The RNA binding protein FgRbp1 regulates specific pre-mRNA splicing via interacting with U2AF23 in *Fusarium*

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Precursor messenger RNA (pre-mRNA) splicing is an essential and tightly regulated process in eukaryotic cells; however, the regulatory mechanisms for the splicing are not well understood. Here, we characterize a RNA binding protein named FgRbp1 in *Fusarium graminearum*, a fungal pathogen of cereal crops worldwide. Deletion of FgRbp1 leads to reduced splicing efficiency in 47% of the *F. graminearum* intron-containing gene transcripts that are involved in various cellular processes including vegetative growth, development, and virulence. The human ortholog RBM42 is able to fully rescue the growth defects of ΔFgRbp1. FgRbp1 binds to the motif CAAGR in its target mRNAs, and interacts with the splicing factor FgU2AF23, a highly conserved protein involved in 3' splice site recognition, leading to enhanced recruitment of FgU2AF23 to the target mRNAs. This study demonstrates that FgRbp1 is a splicing regulator and regulates the pre-mRNA splicing in a sequence-dependent manner in *F. graminearum*.

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In eukaryotic RNA processing, pre-mRNA splicing is a crucial step where introns are removed and exons are joined to form mature mRNAs. In addition, pre-mRNAs are also subject to alternative splicing to generate more than one mature mRNAs from a single gene. Both constitutive and alternative pre-mRNA splicing are catalyzed by a large ribonucleoprotein complex called the spliceosome, which is composed of five core small nuclear ribonucleoprotein (snRNP) particles named U1, U2, U4, U5, and U6, along with many associated protein cofactors. Assembly of the spliceosome occurs in a stepwise fashion. First, U1 snRNP, splicing factor 1 (SF1), and U2 auxiliary factor (U2AF) recognize the 5' splice site (5'SS), branch point sequence (BPS) and 3' splice site (3'SS), respectively, and assemble into an early spliceosome (E complex). Subsequently, SF1 is displaced by the U2 snRNP to form the pre-spliceosome (A complex), which associates with the preformed U4/U6.U5 tri-snRNP to generate the pre-B complex where the tri-snRNP is not yet stably bound. Next, the disruption of the U1/5' SS helix allows stable tri-snRNP integration and U1 snRNP destabilization, yielding the pre-catalytic spliceosome (B complex). The B-complex is then converted into an activated spliceosome (B\* complex), which no longer contains U1 and U4. Subsequent remodeling transforms B\* into a catalytically competent B++ complex (i.e., catalytically activated spliceosome), which catalyzes the first step of splicing, yielding the C complex. The resulting C complex is then remodeled into step I catalytically active spliceosome (C* complex), which performs the second catalytic step to produce mature mRNAs.

The U2AF heterodimer consists of two subunits that are conserved from fission yeast to humans (U2AF65 and U2AF35 in humans and U2AF59 and U2AF23 in fission yeast). The large subunit U2AF65 binds the polyprymidine tract (Py-tract) immediately downstream of the BPS, while U2AF35 directly comes in contact with AG dinucleotide of the 3'SS to further guide U2AF65 to those relatively weaker Py-tracts. Mutations in U2AF lead to serious diseases, including the Myelodysplasia Syndrome (MDS)6-8. Recently, high-throughput RNA sequencing (RNA-seq) assays showed that mutations in U2AF35 alter hematopoiesis due to the mis-splicing of hundreds of gene transcripts resulting from changes in 3' SS recognition9,10. In addition to U2AF, mutations in other spliceosome components and splicing regulatory factors are also responsible for various human illnesses, including genetic diseases, neurodegenerative disorders, and cancer.

Fusarium graminearum is the predominant causal agent of Fusarium head blight (FHB), an economically devastating disease of small grain cereal crops worldwide12. In addition to yield losses caused by FHB, mycotoxins such as deoxynivalenol (DON) and zearalenone produced by the pathogen in infected grains seriously threaten the health of humans and animals13,14. It would not be difficult to anticipate that pre-mRNA splicing is critical for various cellular processes in F. graminearum since the genome of this pathogen contains 77% intron-bearing genes, and 98% of them are constitutively spliced15,16. A recent study in F. graminearum revealed that FgPrp4, the only kinase among the spliceosome components, regulates the intron splicing in over 60% of gene transcripts by phosphorylation of other spliceosome components. The FgPrp4 mutant showed severe growth defects and a total loss of pathogenicity on wheat17. Moreover, the serine/arginine-rich splicing factor FgSrpl, which interacts with FgPrp4 in vivo, also regulates the intron splicing of a subset of genes involved in fungal development and infection18. Consistent with these findings, the splicing factor Num1 in Ustilago maydis is required for the polarized growth of fungal hyphae, and affects the cell cycle and cell division19. In spite of recent reports highlighting the critical roles of pre-mRNA splicing, the mechanisms by which the splice sites are identified and regulated are still largely unknown in fungi.

RNA-binding proteins (RBPs) are critical for co-transcriptional and post-transcriptional gene expression in eukaryotic organisms. Many RBPs are characterized by the presence of various RNA-binding domains including the RNA recognition motif (RRM), K-homology (KH) domain, the zinc finger domain, the DEAD-box domain, and the Pumilio/FBF domain20. The F. graminearum genome encodes more than 100 predicted RBPs with these domains, but the detailed functions of these RBPs remain uninvestigated. In this study, we identify an uncharacterized RBP (named FgRbp1 hereafter) containing a single RRM domain in F. graminearum. The gene deletion mutant displays severe defects in vegetative growth, sexual reproduction and virulence. Using a combination of genetics, biochemistry and high-throughput sequencing approaches, we learned that FgRbp1 is involved in the regulation of pre-mRNA splicing by interacting with a small subunit of U2AF. Moreover, RNA affinity selection assay indicated that FgRbp1 can enhance the recruitment of FgU2AF23 to its target mRNAs. RNA immunoprecipitation followed by deep sequencing ( RIP-seq) assay identified 219 putative pre-mRNA targets for FgRbp1, which are involved in various cellular processes, and FgRbp1 directly binds the CAAGR motif deposited in the pre-mRNA targets. Phylogeny analysis showed that orthologs of FgRbp1 are highly conserved in diverse eukaryote lineages. Consistently, we found that the human ortholog RBM42 can rescue the growth defects in ΔFgRbp1. Results from our study provide a strong argument that FgRbp1 orthologs can regulate pre-mRNA splicing in F. graminearum, and likely in diverse eukaryotic organisms.

Results
Identification of the RNA-binding protein FgRbp1 in F. graminearum. The RRM is one of the most abundant RNA-binding domains in RBPs. The F. graminearum genome contains 67 RBPs with the RRM domain (Supplementary Data 1). As listed in Supplementary Data 1, there are ten proteins with no functional implications while the remaining RBPs are annotated. Among these ten RBPs, nine of these proteins have no other characteristic domains except the RRM domain, and Fg_13028 contains a RRM and a Pumilio domain (Supplementary Fig. 1a). Therefore, we were interested in characterizing these uncharacterized RBPs. To initiate our study, we carried out gene deletion for each of these uncharacterized RBPs using homologous recombination strategy. At least three deletion mutants for each gene were obtained by transforming the wild-type strain PH-1, and colony morphologies of these mutants were shown in Supplementary Fig. 1b. Among the ten RBP deletion mutants, ΔFg11902 strain exhibited a severe growth defect, and we designate the putative encoded protein as FgRbp1. The ΔFg11902 strain (herein ΔFgRbp1) was further verified by Southern blot assays (Supplementary Fig. 2). The growth defects of ΔFgRbp1 were restored by genetic complementation with the full-length FgRbp1 gene in the complemented strain ΔFgRbp1::FgRbp1-GFP (Fig. 1a).

The FgRBP1 gene is predicted to encode a 351-amino-acid protein containing one RRM domain, which is franked by extended N- and C-terminal tails (Supplementary Fig. 1a). Phylogenetic analysis indicates that FgRbp1 orthologs are conserved in fungi, metazoas and plants, but not in Saccharomyces (Supplementary Fig. 3). Amino acid alignment showed that the RRM domain shares a high level of similarity, while the N- and C-terminus vary significantly among different species (Supplementary Fig. 4). Since the RRM domain of FgRbp1 is similar to that of human counterpart (RBM42) with 51% amino acid identity, we generated RBM42-GFP construct with fungal constitutive promoter RP27 and transformed into ΔFgRbp1 to test whether FgRbp1 and RBM42 are functionally conserved. Significantly, RBM42-GFP fully restored the growth defects in ΔFgRbp1 to the
RBM42-GFP expressed in localizes in the nucleus (Fig. 2a, left panel). As anticipated, the ΔRM does not alter the nuclear localization of FgRbp1 (Fig. 2a, right panel). To further elaborate the molecular function of FgRbp1, we screened a F. graminearum cDNA library with the yeast two-hybrid (Y2H) approach using FgRbp1 as the bait and identified 87 potential FgRbp1-interacting proteins (Supplementary Data 1). One of these proteins was FgU2AF23 (the ortholog of human U2AF35), which is essential for the determination of 3' splice sites in pre-mRNA splicing. Given that FgRbp1 is an RNA-binding protein that may participate in pre-mRNA processing, we were interested in this potential interaction. To confirm this interaction between FgRbp1 and FgU2AF23, we first cloned the full-length cDNA sequence of FgU2AF23 into prey plasmid pGADT7 and subsequently validated this interaction using the Y2H assay (Fig. 2b). Concurrently, Y2H assays with a series of truncated FgRbp1 constructs revealed that the uncharacterized N-terminal domain is essential for FgRbp1-FgU2AF23 interaction, while the deletion of the RRM domain weakened the interaction (Fig. 2b). Since the N-terminal domain of FgRbp1 is necessary for the interaction, we therefore generated a FgRbp1ΔN-GFP complemented construct (a truncated FgRbp1 lacking N-terminal domain [1-213 aa] fused with GFP) and transformed into ΔFgRbp1 to investigate the function of the uncharacterized N-terminus. The resulting strain ΔFgRbp1::FgRbp1ΔN-GFP displayed reduced mycelial growth on PDA, although the growth defect was not as severe as ΔFgRbp1 (Fig. 2c). FgRbp1 and FgU2AF23 interaction, along with the fact that U2AF35 and U2AF59 form a heterodimer complex, led us to ask whether FgRbp1 interacts with FgU2AF59. The result of Y2H assay showed that FgRbp1 does not interact with FgU2AF59 (Supplementary Fig. 6).

Next, subcellular localization assay showed that FgU2AF23 colocalizes with FgRbp1 in the nucleus, revealing the spatial position of their interaction (Fig. 2d). Moreover, we carried out a co-immunoprecipitation (Co-IP) experiment, and the result further verified the interaction between FgRbp1 and FgU2AF23 (Fig. 2e). To test whether the interaction is mediated by RNA, we treated the protein extracts for Co-IP with RNase A prior to immunoprecipitation. Notably, the FgRbp1-FgU2AF23 interaction was RNase A insensitive (Fig. 2e), indicating that this interaction is RNA-independent. We also conducted bimolecular fluorescence complementation (BiFC) assay to further confirm the interaction. As shown in Fig. 2f, the recombined YFP fluorescence signals from FgRbp1-YFPN-terminal and FgU2AF23-YFPC-terminal showed co-localization with the RFP signals of nuclear marker histone 1 (H1), suggesting that they interacted with each other in the nucleus in vivo. Taken together, these results convincingly demonstrated that FgRbp1 physically interacts with FgU2AF23 in F. graminearum.

**FgRbp1 is critical for intron splicing efficiency.** U2AF23, the small subunit of U2AF heterodimer, is essential for the determination of 3' splice sites in pre-mRNA splicing. The interaction of FgRbp1 with FgU2AF23 suggests that FgRbp1 participates in pre-mRNA splicing. To test this, we performed RNA-seq in the wild-type and ΔFgRbp1 mutant strains with three independent biological replicates. To evaluate the splicing efficiency of introns, we calculated the intron retention rate (the ratio of normalized read number within introns to normalized read number of exon regions for each intron-containing gene) of each measured intron based on a previously established approach, and found that the intron retention rate in ΔFgRbp1 is significantly higher than that in the wild type (p<2.2e-16) (Fig. 3a). In ΔFgRbp1, 8689 introns (accounting for 70% introns detected) showed the intron retention rate twofold higher than those in the wild type (corresponding to 4849 genes that are defined as intron retention genes hereafter) (Supplementary Data 3a). Significantly, 52% of these introns have over fourfold increase in intron retention rate (Fig. 3b, Supplementary Data 3a). These results indicate that FgRbp1 regulates pre-mRNA splicing in F. graminearum.

**Identification of FgRbp1 targets by RIP-seq.** Since FgRbp1 contains a RRM domain, we questioned whether FgRbp1
Fig. 2 Nuclear localization of FgRbp1 and its physical interaction with the splicing factor FgU2AF23. a Subcellular localization of FgRbp1 (left panel) and FgRbp1ΔRRM (right panel) in F. graminearum. Both FgRbp1-GFP and FgRbp1ΔRRM-GFP localize to the nucleus and co-localize with the RFP-tagged histone H1. Bar = 10 μm. b Yeast two hybrid assay verifies interaction of FgRbp1 with FgU2AF23, demonstrating the essential role of the N-terminal domain of FgRbp1 in the interaction. The domain arrangement within FgRbp1 and the number of amino acids of corresponding domains are depicted in the schematic at the top. Tenfold serial dilutions of yeast cells transferred with the bait (a series of truncated FgRbp1) and prey construct (pGADT7-FgU2AF23) were assayed for growth on SD-Leu-Trp-His-Ade plates. A pair of plasmids, pGBKT7-53 and pGADT7-T was used as the positive control, while pGBKT7-Lam and pGADT7-T was used as the negative control. c Colony morphology of PH-1, ΔFgRbp1, and ΔFgRbp1::FgRbp1ΔN-GFP on PDA at 25 °C for 3 d. d FgRbp1-GFP co-localizes with FgU2AF23-RFP in the nucleus. Nuclei in hyphae were stained with 4',6-diamidino-2-phenylindole (DAPI). Bar = 10 μm. e The interaction between FgRbp1 and FgU2AF23 is confirmed by co-immunoprecipitation assay. The sensitivity of the interaction to RNase A was determined by treating the lysates with RNase A prior and during immunoprecipitation. To assess RNase A treatment efficiency, the RNA from the 1/10 volume lysate of each strain was extracted with Trizol reagent after immunoprecipitation and assessed on the agarose gel. f Bimolecular fluorescence complementation (BiFC) analysis verifies the FgRbp1-FgU2AF23 interaction in vivo. Transformants with FgRbp1-YFPN and FgU2AF23-YFPC constructs were examined under the confocal microscope. The strains bearing a single construct (FgRbp1-YFPN or FgU2AF23-YFPC) were used as negative controls. The RFP-tagged histone H1 was used to visualize the nucleus. Bar = 10 μm.
regulates pre-mRNA splicing via binding directly to its target pre-mRNAs. To test this, we performed the RNA immunoprecipitation (RIP) assay followed by sequencing (RIP-seq) using ΔFgRbp1::FgRbp1-FLAG strain (a complementary strain expressing FLAG-tagged FgRbp1 in ΔFgRbp1). By RIP-seq, we identified 384 FgRbp1 binding peaks ($P < 0.0005$, fold change $>1.5$), of which 272 peaks were corresponding to 219 protein-coding genes (Supplementary Data 4a), and the remaining peaks were mapped to noncoding RNAs. We analyzed the distribution of these peaks along protein-coding transcripts, and found that more than half of these peaks reside in the coding sequences and 25% and 12% of the peaks reside in the 3′-untranslated regions (UTRs) and 5′-UTRs, respectively, and the remaining peaks (6%) reside in intronic regions (Fig. 3c).

To explore the relationship between RNA binding and pre-mRNA splicing associated with FgRbp1, we overlapped FgRbp1-bound mRNAs with the intron-retention mRNAs in ΔFgRbp1 and found that 170 out of 219 FgRbp1-bound mRNAs (78%) have the intron splicing defect in their pre-mRNAs (Fig. 3d). This suggests a tight relationship between FgRbp1 binding and intron splicing. In addition, pathway analysis using KEGG database revealed similar enrichment pathways in these two classes of genes. They both showed significant enrichment in ribosome and oxidative phosphorylation pathways, which are the top three pathways in enrichment analysis (Fig. 3e, f), further implying that the binding of FgRbp1 to mRNAs and pre-mRNA splicing are closely associated.

**FgRbp1 binds the CAAGR cis element in target mRNAs.** To identify the FgRbp1-binding cis-element in the target mRNAs, we performed an unbiased search for consensus motifs enriched in peak sequences from 219 potential target pre-mRNAs (corresponding to 272 peaks) by using the DREME algorithm in the MEME suite.

The results showed that the most significantly enriched motif is CAAGR ($e$-value = $9.2e-010$) (Fig. 4a), which is present in ~70% of the peaks corresponding to 75% of transcripts (Supplementary Data 4a). To verify the interaction of FgRbp1 with this cis element, we carried out electrophoretic mobility shift assays (EMSAs) by using purified GST-FgRbp1 fusion protein (Fig. 4b) and the synthetic RNA of six repeats of 5′-CAAGA-3′ or
5'-CAAGG-3'. As shown in Fig. 4c, retardation of RNA migration was observed for both 5'-CAAGA-3' and 5'-CAAGG-3' repeated motifs, whereas GST or GST-FgRbp1ΔRRM (a truncated FgRbp1 lacking RRM domain) was unable to bind to the motif at all. As a control, FgRbp1 was unable to bind to a scrambled RNA sequence 5'-ACGAA-3', nor a mutated RNA sequence 5'-GUUCU-3' with six repeats (Fig. 4c), indicating the binding specificity of FgRbp1 to the CAAGR motif. Together, these results indicate that the CAAGR motif deposited in the target mRNAs is one of cis-elements for FgRbp1 and that the RRM domain is essential for the binding of FgRbp1 to this cis-element.

FgRbp1 binds and regulates splicing of ribosomal protein genes. Among the 170 overlapped mRNAs in FgRbp1-bound mRNAs and intron retention mRNAs, 68 mRNAs encode ribosomal proteins (Fig. 3e, f). Sequence analysis demonstrated that peaks of 64 target ribosomal protein genes (RPGs) contain the CAAGR motif (Supplementary Data 4b), thereby the motif may direct the specificity of FgRbp1 to these RPGs. Ribosomal proteins (RP) are fundamental components of the ribosome, contributing to the assembly of the ribosome and its ability to synthesize proteins. Inactivation of RP has been linked to developmental abnormalities in a variety of human organ systems, including short stature, craniofacial defects, thumb malformation and heart defects22. Similarly, these essential RPGs play crucial roles in mycelium development in F. graminearum based on the fact that we failed to generate any viable RP deletion mutants. Based on these outcomes, we conclude that the collective splicing defects of these RPGs are contributing to the severe growth defects in ΔFgRbp1.

To verify the splicing defect in these RPGs observed by RNA-Seq, we measured the intron splicing ratio (the ratio of spliced RNA to unspliced RNA) in nine RPGs randomly selected from 68 using the qRT-PCR approach described previously19,23,24 (for details, see “Methods”). Since each RPG has multiple introns with splicing defects, we then chose the intron with the highest retention rate in each RPG for verification. As shown in Fig. 5a, the splicing ratios of nine RPGs decreased dramatically in ΔFgRbp1 when compared with those of the wild-type strain, while the complemented strain ΔFgRbp1::FgRbp1-GFP bearing full length FgRbp1 restored the splicing ratios to the wild-type levels. Meanwhile, the RRM-depleted complemented strain (ΔFgRbp1::FgRbp1ΔRRM-GFP) showed similar splicing defects as ΔFgRbp1, indicating that the RRM domain is necessary for the splicing regulation of FgRbp1 (Fig. 5a). The N-terminal domain-depleted complemented strain (FgRbp1::FgRbp1ΔN-GFP) also showed decreased splicing ratio compared with PH-1, although the decline in the complemented strain was not as great as that in ΔFgRbp1 (Fig. 5a), suggesting that the N-terminal domain also plays a role in splicing regulation for FgRbp1. Consistently, the abundances of two ribosomal proteins RPS20 and RPS3 in ΔFgRbp1 were reduced to 25% and 42% of wild-type level, respectively (Fig. 5b). Moreover, qRT-PCR assays also demonstrated that the gene expression levels of all nine tested genes are not significantly altered in ΔFgRbp1 when compared to those in the wild-type strain (Fig. 5c), suggesting the decrease in protein abundance is not due to the decrease in transcription, but rather due to the ineffective pre-mRNA splicing. Moreover, the RNA-seq data also revealed that all 68 RPGs with splicing defects showed no significant fold change in gene expression levels, except for one gene FSGS_06724 that exhibits a 2.4 fold increased expression in ΔFgRbp1 compared with the wild-type strain (Supplementary Data 3b).

Next, the binding of FgRbp1 to the peak sequences of RPGs in vivo were confirmed by RIP-qPCR (Fig. 5d). To validate whether FgRbp1 directly binds to these RPG pre-mRNAs, we carried out EMASs by using purified GST-FgRbp1 and in vitro transcribed ~250-bp mRNA fragments of three RPGs (FgRPL25, FgRPL39 and FgRPS20) containing the peaks identified in the RIP-seq (in vitro transcribed mRNA sequence contains the sequence confirmed in Fig. 5d). As shown in Fig. 5e, the migration of fragment mRNAs through the gel was obstructed by GST-FgRbp1 whereas no obstruction was observed by GST or GST-FgRbp1ΔRRM. These results indicate that FgRbp1 directly binds to these RPG pre-mRNAs.

FgRbp1 regulates splicing of the ribosomal protein genes in a sequence-dependent manner. We next asked if the CAAGR motif in RPGs were required for the effect of FgRbp1 on splicing. Thus, we further used FgRPS20 as a model to investigate the role of FgRbp1–CAAGR interaction in regulating pre-mRNA splicing. FgRPS20 contains 5'-CAAGAACCTCAAGA-3' sequence (having two repeated 'CAAGA' sequences) located at the downstream exon of the first intron (127 nt downstream of intron1) (Fig. 5f). We then designed two constructs, the wild-type construct containing exon1-intron1-exon2 of FgRPS20 fused with GFP and the
mutated construct which is consistent to the wild-type construct except for the deletion of 5’-CCAAGAACCTCAAGA-3’ sequence (Fig. 5f). Since FgRPS20 is predicted to be essential for fungal growth (i.e., we were unable to obtain FgRPS20 deletion mutant after extensive effort), we transformed the wild-type strain PH-1 with each construct and assayed the splicing ratio of intron 1 in these transformants by qRT-PCR. As shown in Fig. 5f, the splicing ratio of FgRPS20 intron 1 was reduced dramatically in the strain containing the mutated construct when compared to that of the wild-type construct. To further confirm that the splicing defect of FgRPS20 intron 1 in the mutated-construct containing strain resulted from the loss of binding ability of FgRbp1 to the
Fig. 5 FgRbp1 regulates the splicing of ribosomal protein genes (RPGs) and facilitates the recruitment of FgU2AF23 to target mRNAs. a qRT-PCR validation of the splicing defects of introns in ribosomal protein genes. Data are presented as means ± standard deviations (SD), n = 3 biologically independent replicates. Different letters indicate a significant difference (P < 0.05) according to one-way ANOVA followed by Fisher’s least significant difference (LSD) test. b Comparison of the protein amount of RPS3 and RPS20 in the wild-type PH-1 and ΔFgRbp1 strains. The intensities of the western blot bands were quantified with the program IMAGE J. Error bars represent SD (n = 3 independent experiments). c Relative gene expression level of nine RPGs in PH-1 and ΔFgRbp1. Error bars represent SD (n = 3 biologically independent replicates). Individual P values are denoted above the comparison lines. d Verification of FgRbp1 binding on the mRNAs of RPGs by RIP-qPCR. ACTIN served as a control. Data are presented as means ± SD (n = 3 biologically independent replicates). e EMSA confirmation of the direct binding of FgRbp1 to the mRNAs of RPGs. In vitro transcribed RNAs containing the peak sequence were incubated with purified proteins described in Fig. 4b. f qRT-PCR analysis of the splicing ratio of FgRPS20 in the strains transformed with WT or mutated construct FgRPS20. PH-1 was transformed with either the wild-type construct (WT) containing exon1-intron1-exon2 of FgRPS20 fused with GFP or the mutated construct (Mu) with the sequence 5′-CCAAGAACCTCAAGA-3′ downstream of the intron deleted. The schematic of the two constructs of FgRPS20 is at the top. The arrows indicate the primers used to measure the splicing ratio of introns. g EMSA assay to test the binding ability of FgRbp1 to mRNA sequence “FgRPS20-WT” or “FgRPS20-Mu”. RNA sequence of FgRPS20-WT is identical to that of exon2 (267-bp) as indicated in d and e, and FgRPS20-Mu is consistent to the FgRPS20-WT except that the motif 5′-CCAAGAACCTCAAGA-3′ was deleted. h RNA affinity selection assay. The biotin-labeled FgRPS20 mRNA were immobilized on streptavidin beads and incubated with single protein: GST-FgRbp1, GST- FgRbp1ΔN, FgU2AF23-His, or pairs of proteins: GST-FgRbp1/ FgU2AF23-His, GST- FgRbp1ΔN/ FgU2AF23-His. The bound proteins were detected by western blot with the anti-GST or anti-His antibody. The fold-change in the Anti-His of pull-down for mixed proteins relative to that of FgU2AF23 was indicated below the band. In b, c and f, bars represent means ± SD of three independent experiments. Different letters indicate a significant difference (P < 0.05) according to the two-tailed unpaired Student’s t test.

FgRbp1 enhances the recruitment of FgU2AF23 to the FgRPS20 mRNA. Given that the unknown N-terminal domain of FgRbp1 was required for the interaction with FgU2AF23 (Fig. 2b), and the deletion of N-terminal domain also led to the intron splicing defect (Fig. 5a), it is reasonable to propose that FgRbp1-FgU2AF23 interaction should contribute to the role of FgRbp1 in splicing. The study of splicing regulation mechanism in human shows that many splicing regulators (e.g., SR protein or other RBPs) can promote exon inclusion or intron splicing by recruiting U1 snRNP to the 5′ splice site or U2 auxiliary factor (U2AF) to the 3′ splice site through protein–protein interactions in early steps of spliceosome assembly25–27. Since U2AF35 (the ortholog of FgU2AF23 in human) is an essential protein in the first step of assembly of spliceosome, we hypothesized that FgRbp1-FgU2AF23 interaction also promotes the recruitment of FgU2AF23 to FgRbp1-bound mRNAs to regulate intron splicing. To assess this, RNA affinity selection assays were conducted using FgRPS20 mRNA as a representative sample. The biotin-labeled FgRPS20 mRNA fragment containing exon1-intron1-exon2 (as shown in Fig. 5f) was in vitro transcribed and immobilized on streptavidin magnetic beads. The biotin-labeled FgRPS20 mRNA were incubated with either in vitro purified GST-FgRbp1 or FgU2AF23-His (Supplementary Fig. 7), or both proteins; while the interaction domain deleted protein GST-FgRbp1ΔN (Supplementary Fig. 7) was used as a control. As observed in Fig. 5h, GST-FgRbp1 alone bound to the FgRPS20 RNA baits, which is consistent with the EMSA result. Meanwhile FgU2AF23-His alone also bound to the RNA baits, which is in line with the previous reports that U2AF35 (human counterpart) or U2AF23 (fission yeast counterpart) binds to AG dinucleotide at the 3′ splice site in intron28,29. However, when two proteins were mixed together and incubated with the RNA baits, the binding of FgU2AF23-his to the RNA was significantly increased by ~60% (Fig. 5h). Moreover, GST-FgRbp1ΔN could also alone bind to the FgRPS20 RNA baits, but the addition of GST-FgRbp1ΔN did not increase the FgU2AF23-his binding to the RNA baits (Fig. 5h), suggesting that this recruitment is dependent on the interaction between the two proteins. These results indicate that FgRbp1 can enhance the recruitment of FgU2AF23 to the target FgRPS20 mRNA via interacting with FgU2AF23.

FgRbp1 regulates the splicing of pre-mRNAs involved in sexual reproduction. Sexual reproduction is a critical part of F. graminearum life cycle, and the perithecia produced from sexual reproduction serve as the key primary inoculum.30 To characterize the function of FgRbp1 in sexual development, we assayed perithecium formation on carrot agar. As shown in Fig. 6a, deletion of FgRbp1 completely abolishes the production of perithecium, while the wild-type strain PH-1 and the complemented strain ΔFgRbp1::FgRbp1-GFP produce abundant perithecia. To explore the cause of sexual infertility in ΔFgRbp1, we screened for mating related genes from the FgRbp1 target mRNAs and found two pivotal genes FgMCM1 and FgGPA1 that have been reported to control F. graminearum sexual production31,32. The RNA-seq data revealed that two introns of FgMCM1 and three introns of FgGPA1 are retained to different extents (Supplementary Data 3a). The qRT-PCR assays substantiated the severe splicing defects of the two genes in ΔFgRbp1 (Fig. 6b), which is in agreement with the loss of perithecium production in the mutant. Meanwhile, the transcription level of FgMCM1 and FgGPA1 remained at a similar level in PH-1 and ΔFgRbp1 (Supplementary Fig. 8a). The peak fragments of FgMCM1 and FgGPA1 transcripts identified in RIP-seq are both located in the 3′-UTR of these two genes (Fig. 6b). FgGPA1 contains a CAAGA motif in the identified 200-bp peak fragment, while FgMCM1 contains no CAAGR motif in the identified 173-bp peak fragment (Supplementary Data 4a). Nevertheless, RIP-qPCR and EMSA confirmed the direct binding of FgRbp1 to the two peak fragments of FgMCM1 and FgGPA1 (Fig. 6c, d). To investigate whether the binding of FgRbp1 to 3′-UTR of FgMCM1 and FgGPA1 is related to the splicing of these two genes, we constructed strains with a mutated 3′-UTR for FgMCM1 (the 173-bp peak sequence in 3′-UTR was deleted) and a mutated 3′-UTR for FgGPA1 (the 200-bp peak sequence in 3′-UTR was deleted) using an in situ replacement strategy. The FgRbp1’s binding region in 3′-UTR of FgMCM1 is responsible for the
splicing of intron 2 of FgMCM1, while the 3’-UTR binding region of FgGPA1 is related to the splicing of intron 2 and intron 4 of FgGPA1 (Fig. 6c). Together, these results indicate that FgRbp1 is critical for sexual reproduction in F. graminearum at least partially through regulating the splicing of two major mating-related mRNAs.

**FgRbp1 is required for full virulence.** The virulence of ΔFgRbp1 was evaluated by point inoculation of conidial suspension on flowering wheat heads. After 15 dpi, ΔFgRbp1 and ΔFgRbp1::FgRbp1::RRM-GFP failed to infect the spikelets, even in the spikelet directly inoculated, while the wild-type and the complemented strains resulted in extensive scab symptoms on wheat heads (Fig. 7a, left). Consistently, ΔFgRbp1 and ΔFgRbp1::FgRbp1::RRM-GFP also exhibited dramatically decreased pathogenicity on wheat heads, whereas the wild-type strain formed a number of penetration structures when observed by electronic scanning microscope (Fig. 7d). Therefore, it is reasonable to conclude that the loss of pathogenicity is due to the defect in penetration capability in ΔFgRbp1.

In plant pathogenic fungi, the Fus3/Kss1 MAPK pathway is well known to control a variety of virulence-related functions, including formation of penetration structure and secretion of plant cell wall degrading enzymes. Thus, we asked whether FgRbp1 regulates the splicing of pre-mRNAs that are responsible for host penetration. The transcription factor FgSte12, which is well known to control a variety of virulence-related functions, including formation of penetration structure and secretion of plant cell wall degrading enzymes. Thus, we asked whether FgRbp1 regulates the splicing of pre-mRNAs that are responsible for host penetration. The transcription factor FgSte12, which is downstream of the Fus3/Kss1 MAPK pathway, was identified as a FgRbp1 target with splicing defect in our RIP-seq and RNA-seq data (Supplementary Data 3, 4). A previous study showed that the FgSTE12 deletion mutant is unable to form infection structures on wheat. The splicing ratios of the two introns of FgSTE12 decreased dramatically in ΔFgRbp1 when compared with those in the wild type (Fig. 7e), although the transcription level of FgSTE12 remained at a similar level in both strains (Supplementary Fig. 8b). RIP-qPCR and EMSA assays confirmed the direct interaction between FgRbp1 and the identified peak fragment in FgSTE12 exon 1 with a CAAGA motif (Fig. 7f, g). Furthermore, the deletion of the CAAGA motif in the peak fragment led to significantly reduced intron splicing ratio of two introns in FgSTE12 (Fig. 7h). Taken together, these results indicate that FgRbp1 controls virulence of F. graminearum partially by...
Fig. 7 FgRbp1 is essential for the formation of fungal penetration structures on wheat. 

a Deletion of FgRbp1 leads to significantly reduced virulence on wheat heads (left panel) and corn silks (right panel). Flowering wheat heads were point-inoculated with the PH-1, ΔFgRbp1, ΔFgRbp1::FgRbp1ΔN-GFP, ΔFgRbp1::FgRbp1ΔRM-GFP and ΔFgRbp1::FgRbp1-GFP. The infected wheat heads were photographed 14 days post-inoculation (dpi). Infected corn silks were examined 5 dpi.

b Cross-sections of inoculated and adjacent wheat spikelets. Spikelets were infected with PH-1 or ΔFgRbp1, each strain bearing FgActin-RFP. The samples were taken at 7 dpi. Inoculated spikelets were indicated with black arrows. BF, bright field; RFP, red fluorescent protein. Bar = 200 µm.

c Penetration of the wild type PH-1 and ΔFgRbp1 into cellophane membrane. After removing colonies of PH-1 and ΔFgRbp1 grown over cellophane membranes on MM (Before) for 3d, the plates were incubated for three additional days to examine for hyphal growth (After).

d Electronic scanning microscope examination of the penetration structures on wheat glumes. Wheat head spikelets were inoculated with conidia of the wild-type PH-1 or ΔFgRbp1, and sampled after 7 dpi. Penetration structures are marked with yellow arrows. Bar = 5 µm.

e Introns of FgSTE12 showed significantly reduced splicing ratio (left panel) in ΔFgRbp1 compared with those in the wild-type PH-1.

f Verification of FgRbp1 binding on the mRNA of FgSTE12 by RIP-qPCR. ACTIN served as a control. Error bars represent means ± SD of three repeated experiments. The schematic of FgSTE12 is shown at the top. The black solid line denotes the binding peak identified in RIP-seq.

g EMSA confirmation of the direct binding of FgRbp1 to the in vitro transcribed mRNAs containing the peak sequence of FgSTE12. The in vitro transcribed mRNA sequence is identical to f. The deletion of the CAAGA motif in the peak fragment resulted in the decreased intron splicing ratio of two introns in FgSTE12. The strain FgSTE12-WT contains the CAAGA motif in the peak fragment, while the strain FgSTE12-Mu was deleted with the CAAGA motif. In e and h, bars represent means ± SD (n = 3 biologically independent replicates). Different letters indicate a significant difference (P < 0.05) according to the two-tailed unpaired Student’s t test.
regulating pre-mRNA splicing of the transcription factor FgSTE12.

Discussion

Splicing of mRNA is an essential process in eukaryotic gene expression. Recognition of the splicing signals and removal of introns in pre-mRNA are largely carried out by the spliceosome. Although components of splicingosomal complexes (e.g., E, A, B, and C) have been well established in the model organism S. cerevisiae, new associated factors are reported occasionally in various eukaryotes. In F. graminearum, the highly conserved spliceosome component Prp4 regulates pre-mRNA splicing likely by phosphorylation of other components of the U4/U6-U5 tri-snRNP (37). However, Prp4 ortholog is absent in all sequenced Saccharomycotina species, except Yarrowia lipolytica. Because Y. lipolytica has over 1500 introns in its genome, and S. cerevisiae only 376 introns, it is reasonable to speculate that Saccharomyces species may have lost the Prp4 ortholog after going through massive intron loss during evolution (38). In this study, we characterize the RRM-containing protein FgRbp1 in F. graminearum and showed that FgRbp1 interacts with an essential splicing factor FgU2AF23 in the nucleus. Deletion of FgRbp1 does not block intron splicing but affects intron splicing efficiency in 47% (4849 out of 10268) of the intron-containing genes in F. graminearum. Significantly, the human RBM42 protein is able to completely restore the growth defects in AfRbp1. Similar to Prp4, FgRbp1 ortholog is found in diverse eukaryotes, but not in S. cerevisiae. These results indicate that there is a greater level of complexity associated with the regululatory mechanism of pre-mRNA splicing in diverse eukaryotes than currently outlined in the budding yeast.

In human, the FgRbp1 ortholog RBM42 was first identified as a heterogeneous nuclear ribonucleoprotein K (hnRNPK) -binding protein playing a role in the maintenance of cellular ATP level under stress by protecting their target mRNAs (40). Proteomic assays later revealed that RBM42 may be an integral component of the B complex (41), U4/U6.U5 tri-snRNP (42) and pre-B complex (43), but the role it plays in these complexes is still unknown. More recently, RBM42 has been verified as a component of human tri-snRNP and pre-B spliceosome by cryo-electron microscopy (cryo-EM) (44). In human tri-snRNP, RBM42 contributes to stabilizing the quasi-pseudoknot (a structure formed by the U4 snRNA nucleotides 63 to 67) by binding to the U4 nucleotides 68 to 70th via the RRM domain. The quasi-pseudoknot brings U6 snRNA nucleotides A36 and A47 closer to enable the U6 pseudo-knot, thereby having a direct role in regulating global pre-mRNA splicing of many non-target genes. Thus, the splicing of these non-target genes are indirectly regulated by FgRbp1. More importantly, FgRbp1 directly binds and regulates the splicing of 68 transcripts coding for ribosomal proteins as demonstrated in our results (Fig. 5; Supplementary Data 3, 4). The mis-splicing of these ribosomal proteins is likely to lead to a decline in the overall translation ability in cells, subsequently affecting splicing of a wide range of genes.

In current study, we identified a cis element CAAGR in RIP-seq data and confirmed that FgRbp1 directly binds to this motif via EMSA. However, not all peak sequences of target genes contain the CAAGR motif. Among the 219 FgRbp1 target mRNAs identified by RIP assay, 165 mRNAs contained this motif, while the remaining 54 mRNAs (including FgMCM1) did not (Supplementary Data 4a). Therefore, we can presume that FgRbp1 resort to other affinity mechanisms for binding to these CAAGR-lacking genes, perhaps via other cis elements or specific RNA secondary structures. We found that most CAAGR binding sites are located within the exons around the intron, with a proportion of sequences located in 3′-UTR (like FgMCM1 and FgGPA1). Theoretically, splicing regulatory elements may function at any location in a pre-mRNA, but most studies focused on 200–300 nucleotides adjacent to the observed splice sites with the most identifiable sequence features (45). In this study, we found that the CAAGR motif (CCAGAAGACCTCAAGA) located in exon2 (127 nt downstream of the adjacent intron1) that bound to FgRbp1 is responsible for the splicing of intron1 (Fig. 5, f, g). This is consistent with the intron retention (IR) regulation observed, in human, i.e., specific RNA binding proteins may modulate the level of particular IR events by binding specific motifs in introns or their flanking exons (46). Several studies showed that remote regulatory sequences (e.g., 3′-UTR) are also important to the splicing outcome via forming RNA secondary structures (47,48). Further investigation is needed to gain clearer understanding of how FgRbp1 regulates splicing via remote regulatory sequences.

U2AF23, the small subunit of U2AF heterodimer, is conserved among diverse eukaryotes, except in S. cerevisiae. Interestingly, S. cerevisiae does not contain an ortholog of FgRbp1. In this study, we identified FgRbp1 as an additional interacting partner of the U2AF small subunit (Fig. 2). The amino acid sequence conservation between FgU2AF23 and FgRbp1 suggests that the interaction of the two proteins may be retained in other organisms. Indeed, the RBM42 protein (human counterpart of FgRbp1) interacts with FgU2AF23 in Y2H assay (Supplementary Fig. 9). Here, we provided evidence in vitro that FgRbp1 enhances the recruitment of FgU2AF23 to its target mRNA FgRPS20, and this recruitment is dependent on the interaction between the two proteins (Fig. 5h). Furthermore, by comparing the degree of splicing defects between the intron-retention genes excluded from RIP target genes (4679 genes) (49) and those overlapped with RIP target genes (170 genes) (49) (Fig. 5h), we found that the extent of splicing defects is more severe in the latter (Supplementary Fig. 10). This suggests that binding of FgRbp1 to target mRNAs presumably improves the splicing efficiency of its substrate mRNAs. Therefore, FgRbp1 may recruit FgU2AF23 to the 3′-splice site of its target gene by directly binding to its target mRNA, thereby promoting the early assembly of spliceosome and contributing to elevated splicing efficiency of target mRNAs. This is similar to the splicing regulation mechanism in mammals.
where some non-snRNP splicing regulators (such as SR protein, YB-1, SAM68) can promote exon inclusion or intron splicing by recruiting U1 snRNP to the 5′ splice site or U2AF to the 3′ splice site in early steps of spliceosome assembly.

Splicing errors resulting from either defects of key splicing factors or mutations of splicing regulatory sequences lead to aberrant splicing of multiple transcripts and contribute to various diseases in humans. Similarly, ΔFgRbp1 mutant exhibited various defects in hyphal growth, sexual development, and pathogenicity. The pathogenicity is meticulously regulated by various pathways, and in *F. graminearum* the Gpmk1 MAPK signaling pathway is involved in regulating pathogenicity via controlling the formation of penetration structure and secretion of plant cell wall degrading enzymes. All in this pathway, the deletion mutants of the upstream sensor FgSbo1, the MAPK module FgSte50-Stel1-Ste7-Gpmk1, and the downstream transcription factor FgSte12 are unable to penetrate the cellophane and plant.

Significantly, all these genes exhibited splicing defects in ΔFgRbp1 while the transcription levels of these genes did not alter (Supplementary Data 3c). In addition, it is worth noting that FgRbp1 influences splicing of DON biosynthesis genes (TRI1, TRI4, TRI5, TRI10), thus significantly reducing DON levels in ΔFgRbp1 compared with that in the wild type (Supplementary Fig. 11). Therefore, we conclude that the collective splicing defects in pathogenicity related genes contribute to the loss of virulence in ΔFgRbp1 on host plant.

**Methods**

**Fungal strains and culture conditions.** *Fusarium graminearum* strain PH-1 (NRRL31084) was used as the wild-type strain for constructing gene-deletion mutants. Fungal strains were cultured at 25 °C on potato dextrose agar (PDA), complete medium (CM) or minimal medium (MM) for mycelial growth tests. The carboxymethyl cellulose (CMC) liquid medium was used for conidiation assays. For trichothecene production analysis, each strain was grown in liquid trichothecene biosynthesis inducing (TBI) medium. For RNA and protein extraction, vegetative hyphae were harvested from yeast extract peptone dextrose (YPD, 1% yeast extract, 2% peptone, 2% glucose) liquid medium. Each experiment was repeated three times.

**Construction of gene deletion and complementation mutants.** Mutant alleles for targeted gene deletion were generated using the double-joint (DJ) PCR method. In brief, the 5′ and 3′ flanking regions of each gene were amplified using primers listed in Supplementary Data 5 and the flanking sequences were then fused with the hygromycin-resistance gene cassette (HPH). The transformation of *F. graminearum* was carried out via polyethylene glycol (PEG) mediated protoplast transformation method.

To obtain *F. graminearum* protoplasts, the fresh mycelia were treated with 30 mg driselase (D9515, Sigma, St. Louis, MO), 200 mg lysozyme (RM1027, YRON, Shanghai, China), and 200 mg cellulose (RM1030, YRON, Shanghai, China). Putative gene-deletion mutants were identified by PCR assays with relevant primers (Supplementary Data 5) and further confirmed by Southern hybridization. For complementation, vectors containing target ORF fused with a geneticin-resistance gene were transformed into corresponding strains. For Co-IP assays, total proteins were extracted and incubated with the GFP-Trap Agarose (ChromTek, Martinsried, Germany) at 4 °C for 2 h with rotation. After six times of washing, proteins eluted from agarose were analyzed by western blot detection with anti-RFP (ab65856, Abcam, Cambridge, UK, 1:10000 dilution) or anti-GAPDH antibody (EM1101, HuaAn Biotechnology, Hangzhou, China, 1:5000 dilution) as a reference. The intensity of immunoblot bands were quantified using the Image J software. Each experiment was repeated twice.

**Bimolecular fluorescence complementation (BiFC) assays.** To produce constructs for BiFC, the full-length FgRbp1 was amplified and cloned into pH2ES vector that carries NYPF and the hygromycin-resistance marker to generate the FgRbp1-NYPF fusion construct as described. Using the same approach, the FgU2AF23-CYPF fusion construct was generated by cloning the full-length FgU2AF23 into pH2ES vector that carries CYPF and the zeocin-resistance marker. Both FgRbp1-NYPF and FgU2AF23-CYPF constructs were verified by sequencing and then transformed in pair into PH-1::H1-RFP strain (a strain that expressed RFP-tagged histone 1 protein in PH-1 to visualize nuclear part). Construct pairs of FgRbp1-NYPF and CYPF, FgU2AF23-CYPF and NYPF were also co-transformed into PH-1::H1-RFP strain to serve as negative controls. Transformsants resistant to both hygromycin and neomycin were isolated and control signals in the mycelia were examined under a Zeiss LSM780 confocal microscope (Göttingen, Niedersachsen, Germany).

**RNA-seq analysis.** To harvest mycelial samples for RNA-seq, 1 ml of conidial suspension (10⁶ conidia/ml) of each strain was inoculated and incubated in 200 ml YEPD liquid medium with agitation (180 rpm) 12 h at 25 °C. Total RNA was isolated from the harvested mycelia with the TRIzol Reagent (Life technologies, US) and shipped to Shino-tech Genomics Corporation (Shanghai, China) for library construction and sequencing with Illumina HiSeq Xv2 sequencer. Three biological replicates for each strain were sequenced. For each sample, 30–50 M clean reads were obtained and mapped to the *F. graminearum* PH-1 genome sequence using Hisat2. For comparison and visualization of splicing efficiency, intron retention rate was determined by calculating the ratio of introns FPKM (Fragments Per Kilobase Million) to the gene’s overall exon FPKM for each tested intron. To filter out weakly expressed genes, only genes with a minimum expression level of 1 count per million were included according to the previous RNA-seq results in *F. graminearum*. The intron retention was termed as FgRbp1::H1-RFP strain (a strain that expressed RFP-tagged histone 1 protein in PH-1 to visualize nuclear part). Construct pairs of FgRbp1-NYPF and CYPF, FgU2AF23-CYPF and NYPF were also co-transformed into PH-1::H1-RFP strain to serve as negative controls. Transformsants resistant to both hygromycin and neomycin were isolated and control signals in the mycelia were examined under a Zeiss LSM780 confocal microscope (Göttingen, Niedersachsen, Germany).
nitrogen and suspended with 4 mL lysis buffer (50 mM Tris- HCl [pH 7.5], 150 mM KC1, 2 mM EDTA, 0.5% NP-40, 0.5 mM dithiothreitol, 1:100 v/v protease inhibitor cocktail (Roche)). Electrophoretic mobility shift assay was carried out using the method of Kao et al. (14). In brief, the hsp90 target DNA, which is a 200-bp cloned fragment, was labeled on one end with biotin using Biotin RNA Labeling Mix (Roche) and transcribed with T7 polymerase (Takara) and Biotin RNA Labeling Mix (Roche). Denatured DNA was annealed to hsp90 cDNA fragment at 95 °C for 5 min, followed by incubation at 4 °C for 30 min. After annealing, the fragments were incubated with 20 μg of sonicated nuclear extract protein from F. graminearum strain N, FgU2AF23-His, or pairs of proteins: GST-FgRbp1, GST-FgRbp1ΔRRM, GST-FgRbp1ΔΔRRM, or GST-FgRbp1ΔΔRRM. GST-FgRbp1 mutants in detail, hyphal structures from infected wheat spikelets at 7 d post inoculation (dpi) were examined with a Hitachi TM-1000 tabletop microscope (Hitachi, Tokyo, Japan). The penetration behavior of each strain was examined on cellobiose membranes. Briefly, each strain was inoculated onto the cellobiose-covered MM medium. After incubation for 3 days at 25 °C, the cellobiose together with the colonies of each strain on the cellobiose were removed. Then, the plates were incubated for three additional days to examine for hyphal growth. The experiment was repeated three times. To determine the DON production, each strain was grown in TBI liquid medium at 28 °C for 7 days in a shaker (150 rpm) in the dark. Then, the DON production for each sample was extracted and quantified by using a DON Quantification Kit Wi5008 (Wi5 Science, Zhenjiang, China).

Statistics and reproducibility. Representative images from three independent experiments are presented in Figs. 2a, d-f, 4b, 7d, and Supplementary figures 5, 7. Electrophoretic mobility shift assay analysis and representative images are shown in Figs. 4c, 5e, 6d, 7g. RNA affinity selection assay was performed three times, and representative images are shown in Supplementary figures 2b and c.

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Measurement of splicing ratio. Total RNAs were extracted from fresh mycelia and treated with DNase to remove residual DNA before reverse transcription. Quantitative real-time PCR was performed to measure RNA levels with three biological repeats. Measurement of splicing ratio was conducted using the previously described protocol (22). In brief, the splicing ratio was calculated by determining the level of spliced RNA normalized to the level of unspliced RNA for each intron tested using the equation 2(Ct(mock-input) - Ct(input sample antibody)) / 2(Ct(mock-input) - Ct(IgG1)). The splicing ratio was calculated as 1.0. The RNA-seq data, RIP-seq data has been deposited in the NCBI SRA database with accession codes PRJNA556958 and PRJNA556748, respectively. All relevant data are available from the corresponding authors upon any reasonable request. Source data are provided with this paper.

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