanized perithecia formed by the *tub1* mutant were visible to naked eye and had ostiole development. These results indicate that *TUB1* is dispensable for initial stages of perithecium formation although it is essential for ascosporogenesis associated with late stages of sexual development.

Majority of ascospores from the *tub1* × *mat1-1* (*TUB1*) outcross had normal morphology and 1:1 segregation for GFP signals in the nucleus. However, approximately 20% of ascospores had abnormal morphology and some of the asci from this outcross had developmental or morphological defects. One explanation is that severe MSUD of *TUB1* occurred in some of the developing asci and

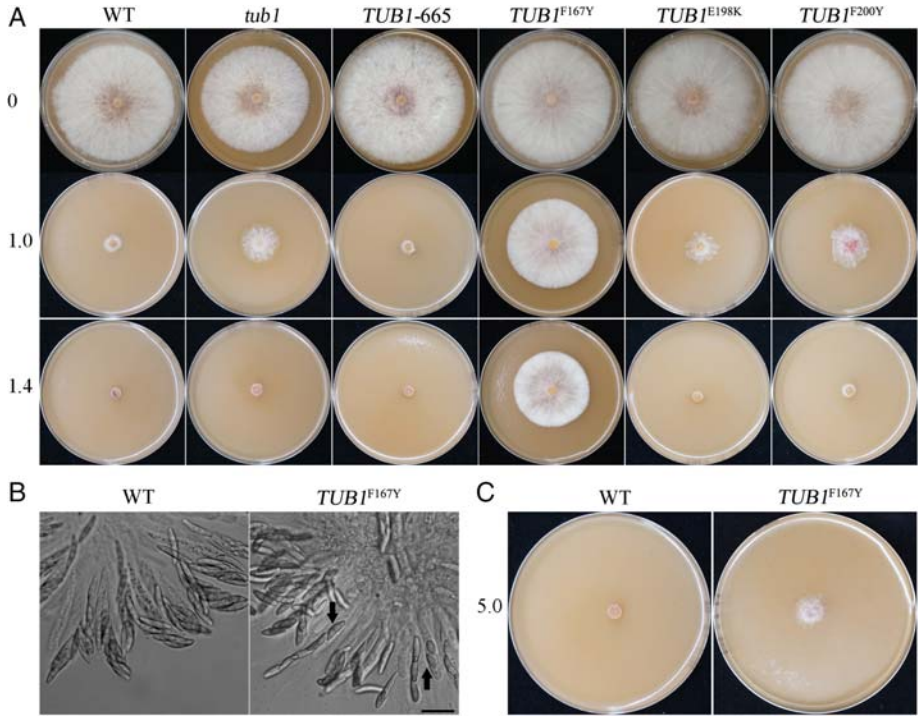

Fig. 7. Effects of the F167Y, E198K and F200Y mutations in *TUB1* on fungicide resistance and ascosporogenesis. **A**. Cultures of PH-1, *tub1* mutant and transformants of *tub1* expressing mutant *TUB1* alleles carrying the marked mutations grown on V8 agar plates with 0, 1.0 or 1.4 μg ml^{-1} carbendazim and photographed after incubation for 3 days. The F167Y mutation increased fungicide resistance. **B.** Perithecia formed by the *tub1*/*TUB1*^{F167Y} transformant were assayed for defects in ascosporogenesis. Asci with defective ascospores were marked with black arrows. Bar = 40 μm. **C.** Three-day-old V8 cultures of PH-1 and the *tub1*/*TUB1*^{F167Y} transformant in the presence of 5.0 μg ml^{-1} carbendazim. [Color figure can be viewed at wileyonlinelibrary.com]

resulted defects in ascus development and ascospore formation. This explanation was supported by observations with the *tub1*/*TUB1*-665 transformant. Although it was normal in sexual reproduction in selfing, abnormal ascospores and asci also were observed when the *tub1*/*TUB1*-665 transformant was crossed as the male with the *mat1-1* mutant. The formation of abnormal asci and ascospores in these outcrosses further indicated that *TUB1* plays important roles in both ascus and ascospore development. The degree of *TUB1* silencing by MSUD may vary among different asci, resulting in variations in defects associated with ascus development and ascospore formation in *F. graminearum.*

TUB1 was constitutively expressed and its up-regulation was not observed during sexual reproduction. Based on RNA-seq data, no differences in alternative splicing or transcription variations in *TUB1* transcripts were observed between hyphae and perithecia. However, we found that whereas *TUB2* transcripts had no A-to-I editing, *TUB1* had three non-synonymous editing sites and the editing levels at these three sites were significantly higher than the average editing level (14.6%) in *F. graminearum* (Liu *et al*., 2016). The highest editing level was 62.1% at T362A and one of the editing sites (N347) is at the α-tubulin/β-tubulin interface (Zhao *et al*., 2014). Therefore, it is tempting to speculate that RNA editing is responsible for the stage-specific functions of Tub1 during sexual reproduction. However, to our surprise, non-editable mutations eliminating these editing events individually had no obvious effects on Tub1 functions in ascosporogenesis. Because editing was not complete at any of these sites, the defects of *tub1*/*TUB1*^{N347D} transformants in ascosporogenesis showed the importance of the unedited Tub1 protein during sexual reproduction. Nevertheless, it remains possible that mutations completely eliminating RNA editing at all these three editing sites may affect the sexual specific function of Tub1 in *F. graminearum.*

In this study, we isolated two RIP progeny from the selfing of PH-1/*TUB1*-GFP transformant that were normal in vegetative growth but defective in sexual reproduction. Both of them carried multiple mutations that were typical of RIP mutations. The isolation of these RIP progeny and identification of RIP mutations further supported that *TUB1* has a stage-specific function that cannot be replaced by *TUB2* in *F. graminearum.* The Tub1 and Tub2 beta-tubulin proteins are highly similar in the amino acid sequences. The functional difference between Tub1 and Tub2 during sexual reproduction may be related to stage-specific microtubule-associated proteins (MAPs) that interact with Tub1. These RIP mutations may affect the interaction of Tub1 with MAPs during sexual reproduction.

Resistance to fungicides targeting at β-tubulins has been observed in various plant pathogenic fungi (Ma and Michailides, 2005). However, unlike in controlling plant diseases caused by other fungi, benomyl and carbendazim fungicides are still used in controlling FHB in China although resistance was first reported in *TUB2* site mutation (Chen *et al*., 2009). Mutations at the conserved fungicide binding sites have been observed in *TUB2* but not *TUB1* in field isolates of *F. graminearum.* In this study, we found that the E198K or F200Y mutation in *TUB1* had no significant effects on fungicide resistance and sexual reproduction. Similar to earlier reports, the *tub1* mutant was slightly increased in carbendazim tolerance (Liu *et al*., 2010a, b; Qiu *et al*., 2012), the *tub1*/*TUB1*^{E198K} or *tub1*/*TUB1*^{F200Y} transformants were similar to the *tub1* mutant in fungicide resistance but similar to the wild type in growth and sexual reproduction. However, the *tub1*/*TUB1*^{F167Y} transformants were increased in carbendazim resistance. Because mutations in *TUB2* alone conferred tolerance but not complete resistance to benomyl fungicides, it is likely that Tub1 is also the target of benomyl fungicides in *F. graminearum.* Nevertheless, the F167Y mutation had a minor effect on ascosporogenesis under laboratory conditions, which may be more severe under field conditions. Interestingly, although their mutations in *TUB2* all conferred fungicide tolerance, the F167Y mutation, but not E198K or F200Y, in *TUB1* had similar effects. Considering the fact that these three residues all in the fungicide binding pocket, it is tempting to speculate that binding of benomyl to Tub1 differs slightly from its binding with Tub2 and mutations at these three residues have different effects in Tub2 and Tub1 beta-tubulins. An earlier study showed that the F200Y mutation in *TUB2* conferred higher degree of carbendazim tolerance than the F167Y mutations in the *tub1* mutant background although these two mutations had similar effects on fungicide resistance in the wild-type background (Qiu *et al*., 2012).

Although many genes could fully complement their corresponding mutants without their own 3′-UTR or terminator sequences in *F. graminearum*, including the *Sch9*, *Cdc2A*, *Cdc2B* and *Cap1* genes (Chen *et al*., 2014; Liu *et al*., 2015; Yin *et al*., 2018). In fact, it is a routine practice in complementation assays with GFP-fusion constructs generated with the yeast gap-repair system that lacks *F. graminearum* 3′-end sequences (Zhou *et al*., 2011; Li *et al*., 2015; Zhang *et al*., 2017). Interestingly, *TUB1*-GFP and *TUB1*-150 complemented the defect of *tub1* mutant in vegetative growth but not its defects in sexual reproduction. However, *TUB1*-200 and *TUB1*-665 could fully complement all the defects of *tub1* mutant. Because the 3′-UTR of *TUB1* transcripts is approximately 170-bp in length, these data indicate that the 3′-UTR and terminator sequences of *TUB1* may be not important for its expression and function during vegetative growth but essential for ascosporogenesis. Differences in the

complementation of *tub1*/*TUB1*-150 and *tub1*/*TUB1*-GFP transformants in vegetative growth and sexual reproduction indicated that *TUB1* may have stage-specific transcription termination or alternative polyadenylation sites (APA) during vegetative growth and sexual reproduction. Although it has not been reported in *F. graminearum,* similar phenomena have been reported in other organisms such as mouse, human, *Arabidopsis* and *Magnaporthe oryzae* (Hornyik *et al*., 2010; MacDonald and McMahon, 2010; Franceschetti *et al*., 2011). It will be important to determine the underlying mechanisms responsible for this stage-specific polyadenylation site recognition during sexual reproduction or differences in polyadenylation requirement between vegetative hyphae and ascogenous tissues.

Experimental procedures

Strains and culture conditions

The wild-type strain PH-1 (Cuomo *et al*., 2007) and transformants or mutants generated in this study were routinely cultured on V8 juice agar plates (20% V8 juice, 0.2% $CaCO_3$, 2% agar). Growth rate on CM agar plates and conidiation in liquid carboxymethylcellulose (CMC) medium (1.5% CMC, 0.1% NH_4NO_3, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract) were assayed. Protoplast preparation and PEG-mediated transformation were performed as described (Hou *et al*., 2002) with hygromycin B (Calbiochem, La Jolla, CA) and geneticin (Sigma, St. Louis, MO) added to the final concentrations of 300 and 350 μg ml^{-1}, respectively, to the TB3 medium (0.3% yeast extract, 0.3% casamino acids and 3% glucose) for transformant selection. Hyphae harvested from liquid YEPD (1% yeast extract, 2% peptone, 2% glucose) were used for DNA and RNA isolation as described (Liu *et al*., 2016). Cell wall and nuclei were stained with 20 μg ml^{-1} Calcofluor white and 20 μg ml^{-1} 4,6-diamidino-2-phenylindole (DAPI) as described (Luo *et al*., 2014).

Assays for defects in sexual reproduction

For selfing, the *tub1* mutant and complementation strains were inoculated on carrot agar plates and fertilized as described (Luo *et al*., 2014). Perithecium formation, cirrhi production and the formation of asci and ascospores were examined 7–14 days post-fertilization (dpf) (Zhang *et al*., 2017). For thick sections, 7-dpf perithecia were fixed in 4% (v v^{-1}) glutaraldehyde and dehydrated in a gradient concentration of acetone solution before being embedded in Spurr resin as described (Cao *et al*., 2017). For out-crossing, 5–7 day-old carrot agar cultures of the female strain were fertilized with 200 μl of conidium suspensions of the male strain (5×10^4 conidia ml^{-1}) as described previously (Zheng *et al*., 2013). Ascospores discharge was assayed as described (Cavinder *et al*., 2012).

Generation of the tub1 *deletion mutants*

The *tub1* mutant deleted of the ORF only was generated in a previous study (Zhao *et al*., 2014). To delete the entire *TUB1* gene, including its promoter and 3′-UTR sequences, by the split-marker approach (Zhou *et al*., 2010), the 0.88 kb upstream and 0.86 kb downstream flanking sequences were amplified with primer pairs 1F/2R and 3F/4R respectively (Supporting Information Table S1). Primer 2R is at 1677 bp upstream from start codon and 3F is at 239 bp downstream from the stop codon. The resulting PCR products were connected to the hygromycin-phosphotransferase (*hph*) cassette amplified from vector pDL2 (Zhou *et al*., 2011) by overlapping PCR and transformed into protoplasts of the wild-type strain PH-1. Hygromycin-resistant transformants were isolated and screened by PCR.

Generation of TUB1-*665,* TUB1-*150 and* TUB1-*200 constructs and transformants*

To generate the complement constructs, PCR products were cloned into pFL2 by the yeast gap repair approach (Bruno *et al*., 2004; Zhou *et al*., 2012a,b). Primer pairs Tub1/NF and Tub1/665R, Tub1/NF and Tub1/150R and Tub1/NF and Tub1/200R used for amplifying *TUB1*gene contains 665, 150 and 200 bp of 3′-flanking sequence. All three construct contain 1.58 kb promoter sequence. Then construct were confirmed by sequencing analysis and transformed into protoplasts of the *tub1* mutant TD4. Geneticin-resistant transformants harbouring the transforming construct were identified by PCR.

Functional characterization of the three RNA editing sites

All the mutations at the three editing sites were introduced into the *TUB1*-665 allele by overlapping PCR and cloned into vector pFL2 by the yeast gap approach (Bruno *et al*., 2004; Zhou *et al*., 2012a,b). The *TUB1*^{N347D}, *TUB1*^{T362A} and *TUB1*^{I368V} edited alleles were generated by introducing the A^{1327}AC to GAC, A^{1372}CT to GCT and A^{1390}TT to GTT mutations, respectively, with primers listed in Supporting Information Table S1. The CCT^{1326}AAC to CCGAAC, CTT^{1371}ACT to CTGACT and TTT^{1389}ATT to TTCATT mutations were introduced into *TUB1*-665 to generate the *TUB1*^{*N347}, *TUB1*^{*T362} and *TUB1*^{*I368} non-editable alleles respectively. The plasmid constructs carrying the expected

mutations in *TUB1* were rescued from Trp + transformants and confirmed by sequencing analysis as described (Zhou *et al*., 2011). After transformation of the *tub1* mutant TD4, transformants resistant to geneticin and hygromycin were isolated and analyzed by PCR for the integration of transforming *TUB1* constructs.

Isolation of RIP progeny and identification of RIP mutations

Ascospore cirrhi were collected from perithecia formed by the selfing plates of PH-1/*TUB1*-GFP transformant T1P10 and resuspended in 1 ml of sterile distilled water in a 1.5 ml centrifuge tube. After streaking ascospore suspensions on 3% (w v^{-1}) water agar, ascospores with morphological defects were isolated by dragging individual ascospores under an inverse microscopy. Single ascospore cultures were then assayed for defects in growth and sexual reproduction as described (Luo *et al*., 2014) (Zhang *et al*., 2017). To identify RIP mutations in ascospore progeny normal in growth but defective in ascosporogenesis, the endogenous *TUB1* gene was amplified with primers Tub1-S1 and Tub1-155/R (Supporting Information Table S1) and sequenced. Primer Tub1-155/R is in the 3′-end sequence (−6 205 203 to −6 205 224 on supercontig 4), which was not present on the ectopically integrated *TUB1*-GFP construct.

Generation of TUB1 *mutant alleles with the F167Y, E198K and F200Y mutations*

The F167Y ($\underline{T}^{787}$TC to $\underline{T}$AT), E198K ($\underline{G}^{880}$AA to $\underline{A}$AA) and F200Y ($\underline{T}^{886}$TT to $\underline{T}$AC) mutations known to confer benomyl resistance in *TUB2* (Chen *et al*., 2009; Qiu *et al*., 2012) were introduced into the *TUB1*-665 allele by overlapping PCR and cloned into vector pFL2 by the yeast gap approach (Bruno *et al*., 2004; Zhou *et al*., 2012a, b) with primers listed in Supporting Information Table S1. The *TUB1*^{F167Y}, *TUB1*^{E198K} and *TUB1*^{F200Y} constructs rescued from yeast transformants were confirmed by sequencing analysis and transformed into the *tub1* deletion mutant TD4. Transformants resistant to both geneticin and hygromycin were isolated and confirmed by PCR analysis for the transforming *TUB1* mutant alleles. Growth on V8 agar with 1.0, 1.4 and 5.0 μg ml^{-1} of Carbendazim (Sigma, St. Louis, MO) was assayed as described (Liu *et al*., 2010a, b).

Bioinformatics analysis

DNA and protein sequence used in this study was downloading from Ensemble Fungi or NCBI. EST data of Tub1 was downloading from GenBank (XM_011329885). mRNA folding structure prediction of *TUB1* was analysis by ViennaRNA website (Gruber *et al*., 2008). Protein modelling and 3D visualization: the 3D-structural models of α- and β-tubulins were presented according to that of α β-tubulins of *Saccharomyces cerevisiae* (PDB ID: 4FFB) (Ayaz *et al*., 2012) and displayed with Chimera 1.8.1 (Yang *et al*., 2012). The 3D structures of Tub1 was predicted with HHpred (Soding *et al*., 2005) using the HHblits multiple sequence alignment method.

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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. The *tub1* deletion constructs and mutants.

A. Schematic diagrams of the gene replacement events in *tub1* mutants TD28 and TD4. **B**. Three-day-old V8 cultures of *tub1* mutants TD28 and TD4.

Fig. S2. Assays for ascospores discharge and ascospore germination in the *tub1*/*TUB1*^{F167Y} transformant.

A. Perithecia of the wild-type strain PH-1 (WT) and *tub1*/*TUB1*^{F167Y} transformant were assayed for ascospore discharge. **B.** Ascospores of PH-1 and of *tub1*/*TUB1*^{F167Y} transformant were photographed after incubating in 5xYEG for 6 h. The *tub1*/*TUB1*^{F167Y} transformant had no obvious defects in ascospore discharge and germination. Bar = 20 μm.

Table S1. Primers used in this study.