INTRODUCTION

Fusarium graminearum is the causal agent of fusarium head blight (FHB), which is a destructive disease of wheat and other small grain crops worldwide (Goswami & Kistler, 2004). In addition to decreasing crop yield and quality, the pathogen contaminates crops with trichothecenes, a group of mycotoxins with serious threats to human and animal health (Alexander et al., 2009; Goswami & Kistler, 2004). Mycotoxins such as deoxynivalenol (DON), nivalenol (NIV), and their acetylated derivatives (3-ADON, 4-ANIV, and 15-ADON) all belong to type B trichothecenes, of which 3-acetyldexynivalenol (3-ADON) is the most frequently detected type in Asia (Alexander et al., 2011; Audenaert et al., 2013; Bennett & Klich, 2003). Notably, trichothecenes have been shown to be a vital virulence factor of Fusarium species (Alexander et al., 2009; Goswami & Kistler, 2004). Our previous study also showed that the inhibition of DON biosynthesis can...
lead to effective control of FHB (Li et al., 2019). In-depth research into the biosynthesis and regulation of trichothece can provide crucial understanding for the development of novel control strategies, not only for Fusarium-incited disease control but also for grain storage management.

The concept of metabolic channelling was first proposed by Srere more than 30 years ago (Srere, 1987). All cytoplasm contains various large molecules, such as proteins, nucleic acids, polysaccharides, even some toxic intermediaries or inhibitors. High concentrations of these molecules could influence many aspects of cellular function and metabolism (Ellis & Minton, 2003). Enzymes involved in the same biochemical pathway conventionally assemble to catalyse several specific consecutive reactions and these enzyme complexes are called metabolons (Clegg, 1984). Normally, the formation of metabolons is in response to a condition requiring adaption in metabolic flux, which results in enhanced conversion (Kohnhorst et al., 2017; Thomas et al., 2017). While these theories are now widely accepted, various underlying molecular mechanisms are still fascinating research topics. To date, a variety of examples in mammalian and human metabolons have been characterized in glycolysis, the tricarboxylic acid cycle, and many other metabolic pathways (An et al., 2010; Haanstra et al., 2016; Haggie & Verkman, 2002; Kohnhorst et al., 2017). In fungal species, there are few examples of characterized metabolons. In Fusarium species, enzymes involved in the de novo trichothecene biosynthetic pathway (from acetyl-CoA to farnesyl pyrophosphate then to DON) have been comprehensively investigated (Figure 1) (Alexander et al., 2011; Gardiner et al., 2009). Previous studies have demonstrated that genes involved in trichothecene biosynthesis reside at different chromosomal loci. Nearly all Tri genes are derived from the core 25-kb region of the Tri5-cluster that includes Tri4, Tri5, Tri6, and Tri10 (Alexander et al., 2011; Kimura et al., 2003). Tri1 and Tri101 genes reportedly have been found at separate loci (Alexander et al., 2011; Brown et al., 2003; Peplow et al., 2003).

Notably, recent studies of the enzymes involved in the DON metabolic pathway in vivo revealed that enzymes Tri1, Tri4, and Hmr1 colocalize in the remodelled endoplasmic reticulum (ER). Based on these studies, a novel and unique subcellular organization containing DON biosynthetic enzymes, the “toxisome”, was proposed in Fusarium species (Boenisch et al., 2017; Menke et al., 2013). The myosin I-actin cytoskeleton, which has been identified as a toxisome component, was also determined to play an indispensable role in toxisome formation in Fusarium species (Tang et al., 2018).

Accumulated evidence suggests that the microtubule (MT) cytoskeleton plays a vital role in the sequential organization of metabolic pathways involved in DON biosynthesis. The MT cytoskeleton is known to interact with the ER and TOXISOME, facilitating the localization of DON biosynthetic enzymes (Figure 1). The interaction of the MT cytoskeleton with the ER has been implicated in the regulation of DON biosynthesis, which is essential for fungal stress adaptation and virulence. The MT cytoskeleton may also play a role in the organization of the TOXISOME, which is a multiprotein complex that facilitates the rapid and efficient production of DON.

**FIGURE 1** Deoxynivalenol (DON) biosynthetic pathway. The de novo DON biosynthetic pathway transforms acetyl-CoA to DON in multiple steps. ACAT, acetyl-CoA acetyltransferase (ACAT1, FGSG_05087; ACAT2, FGSG_09321); Hmcs, hydroxymethylglutaryl-CoA synthase (FGSG_09266); Hmcr, 3-hydroxy-3-methylglutaryl-CoA reductase (FGSG_09197); MVK, mevalonate kinase (FGSG_05912); PMK, phosphomevalonate kinase (FGSG_09764); MDV, diphosphomevalonate decarboxylase (FGSG_10424); FDPS, farnesyl pyrophosphate synthetase (FGSG_06784); Tri5, trichodiene synthetase (sesquiterpene cyclase, FGSG_03537); Tri4, trichodiene oxygenase (cytochrome P450, FGSG_03535); Tri101, trichothecene 3-O-acetyltransferase (FGSG_07896); Tri11, isotrichodermin C-15 hydroxylase (FGSG_03540); Tri3, trichothecene 15-O-acetyltransferase (FGSG_03534); Tri1, calonectrin oxygenase (FGSG_00071); Tri8, trichothecene 3-O-esterase (FGSG_03532). Hmcr, Tri4, and Tri1 are localized in the toxisome.
enzymes (An et al., 2010; Volker et al., 1995). A previous study showed that the purine biosynthetic metabolons, the purinosomes, were embedded with the MT networks in HeLa cells and that the MT cytoskeleton guided the spatial distribution of purinosomes through the embedded locate mode (An et al., 2010). In our current study, we questioned whether F. graminearum toxisome is associated with the MT cytoskeleton and how this association regulates mycotoxin biosynthesis. To test this hypothesis, we fused calonectrin oxygenase (Tr1) with green fluorescent protein (GFP) as a toxisome marker and investigated the structural and functional relationships between the toxisome and the MT cytoskeleton.

Saccharomyces cerevisiae and most other phytopathogens have three types of tubulins (α, β, and γ) (Duan et al., 2015; Liu et al., 2013; Neff et al., 1983; Schatz et al., 1986), whereas F. graminearum contains four types of tubulins: α1, α2, β1, and β2 (Hu et al., 2015; Zhou et al., 2016). To visualize and manipulate the MT cytoskeleton in the presence of toxisomes, we used F. graminearum tubulin β1 chain (β1 tubulin) fused with red fluorescent protein (RFP) as an MT marker and the hyphae were treated with a high concentration of carbendazim, which targets the MT and cytoskeleton and interferes with its formation during mitosis. After treatment for 24 hr with 1.2 μg/ml carbendazim (approximately EC95 against mycelial growth), Tr1-RFP only displayed weak signals when compared with the control samples (Figure 3b, left panel; Figure S4). The expression of Tr1-GFP was verified by the western blot assay, and the intensity of the GFP band was dramatically reduced compared with the control samples (Figure 3b, left panel; Figure S3). Among these transformants, FgPMK-S15 showed the lowest FgPMK expression (15% of the wild-type strain PH-1), and was thus selected for further experiments. The mutant exhibited slightly reduced growth on potato dextrose agar (PDA) (Figure 2g) and, as expected, FgPMK-S15 mycelia formed only faint toxisomes in toxin-inducing conditions (Figure 2h). The translation level of Tri1-GFP protein in FgPMK-S15 was assayed by western blot analysis, and the result was consistent with fluorescent signals (Figure 2i). The mutant FgPMK-S15 also exhibited significantly reduced DON production (Figure 2). Taken together, these results suggest that mevalonate biosynthetic enzymes ACAT2 and PMK localize in toxisome under toxin-inducing conditions and play an important role in DON biosynthesis.

2 | RESULTS

2.1 | Two enzymes in mevalonate metabolism are localized in the F. graminearum toxisome

While enzymes involved in DON biosynthesis have been studied intensively, the subcellular localization for de novo DON biosynthesis, the toxisome in F. graminearum, was only proposed recently (Menke et al., 2013). Other components were also found localized in the toxisome, including a mevalonate biosynthetic enzyme Hmcr, two trichothecene biosynthetic pathway enzymes Tr1 and Tr4, ribosomal protein Asc1, and cytoskeleton proteins myosin I and actin (Boenisch et al., 2017; Tang et al., 2018). To further characterize the toxisome components, we selected four mevalonate biosynthetic enzymes and tagged them with GFP in the wild-type strain PH-1: two acetyl-CoA acetyltransferases, ACAT1 (FGSG_05087) and ACAT2 (FGSG_09321), phosphomevalonate kinase PMK (FGSG_09764), and diphosphomevalonate decarboxylase MDV (FGSG_10424). The strains expressing ACAT1-GFP, ACAT2-GFP, and MDV-GFP showed cytoplasmic fluorescence only during growth in toxin-noninducing conditions (Figure 2a,b,d). In toxin-inducing conditions, ACAT2-GFP was partially localized to yet-to-be-determined spherical structures (Figure 2b). We stained the ACAT2-GFP strain with the vacuole-labelling dye 7-amino-4-chloromethylcoumarin and verified that ACAT2 does not localize to the vacuoles (Figure S1). Interestingly, stronger fluorescence from PMK-GFP was localized to the spherical structures under toxin-noninducing and toxin-inducing conditions (Figure 2c). Furthermore, ER-tracker blue and 4',6-diamidino-2-phenylindole staining indicated that PMK-GFP is not only associated with the ER but also with the perimeter of the nucleus (Figure S2). To determine whether these two enzymes (ACAT2 and PMK) localize to the toxisomes in toxin-inducing conditions, we tagged Tr1 with an RFP in ACAT2-GFP and PMK-GFP strains. Tr1-RFP colocalized with ACAT2-GFP and PMK-GFP in trichothecene biosynthesis induction (TBI) medium to some degree, as shown in Figure 2e,f.

To verify the role of these mevalonate biosynthetic enzymes in the toxisome assembly, we performed FgPMK knockdown by transforming the recombinant plasmid pSilent-FgPMK, containing the hairpin RNA of an FgPMK fragment (485 bp), into the wild-type strain PH-1. Among the 12 tested transformants, eight showed decreased expression of FgPMK in comparison with the wild-type progenitor (Figure S3). Among these transformants, FgPMK-S15 showed the lowest FgPMK expression (15% of the wild-type strain PH-1), and was thus selected for further experiments. The mutant exhibited slightly reduced growth on potato dextrose agar (PDA) (Figure 2g) and, as expected, FgPMK-S15 mycelia formed only faint toxisomes in toxin-inducing conditions (Figure 2h). The translation level of Tr1-GFP protein in FgPMK-S15 was assayed by western blot analysis, and the result was consistent with fluorescent signals (Figure 2i). The mutant FgPMK-S15 also exhibited significantly reduced DON production (Figure 2). Taken together, these results suggest that mevalonate biosynthetic enzymes ACAT2 and PMK localize in toxisome under toxin-inducing conditions and play an important role in DON biosynthesis.

2.2 | Microtubule-associated toxisome assembly

To investigate whether the MT cytoskeleton is involved in the toxisome assembly, we first constructed a strain bearing β1-RFP and Tr1-GFP in the wild-type progenitor. As indicated in Figure 3a, toxisomes were found to be associated with MT filaments in F. graminearum hyphae. Furthermore, we examined how toxisome assembly responds to the small-molecule fungicide carbendazim, which targets the MT cytoskeleton and interferes with its formation during mitosis. After treatment for 24 hr with 1.2 μg/ml carbendazim (approximately EC95 against mycelial growth), Tr1-RFP only displayed weak signals when compared with the control samples (Figure 3b, left panel; Figure S4). The expression of Tr1-GFP was verified by the western blot assay, and the intensity of the GFP band was dramatically reduced in the samples treated with carbendazim (Figure 3b, right panel). Accordingly, there was a significant reduction in DON production after treatment with carbendazim (Figure 3c). Collectively, the assembly of toxisome is associated with MT, and the disruption of MT by carbendazim impaired the cluster assembly of toxisome and DON biosynthesis in toxin-inducing conditions.
2.3 | α1 tubulin participates in toxisome assembly

To better define the impact of the MT cytoskeleton in toxisome assembly, we investigated the role of each MT component. First, we deleted α1 tubulin in the wild-type strain PH-1, and Tri1-GFP plasmid was subsequently introduced into the deletion mutant ∆Fgα1. All transformants were screened under a confocal microscope and further verified by western blot assay (data not shown). The results showed that ∆Fgα1 showed drastically impaired hyphal growth when compared with PH-1. When treated with 0.5 μg/ml carbendazim, ∆Fgα1 exhibited increased drug sensitivity against the fungicide (Figure 4a). As shown in Figure 4b,c, the Tri1-GFP signals sharply
decreased in ΔFGα1. Furthermore, western blot assays showed that the expression of Tri1-GFP protein was significantly lower in ΔFGα1 compared with PH-1 in TBI medium (Figure 4f). Furthermore, when we measured DON production in equal mycelial mass, significantly less mycotoxin was produced in ΔFGα1 in comparison with PH-1 (Figure 4g). These data on the α1 tubulin deletion mutant are in line with the wild-type phenotypes when treated with carbendazim described earlier, and indicate that α1 tubulin plays a vital role in organizing the toxisome assembly in F. graminearum.

2.4 | α2 and β1 tubulin are dispensable for DON biosynthesis

To test whether or not α2 tubulin is necessary for toxisome assembly, we deleted α2 tubulin in the wild-type strain PH-1. The results revealed that ΔFGα2 showed hyphal growth similar to PH-1 but exhibited increased sensitivity to the MT inhibitor carbendazim (Figure 4a). When the toxisome assembly in ΔFGα2;Tri1-GFP mutant was examined, the Tri1-GFP signals showed no difference in ΔFGα2 compared with the wild type (Figure 4b,d). Western blot assays also confirmed that the expression of the Tri1-GFP protein in ΔFGα2 was similar to the wild-type progenitor (Figure 4f). Furthermore, DON production in ΔFGα2 was similar to the wild type (Figure 4g). We concluded that α2 tubulin is not critical for toxisome assembly in F. graminearum.

We also constructed a deletion mutant of β1 tubulin to investigate toxisome association. The mutant ΔFGβ1 showed similar hyphal growth compared with PH-1 and decreased sensitivity to the MT inhibitor carbendazim (Figure 4a). In addition, the Tri1-GFP signals and the expression of Tri1-GFP protein showed no difference between ΔFGβ1 and the wild-type progenitor (Figure 4b,e,f). Furthermore, DON production was similar in ΔFGβ1 and the wild type (Figure 4g). We concluded that β1 tubulin is not involved in DON biosynthesis in F. graminearum.

2.5 | β2 tubulin-associated toxisome assembly

Previous studies showed that the carbendazim-resistant mutations in β2 tubulin significantly increased DON production in F. graminearum (Zhang et al., 2009; Zhou et al., 2020), suggesting that β2 tubulin is involved in the regulation of DON biosynthesis. In this study, we further investigated whether β2 tubulin, one of the key
MT components, is involved in toxisome assembly. As anticipated, the $\beta_2$ tubulin deletion mutant $\Delta Fg\beta_2$ showed significantly reduced hyphal growth and increased drug sensitivity to the tubulin inhibitor carbendazim similar to that observed in $\Delta Fg\alpha_1$ (Figure 5a). Using fluorescent live cell imaging, we found that toxisome assembly was severely impaired in $\Delta Fg\beta_2$ with weak fluorescent signals loosely dispersed in hyphae (Figure 5b). In addition, western blot analysis of Tri1-GFP protein in the wild-type PH-1 and $\Delta Fg\beta_2$ was in agreement with the fluorescent signals (Figure 5c). Furthermore, DON production in the mutant was drastically lower than wild type in toxin-inducing conditions (Figure 5d). These results show that $\beta_2$ tubulin is indispensable for toxisome assembly.

2.6 $\alpha_1$- $\beta_2$ tubulin dimer facilitates toxisome assembly by providing a scaffold

In the current study, $\Delta Fg\alpha_1$ and $\Delta Fg\beta_2$ mutants showed consistent defects in hyphal growth, drug sensitivity, toxisome assembly, and DON biosynthesis. Therefore, we hypothesized that (a) $\alpha_1$ tubulin is associated with $\beta_2$ tubulin and (b) $\alpha_1$ and $\beta_2$ tubulin are associated with toxisome components in *F. graminearum*. To test our hypotheses, we first constructed a strain bearing $\alpha_1$-GFP and $\beta_2$-RFP. Using fluorescent live cell imaging, we showed that $\alpha_1$-GFP colocalizes with $\beta_2$-RFP (Figure S5). Subsequently, we constructed a strain that was dual-labelled with either $Fg\alpha_1$-3×FLAG and Tri1-GFP or
ZHOU et al.

Fgβ2 and Tri1-GFP, followed by culturing in TBI medium for 48 hr. The mycelial samples then were harvested for protein extraction and coimmunoprecipitation (Co-IP) assays. The results verified the interactions between Tri1 and β2 tubulin as well as Tri1 and α1 tubulin (Figure 6a,b). Furthermore, we demonstrated that Tri1 does not interact with α2 or β1 tubulin (Figure 6c,d), which is consistent with their roles in toxosome assembly and DON biosynthesis. These results provide a strong argument for the critical role α1–β2 tubulin heterodimer plays in toxosome assembly, and we postulate that it serves as the scaffold for the spatial organization and structure stabilization of toxisomes in Fusarium species.

3 | DISCUSSION

The formation of metabolic multienzyme complexes, metabolons, is thought to promote metabolic processes and provide a coordinated system for the cell to effectively control metabolism (Kohnhorst et al., 2017; Thomas et al., 2017). Recently, the existence of metabolons in mammalian cells has come to light with the development of advanced cell-based techniques. Accumulating evidence has indicated that various metabolons exist in cell metabolisms, such as glycosome in glycolysis, pyruvate dehydrogenase complex (PDC) in the tricarboxylic acid (TCA) cycle, and purinosome in the purine
biosynthesis pathway (An et al., 2010; Haanstra et al., 2016; Haggie & Verkman, 2002). Additionally, systems biology combined with omics strategies has shown that metabolons often interact with other subcellular components, particularly MTs, to maximize their functions (French et al., 2016; Ori et al., 2016; Rhee et al., 2013). In HeLa cells, purinosomes were embedded in the MT network and this spatial distribution significantly accelerated enzyme activity (An et al., 2010). In Fusarium species, a novel secondary metabolism complex containing several DON biosynthesis enzymes, the toxosome, was found recently (Boenisch et al., 2017; Menke et al., 2013). Moreover, myosin I-actin cytoskeleton was shown to play a vital role in toxosome formation (Tang et al., 2018). Because the biochemical and physiological connections between toxosome and the MT cytoskeleton have not been investigated, our key motivation for this study was to better define their functional correlation.

In eukaryotic cells, the MT cytoskeleton is associated with various cellular processes, including cell motility, mitosis, polarity, and vesicle traffic. By conducting a fluorescent live cell imaging of the MT cytoskeleton in the presence of toxosomes, we provide clear evidence that toxosomes are associated with the network of MTs (Figure 2a). Our studies found that DON production was significantly increased in β2 tubulin carbendazim-resistant mutants (Zhou et al., 2020), indicating that β2 tubulin is involved in DON biosynthesis. In addition, Tang and colleagues showed that carbendazim treatment (approximately EC50 against mycelial growth) increased DON biosynthesis in F. graminearum (Tang et al., 2018). We obtained similar results when we treated our wild-type strain with carbendazim in this study (Figure S6). However, we found that disruption of the MT network by the addition of a high concentration of carbendazim (approximately EC95 against mycelial growth) was sufficient to dissociate toxosomes in F. graminearum (Figure 2b). Furthermore, the architecture and function of MTs are regulated by microtubule-associated proteins (MAPs). Among various MAPs, the end-binding protein 1 (EB1) has been well studied in mammalian cells, yeast, and phytopathogens including F. graminearum. In yeasts, the homologs of EB1 have been used to regulate microtubule dynamics, chromosome stability, and cell polarization (Schwartz et al., 1997). In F. graminearum, the MT network exhibited unstable organization in the absence of EB1 (Liu et al., 2017). Similarly, the homologs of EB1 are involved in
regulating MT integrity and cell formation in Schizosaccharomyces pombe (Beinhauer et al., 1997). As expected, toxisome assembly and DON biosynthesis were severely impaired in the EB1 deletion mutant (Liu et al., 2017). All these results indicate that the integrity of the MT network is important for toxisome assembly and DON production in Fusarium species. Therefore, we hypothesized that the spatial distribution of toxisomes is closely connected with MT networks. Our experiments using carbendazim treatment and MT mutants clearly demonstrated that MTs serve critical roles in DON biosynthetic enzyme organization under toxin-inducing conditions. When the integrity of the MT cytoskeleton was impaired, the proper proximity of DON biosynthetic enzymes to one another would be disrupted, which would lead to a significant decrease in DON metabolic efficiency. Thus, this cell-based toxisome activity experiment indeed supports our hypothesis that MTs are functionally necessary for toxisome assembly in F. graminearum.

Previous studies in S. cerevisiae showed that β-tubulin is directly associated with α tubulin (α1 and α2) to form a tubulin heterodimer, which polymerizes to form MTs (Mandelkow & Mandelkow, 1989; McKean et al., 2001). Four tubulin subunits (α1, α2, β1, and β2) are found in F. graminearum, and previous studies showed that α1 and β2 tubulin are involved in DON biosynthesis (Hu et al., 2015; Wang et al., 2019). In addition, Fgα1 was captured by Fgβ2 in our affinity capture–mass spectrometry (ACMS) assays (authors’ unpublished data). In this study, we further investigated and confirmed the association of the toxisome with each tubulin subunit. The dissociation of the toxisome was evident in the absence of α1 or β2 tubulin whereas deletion of α2 or β1 tubulin did not affect toxisome assembly. In addition, Tri1-GFP translation and DON production were also significantly reduced in the absence of α1 or β2 tubulin, which is consistent with toxisome assembly. Furthermore, we demonstrated that α1 tubulin is directly connected with β2 tubulin in F. graminearum. These results indicate that α1 tubulin associates with β2 tubulin to form the α1-β2 tubulin heterodimer, thereby participating in toxisome assembly and DON biosynthesis in F. graminearum.

Numerous proteins have been reported to bind with MTs or their subunits to some degree, which can alter their activity in comparison with non-MT bound proteins. These proteins include glycolytic enzymes, purine biosynthesis enzymes, mitochondrial outer membrane protein voltage-dependent anion channel (VDAC), and hypoxia-inducible factor (HIF)-1. For example, hexokinase (HK) and pyruvate kinase (PK) have been demonstrated to colocalize with MTs in animal cells, which resulted in greater enzyme activity (Walsh et al., 1989; Wagner et al., 2001). In HeLa cells, purine biosynthetic enzymes embedded in the MT network in purine-depleted conditions, which accelerated the rate of de novo purine synthesis (An et al., 2010). Results showed that the transport of ions and metabolites across the outer membrane of the mitochondria is controlled by the VDAC, and tubulin dimers can regulate the switching of VDAC (Lemasters & Holmuhamedov, 2006; Shoshan-Barmatz et al., 2010). Additionally, a glycolytic metabolon is known to be stabilized by the actin cytoskeleton in S. cerevisiae (Araiza-Olivera et al., 2013). In the present study, we showed that both α1 and β2 tubulin interact with Tri1, an important component of the toxisome. We postulate that this interaction stabilizes the structure of toxisome, thereby facilitating DON biosynthesis. When the structure of α1-β2 tubulin heterodimer was impaired, the toxisome could not form in F. graminearum, which is deleterious to maintaining the proximity and high activity of all DON biosynthetic enzymes.

The ER is a complex organelle that is involved in lipid and protein biosynthesis and calcium regulation as well as interactions with other organelles. The structure and distribution of ER is regulated by various membrane proteins and interactions with cytoskeleton and other organelles. Recent reports have shown that toxisome enzymes are colocalized at the reorganized ER under toxin-inducing conditions in F. graminearum (Boenisch et al., 2017). However, the molecular mechanism for ER reorganization remains to be elucidated. In HeLa cells, the tubule-to-sheet transition in ER was regulated by MTs, and nocodazole (an MT depolymerization drug) treatment could disrupt this transition (Lu & Kirchhausen, 2012). Additionally, ER tubules can be formed by membranes sliding along MTs or attached to polymerizing MTs (Waterman-Storer & Salmon, 1998). Based on this evidence, we speculated that MTs might be involved in ER structure formation in F. graminearum. The interactions between MT and ER under toxin-inducing conditions remains unknown, however, and need further research.

In summary, our study supports a model of MT heterodimer interacting with Tri1, in a "piggy-backing" way, and α1-β2 tubulin and Tri1 serve as scaffold and anchor, respectively, to further organize toxisome assembly and stabilize toxisome structure, which ultimately activates DON biosynthesis in F. graminearum (Figure 7). However, the complex protein–protein interaction network among various toxisome components and their association with α1-β2 tubulin heterodimer remains inconclusive. Thus, it would be worthwhile studying further the protein–protein interaction network of the toxisome and its interactions with MTs to improve our insight into DON regulation mechanism in Fusarium species.

4 | EXPERIMENTAL PROCEDURES

4.1 Strains and culture assays

F. graminearum wild-type strain PH-1 was used for the construction of the derived mutants in this study. All strains used in this study were grown and evaluated at 25 °C on PDA for mycelial growth and fungicide sensitivity assays. Mung bean broth medium was used for conidia culture (Zhang et al., 2016). For DON production analysis or toxisome observation, all strains were grown in TBI medium at 28 °C with agitation (175 rpm) in the dark for 7 days or 2 days, respectively (Tang et al., 2018). All experiments were repeated three times independently.
4.2 | Strain construction

The strains ΔFgαι, ΔFgβι, and ΔFgβι were constructed using the protocol described previously (Liu et al., 2013; Qiu et al., 2012). All targeted open reading frames (ORFs) were replaced with the HPH-HSV-tk fragment, and all transformants were analysed by PCR assays with corresponding primers and by quantitative PCR assays. Fgα1-3 × FLAG and Fgβ1-RFP fusion cassette were constructed following the protocol described previously (Zhang et al., 2016). Tri1-GFP fusion cassette was constructed as described previously and then transformed into the corresponding mutants (Bruno et al., 2004).

4.3 | Analysis of DON production

To measure DON production, PH-1 and all mutants were grown in liquid TBI medium. After incubation at 28 °C for 7 days in the dark, the mycelia and 1 ml of TBI medium were harvested. Thereafter, DON was assayed with a competitive enzyme-linked immunosorbent assay detection plate kit (Wise) according to the protocol described in previous studies (Duan et al., 2018; Li et al., 2019).

4.4 | Microscopic examinations

The localization of Tri-GFP protein was observed with a TCS SP5 confocal microscope (Leica). For examination of toxisome assembly patterns in PH-1 and derived mutants, all strains labelled with Tri1-GFP were cultured in TBI medium for 2 days before observation. The following parameters for confocal microscopy were used: Plan-Neofluar 100×/1.30 oil DIC objective, laser at 488 nm at 30% power for green fluorescence or at 561 nm at 40% power for red fluorescence, pinhole 100 μm, and digital gain 1.00.

4.5 | Western blotting analysis

All transformants were cultured in liquid TBI medium for 2 days, thereafter the mycelia were harvested for protein extraction. The protein extraction and western blotting analysis were performed as previously described (Gu et al., 2015; Zhou et al., 2020). Ten microlitres of protein sample was analysed by western blotting. The monoclonal anti-GFP antibody 300943 (Zenbio) was used at a 1:1,000 dilution ratio to detect Tri-GFP fusion protein. All samples were also assayed with monoclonal anti-actin antibody 700068 (Zenbio) as a
reference. The intensity of immunoblot bands was quantified using Gel-Pro analyser software. All experiments were repeated three times.

4.6 | Co-IP assays

The Tr11-GFP or α1-3 × FLAG-fusion constructs were verified by DNA sequencing and transformed into PH-1 or corresponding mutants. Transformants expressing Tr11-GFP and β2 tubulin or Tr11-GFP and α1-3 × FLAG were confirmed by western blotting analysis. In addition, the transformants expressing a single tag protein were used as references. For Co-IP assays, magnetic beads (Bio-Rad) were first incubated with the monoclonal anti-GFP antibody 300943 following the manufacturer’s protocol. Thereafter, the magnetic beads were incubated with total protein samples. Protein samples (10 μl) eluted from magnetic beads were analysed by western blotting with a polyclonal anti-β2 tubulin or Tr11-GFP and α1-3 × FLAG were confirmed by western blotting analysis. In addition, the transformants expressing a single tag protein were used as references. For Co-IP assays, magnetic beads (Bio-Rad) were first incubated with the monoclonal anti-GFP antibody 300943 following the manufacturer’s protocol. Thereafter, the magnetic beads were incubated with total protein samples. Protein samples (10 μl) eluted from magnetic beads were analysed by western blotting with a polyclonal anti-β2 antibody IF11 (Zhou et al., 2016) or a polyclonal anti-FLAG A9044 (Zenbio). Total protein samples were further assayed with monoclonal anti-actin antibody 700068 as a reference. All experiments were repeated twice.

ACKNOWLEDGMENTS

The research was supported by the National Natural Science Foundation of China 31730072 (to M.Z.) and the National Natural Science Foundation of China 31772190 (to Y.D.). The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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