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ORIGINAL ARTICLE

The SR-protein FgSrp2 regulates vegetative growth, sexual reproduction and pre-mRNA processing by interacting with FgSrp1 in *Fusarium graminearum*

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Abstract

Serine/arginine (SR) proteins play significant roles in pre-mRNA splicing in eukaryotes. To investigate how gene expression influences fungal development and pathogenicity in *Fusarium graminearum*, a causal agent of Fusarium head blight (FHB) of wheat and barley, our previous study identified a SR protein FgSrp1 in *F. graminearum*, and showed that it is important for conidiation, plant infection and pre-mRNA processing. In this study, we identified another SR protein FgSrp2 in *F. graminearum*, which is orthologous to *Schizosaccharomyces pombe* Srp2. Our data showed that, whereas yeast Srp2 is essential for growth, deletion of *FgSRP2* resulted in only slight defects in vegetative growth and perithecia melanization. FgSrp2 localized to the nucleus and both its N- and C-terminal regions were important for the localization to the nucleus. FgSrp2 interacted with FgSrp1 to form a complex in vivo. Double deletion of *FgSRP1* and *FgSRP2* revealed that they had overlapping functions in vegetative growth and sexual reproduction. RNA-seq analysis revealed that, although deletion of *FgSRP2* alone had minimal effects, deletion of both *FgSRP1* and *FgSRP2* caused significant changes in gene transcription and RNA splicing. Overall, our results indicated that FgSrp2 regulates vegetative growth, sexual reproduction and pre-mRNA processing by interacting with FgSrp1.

Keywords SR protein · *Fusarium graminearum* · Alternative splicing · RNA splicing · *NPL3*

Introduction

Fusarium head blight (FHB), caused by the ascomycete fungal pathogen *Fusarium graminearum,* is a devastating disease worldwide on cereals, such as wheat and barley (Bai and Shaner 2004; Goswami and Kistler 2004). In addition to reducing grain yield, FHB causes a food safety risk and health hazard to humans and animals due to the contamination of food and feed with the *F. graminearum* trichothecene toxin deoxynivalenol (DON) (Goswami and Kistler 2004). For these reasons, *F. graminearum* is considered to be one of the most important fungal plant pathogens in the world (Dean et al. 2012). To develop new approaches to control FHB, the cellular and molecular bases of pathogenicity in *F. graminearum* have been extensively studied during the last decade by using approaches of genomics, transcriptomics, and reverse genetics (Jia and Tang 2015; Kazan and Gardiner 2018; Kim, et al. 2015b; Liu, et al. 2016; Son, et al. 2017; Chen et al. 2019; Li et al. 2019; Lv et al. 2019; Xu et al. 2019; Yang et al. 2018; Zhang et al. 2019). These studies totally contribute to our knowledge of the key factors

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Yimei Zhang and Yafeng Dai contributed equally to this work.

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and regulation of hyphal growth, development, pathogenicity and secondary metabolism in *F. graminearum*.

In eukaryotic cells, the splicing of precursor messenger RNAs (pre-mRNAs) is an essential step of gene expression. Pre-mRNA splicing is carried out by the spliceosome, a large dynamic RNA–protein complex that recognizes splicing signals and catalyzes the removal of intronic sequences (introns) to assemble exonic sequences (exons) into mature mRNA (Black 2003). Pre-mRNA splicing is mainly regulated by two families of splicing factors: heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine/arginine-rich (SR) proteins. Whereas hnRNPs commonly act as splicing repressors, most SR proteins function as splicing activators via binding pre-mRNA at exonic splicing enhancers to facilitate exon recognition by the spliceosome (Busch and Hertel 2012; Zhou and Fu 2013). SR proteins play significant roles in constitutive RNA splicing and are also important regulators of alternative splicing (AS) (Erkelenz et al. 2013; Zhou and Fu 2013). In addition, SR proteins are involved in mRNA export, mRNA decay and translation (Huang and Steitz 2001, 2005; Huang et al. 2004).

Typical SR proteins have a domain organization containing one or two RNA recognition motifs (RRMs) at the N-terminus and an arginine (R)-rich region at the C-terminus (Plass et al. 2008). The RRM domain provides RNA-binding specificity while the R-rich region mediates protein–protein interactions that facilitate recruitment of the spliceosome (Long and Caceres 2009). The R-rich region also acts as a nuclear localization signal (NLS) to affect the subcellular localization of SR proteins (Long and Caceres 2009). SR proteins have a variable content of arginine/serine (RS) repeats in the R-rich regions. The density of RS repeats in these regions correlates with the conservation of the branch site signal (Plass et al. 2008). In metazoans, the R-rich regions of SR proteins display a high density of RS repeats, whereas in fungi these regions contain RX repeats, where X can be S (serine), D (aspartic acid), E (glutamic acid) or G (glycine) (Plass et al. 2008).

The number of SR protein family members correlates with the complexity of AS in eukaryotes. Plants and metazoans have a large number of SR proteins, whereas fungi generally contain 1–3 SR proteins (Plass et al. 2008). For example, 12 SR proteins (SRSF1-12) have been identified in human (Busch and Hertel 2012). Only two SR proteins Srp1 and Srp2 have been identified in fission yeast *Schizosaccharomyces pombe* (Tang et al. 2002). Although three SR-like proteins have been identified in budding yeast *Saccharomyces cerevisiae*, only one of those, Npl3, promotes pre-mRNA splicing (Kress et al. 2008).

In filamentous fungi, SR proteins have not been thoroughly characterized. *swoK*, an ortholog of *S. pombe srp1*, has been shown to be important for cell polarity in *Aspergillus nidulans* (Shaw and Upadhyay 2005), but its function in pre-mRNA splicing is unknown. Recently, we identified *FgSRP1,* the ortholog of *srp1,* in *F. graminearum* and revealed that it is important for conidiation, plant infection and pre-mRNA processing (Zhang et al. 2017). In this study, we identified and characterized another SR protein-encoding gene *FgSRP2* (FGRAMPH1_01G27477 or FGSG_09282) in *F. graminearum*, which is orthologous to *S. pombe srp1* and *S. cerevisiae NPL3*. The relationship of the *FgSRP2* and *FgSRP1* was determined, and we performed strand-specific RNA-seq analysis to reveal their roles in gene transcription and RNA splicing.

Materials and methods

Strains and growth conditions

The wild-type *F. graminearum* strain PH-1 (Cuomo et al. 2007) and all transformants used in this study were routinely cultured on PDA plates at 25 °C (Wang et al. 2011). Potato dextrose agar (PDA) plates were used to assay growth rate and colony morphology at 25 °C for three days as described (Hou et al. 2002; Zhou et al. 2010). Conidiation was measured in 5-day-old cultures grown in liquid carboxy-methylcellulose (CMC) medium (Hou et al. 2002), and sexual reproduction on carrot agar plates was assayed as previously described (Wang et al. 2011; Zheng et al. 2013). Protoplasts were prepared and used for PEG-mediated transformation (Hou et al. 2002) with a final concentration of 300 μg/ml hygromycin B (CalBiochem, La Jolla, CA, USA) or 400 μg/ml geneticin (Sigma, St. Louis, MO, USA) for selection. DON production was assayed with rice grain cultures (Seo et al. 1996). For assaying sensitivities to various stresses, vegetative growth was assayed on CM plates supplemented with 0.05% H_2O_2, 0.7 M NaCl, 300 μg/ml Congo red, or 0.01% SDS for 2 days (Wang et al. 2011).

Generation of *Fgsrp2* deletion mutants

The split marker method was used to generate the *FgSRP2* replacement constructs (Catlett et al. 2003). The upstream and downstream flanking fragments were amplified with the primer pairs FgSRP2/1F-2R and FgSRP2/3F-4R, respectively, and fused with hygromycin phosphotransferase (hph) fragments amplified with the primers HYG/F-YG/R and YG/F-HYG/R from pCB1003 plasmid. All primers used for PCR are listed in Table S1. The resulting two fused fragments were co-transformed into the protoplasts of PH-1 as described (Wang et al. 2011). Putative *Fgsrp2* deletion mutants were confirmed by PCR as described (Wang et al. 2011).

Generation of transformants expressing the wild-type and mutant alleles of *FgSRP1*

For complementation assays, a 2.3-kb fragment of *FgSRP2* containing the entire ORF and 0.8-kb native promoter amplified with primers FgSRP2hb/F and FgSRP2hb/R (Table S1) was co-transformed with XhoI-digested pFL2 (carrying the geneticin resistance marker) into yeast strain XK1-25 as described (Bruno et al. 2004). The resulting *FgSRP2*-GFP construct was transformed into *Fgsrp2* deletion mutant M4. Transformants resistant to both hygromycin and geneticin were verified by PCR and examined for GFP signals with an Olympus BX-51 epifluorescence microscope (Olympus, Tokyo, Japan).

The same approach was used to generate GFP fusion constructs of *FgSRP1* mutant alleles. The N-terminal (1–174aa) and C-terminal (175–312aa) regions of *FgSRP2* together with the native promoter were amplified and cloned into pFL2 to generate the *FgSRP2*$^{\Delta C}$- and *FgSRP2*$^{\Delta N}$-GFP fusion constructs. The deletion of NLS (186-193aa) was introduced into *FgSRP2* by overlapping PCR with primers listed in Table S1. All the mutant constructs of *FgSRP2* rescued from Trp^{+} yeast transformants were verified by sequencing and transformed into the *Fgsrp2* mutant M4 or wild type PH-1 (Table 1).

Plant infection assays

For plant infection assays, conidia harvested from 5-day-old CMC cultures were resuspended to 10^5 spores/ml in sterile water. Flowering wheat heads of cultivar Xiaoyan 22 were inoculated with 10 µl of conidium suspensions in the fifth spikelet from the base of the inflorescence as described (Gale et al. 2007). Wheat spikelets with typical head blight symptoms on each head were examined 14 days post-inoculation (dpi) and disease indices were estimated as described (Chen et al. 2014).

qRT-PCR assays

For assaying the expression of *FgSRP1* and *FgSRP2* genes, RNA samples were isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from vegetative hyphae harvested from YEPD cultures. The ReverAid First cDNA synthesis kit (Thermo Fisher Scientific) was used for cDNA synthesis. Relative expression levels of each gene were assayed by qRT-PCR with the β2-tubulin gene (Zhao et al. 2014) as the internal control and calculated by the $2^{-\Delta\Delta Ct}$ method. Data from three biological replicates were used to estimate the mean and standard deviation.

Bimolecular fluorescence complementation (BiFC) assays

To generate the *FgSRP2*-CYFP fusion construct, the *FgSRP2* fragment was amplified with primer pairs FgSRP2PHZ68/F and FgSRP2PHZ68/R (Table S1) and co-transformed with the XhoI-digested pHZ68 vector that carries CYFP and Zeocin resistance marker (Invitrogen, Lot No. 1771594) as described (Zhao and Xu 2007). The *FgSRP1*-NYFP fusion construct with pHZ65 vector carrying hygromycin B resistance marker was generated with the same approach. All the fusion constructs were confirmed by PCR and sequence analysis. *FgSRP1*-NYFP was co-transformed with *FgSRP2*-CYFP into the wild-type PH-1. The resulting transformants were identified by PCR and examined for YFP signals.

Table 1 Wild-type and transformants of *Fusarium graminearum* strains used in this study

Strain	Brief description	References
PH-1	Wild type	Cuomo et al. (2007)
S47	Suppressor mutant of *Fgprp4* deletion mutant	Gao et al. (2016)
M5	*Fgsrp1* deletion mutant of PH-1	Zhang et al. (2017)
M2	*Fgsrk1* deletion mutant of PH-1	Wang et al. (2018)
M4	*Fgsrp1* deletion mutant of PH-1	This study
DK2	*Fgsrp1srp2* deletion mutant of PH-1	This study
C1	*Fgsrp2*/*FgSRP2*-GFP transformant of M4	This study
NC1	WT/*FgSRP2*$^{\Delta C}$-GFP transformant of PH-1	This study
CC1	*Fgsrp2*/*FgSRP2*$^{\Delta N}$-GFP transformant of M4	This study
NLS5	*Fgsrp2*/ *FgSRP2*$^{\Delta NLS}$-GFP transformant of M4	This study
SP3	*Fgprp4*/*FgSRP2*-GFP transformant of S47	This study
SD5	*Fgsrk1*/*FgSRP2*-GFP transformant of M2	This study
CSR3	*FgSRP1*-mCherry and *FgSRP2*-GFP transformant of PH-1	This study
BSR7	*FgSRP1*-YFPN and *FgSRP2*-YFPC transformant of PH-1	This study
WSR8	*FgSRP1*-3xFLAG and *FgSRP2*-GFP transformant of PH-1	This study

Co-immunoprecipitation (co-IP) assays

To generate the *FgSRP1*-3×FLAG construct, *FgSRP1* gene was amplified and cloned into pFL7 (Liu et al. 2011) by the yeast gap repair approach (Bruno et al. 2004). The *FgSRP2*-GFP fusion construct was generated with pFL2 using the same approach. The resulting constructs were verified by sequence analysis and transformed in pairs into strain PH-1. Transformants expressing both GFP and FLAG were identified by PCR and western blot analysis. For co-immunoprecipitation assays, total proteins were isolated and incubated with anti-GFP beads, then detected with the anti-GFP (Roche, Indianapolis, IN), anti-FLAG, and anti-histone H3 (Sigma-Aldrich) antibodies as described (Liu et al. 2015).

RNA-seq analysis

Vegetative hyphae of PH-1 and *Fgsrp2* and *Fgsrp1srp2* mutants were harvested from 16-h YEPD cultures. Total RNA samples were extracted with the Qiagen RNeasy Micro kit and poly(A) + mRNA was enriched with immobilized oligo (dT) as described (Liu et al. 2017). Two biological replicates were prepared for each strain. Strand-specific RNA-seq libraries were prepared with the NEBNext® Ultra™ Directional RNA Library Prep Kit and sequenced with Illumina HiSeq-2500 with a 2×150 bp paired-end read mode at the Novogene Bioinformatics Institute (Beijing, China). For each library, at least 20 Mb of paired end reads were obtained. RNA-seq data were deposited at NCBI SRA database under Accession Nos. SRR10256958 to SRR10256963.

The reference genomic sequence and gene annotation (version 44) of *F. graminearum* strain PH-1 (Cuomo et al. 2007; King et al. 2015) were downloaded from Ensembl Fungi (https://fungi.ensembl.org/Fusarium_graminearum/Info/Index). RNA-seq reads were mapped onto the reference genome using HISAT2 (Kim et al. 2015a). The number of reads aligned to each predicted gene was calculated by FeatureCounts (Liao et al. 2014). Differential expression analysis of genes was performed with edgeRun (Dimont et al. 2015) according to previous methods (Zhang et al. 2017). Genes with an FDR below 0.05 and $\log_2$ fold-change at least 1 were considered to be differentially expressed genes. Differential AS events were detected using CASH v2.2.1 (Wu et al. 2018). We assembled transcripts from the RNA-seq mappings of all samples using StringTie (Pertea et al. 2015), and merged the assembled transcripts with the reference gene annotation. The merged transcript GTF file was then used as the input for running CASH.

Results

Identification of *FgSRP2* in *F. graminearum*

A BLAST search of the predicted protein database of *F. graminearum* at Ensembl Fungi (https://fungi.ensembl.org/Fusarium_graminearum/Info/Index) using the *S. pombe* Srp2 sequence revealed only one protein (FGRAMPH1_01G27477) similar to Srp2 with an E-value below 1e−5. Using FGRAMPH1_01G27477 sequence to search the *S. pombe* protein database confirmed that they were reciprocal best BLAST hits. We identified the reciprocal best hits of Srp2 from other representative ascomycete fungi, including *S. cerevisiae* and performed a phylogenetic analysis to determine their relationships. The resulting phylogenetic tree of Srp2 homolog sequences was in accord with that of the species (Fig. 1a), confirming the orthologous relationship of FGRAMPH1_01G27477 to the *S. pombe srp2* and *S. cerevisiae NPL3*. Based on these results, we named FGRAMPH1_01G27477 as *FgSRP2*.

FgSRP2 encodes a protein with a domain structure similar to that of *S. pombe* Srp1 and *S. cerevisiae* Npl3: two RRM (RNA recognition motif) domains followed by a long arginine (R)-rich region (Fig. 1b; Fig. S1). BLASTp searches revealed that FgSrp2 had 58% and 49% sequence similarity to that of Srp1 and Npl3, respectively, in the RRM domain regions. According to our previous RNA-seq data (Jiang et al. 2019; Liu et al. 2016), *FgSRP2* was expressed in conidia, vegetative hyphae, infectious hyphae and during sexual reproduction, but the expression level was highest in vegetative hyphae (Fig. 1c).

FgSRP2 is required for vegetative growth but dispensable for infectious growth

To determine its function in *F. graminearum*, we generated the *Fgsrp2* deletion mutant by the split-marker approach (Catlett et al. 2003). Nine *Fgsrp2* deletion mutants were identified by PCR. Because all the mutants had the same phenotype, only data for one (M4) (Table 1) is presented below. Compared with the wild-type strain PH-1, the *Fgsrp2* mutant was reduced approximately 11% in growth rate on PDA plates, although it grew slightly faster than the *Fgsrp1* mutant (Fig. 2a; Table 2). The defects of the mutant in response to various stress treatments were also examined, and the results showed that the *Fgsrp2* mutant displayed increased sensitivity to H_2O_2, sodium dodecyl sulfate (SDS) and Congo Red (Fig. 2b,c), indicating that *FgSRP2* also plays a role in response to oxidative stress and cell membrane and cell wall integrity stress.

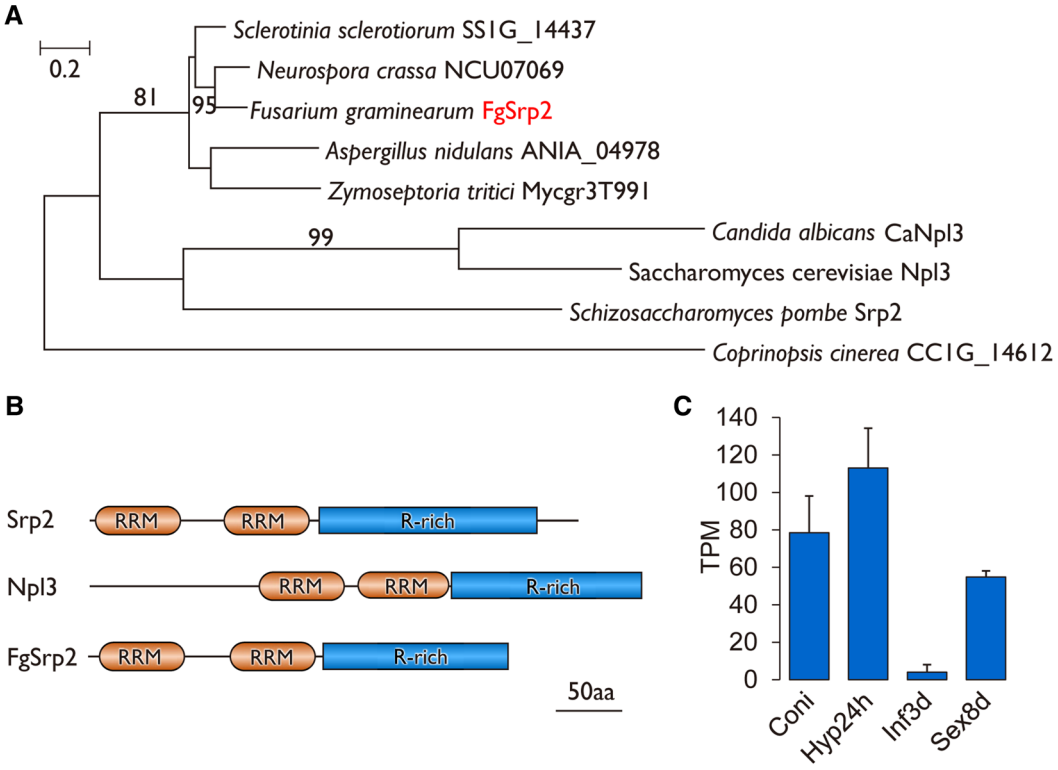

Fig. 1 Identification of Srp2 ortholog in *F. graminearum*. **a** The maximum likelihood tree of Srp2 orthologs. The phylogenetic tree was constructed using PhyML 3.1 (Guindon et al. 2009) with the full-length sequences of proteins. Numbers on branches indicate SH-like approximate likelihood ratio test (SH-aLRT) probabilities (only values > 50% are indicated). Scale bar corresponds to 0.2 amino acid substitutions per site. **b** Comparison of domain structures of *F. graminearum* FgSrp2, *S. cerevisiae* Npl3, and *S. pombe* Srp2. RRM, RNA recognition motif domain; R-rich, arginine-rich region. **c** The expression level (Transcripts Per Kilobase Million, TPM) of *FgSRP2* estimated with RNA-seq data of conidia (Coni), 24-h hyphae (Hyp24h), infected wheat heads at 3 dpi (Inf3d), and perithecia collected at 8 dpf (Sex8d). Error bars indicate standard deviation calculated from two or three biological replicates of RNA-seq data

In infection assays with flowering wheat heads, the *Fgsrp2* mutant caused head blight symptoms in the inoculated florets and spread to other spikelets on the same heads similar to the wild type (Fig. 2d). At 14 days post-inoculation (dpi), the average disease index of *Fgsrp2* mutant was 11.5, comparable with that of PH-1 (11.3) (Table 2). We also assayed DON production in rice grain cultures. In comparison with the wild type, the DON production in the *Fgsrp2* deletion mutant was not reduced (Table 2), suggesting that deletion of *FgSRP2* does not affect DON biosynthesis in *F. graminearum*.

FgSRP2 is required for perithecium pigmentation

Since ascospores are the primary inoculum for epidemics of FHB, we also assayed sexual reproduction with the *Fgsrp2* deletion mutant on carrot agar plates as described (Liu et al. 2015). Similar to the *Fgsrp1* mutant, the *Fgsrp2* deletion mutant produced abundant perithecia, but the perithecia were not fully melanized in comparison with wild-type perithecia (Fig. 2e). However, in contrast to the *Fgsrp1* mutant, which formed abnormal ascospores with two cells, *Fgsrp2* ascospores were morphologically normal (four-celled). Therefore, *FgSRP2* is required for perithecium pigmentation. In addition, whereas the *Fgsrp1* mutant rarely produced conidia, the *Fgsrp2* mutant was normal in conidiation (Table 2).

FgSrp2-GFP is localized to the nucleus

To determine the subcellular localization of *FgSRP2*, the *FgSRP2*^{WT}-GFP fusion construct was generated and transformed into the *Fgsrp2* deletion mutant. The resulting *Fgsrp2*/*FgSRP2*-GFP transformant had the wild-type phenotype (Fig. 2), indicating that fusion with GFP has no effect on FgSrp2 functions and that deletion of *FgSRP2* is directly responsible for the defects observed in the *Fgsrp2* mutant. When examined by epifluorescence microscopy, GFP signals were observed only in the nucleus in both conidia and hyphae (Fig. 3a). The localization of FgSrp2-GFP to the

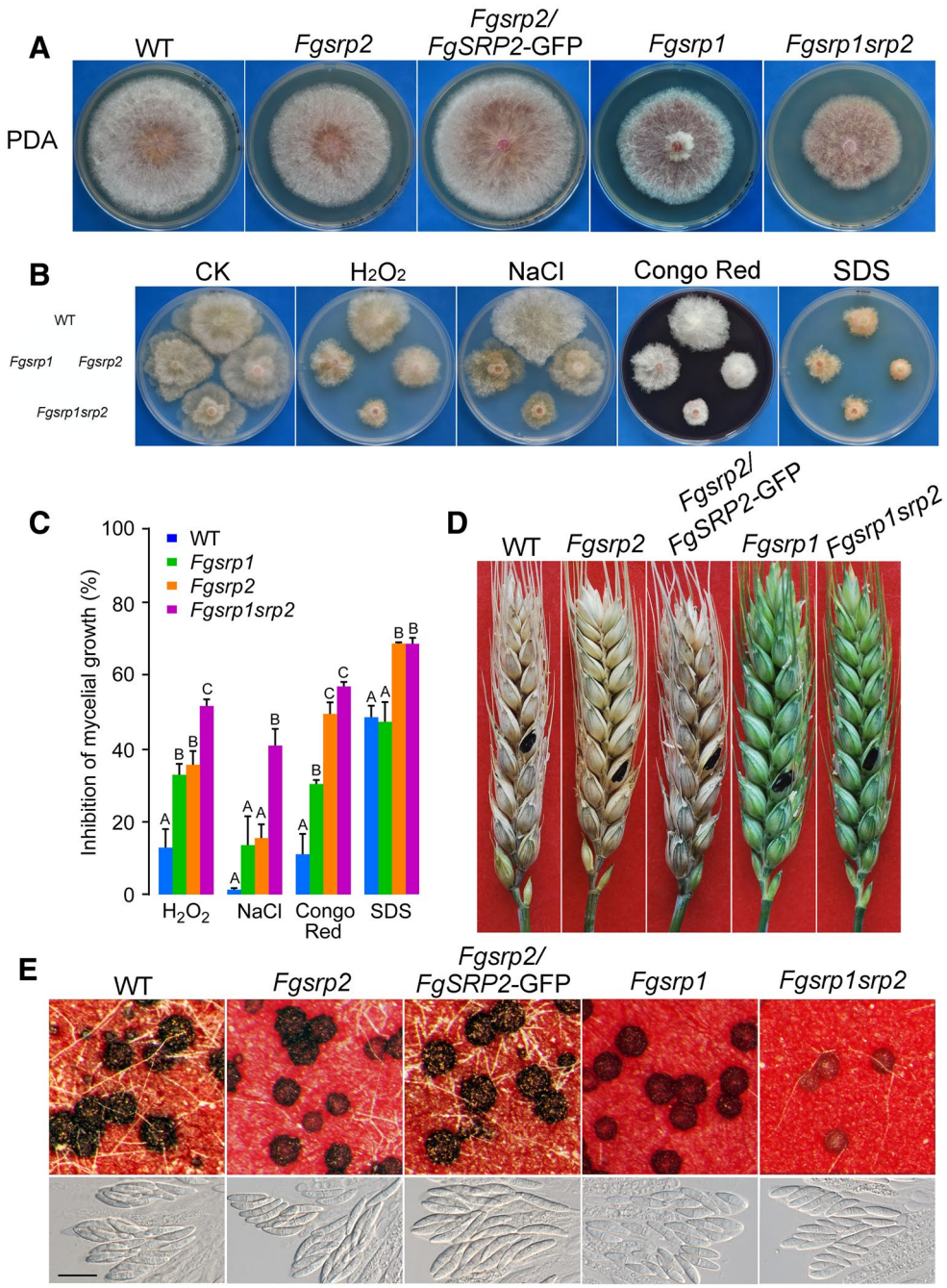

Fig. 2 Phenotypes of different mutants in growth, stress sensitivity, plant infection, and sexual reproduction. **a** The wild type (PH-1), single (*Fgsrp1* and *Fgsrp2*) and double (*Fgsrp1srp2*) deletion mutants, and complemented transformant (*Fgsrp2*/*FgSRP2*-GFP) were cultured on PDA plates for three days. **b** The same set of strains were cultured on CM plates with or without 0.05% H_2O_2, 0.7 M NaCl, 300 µg/ml Congo red, or 0.01% SDS for 2 days. C. Mean and standard deviation of mycelial growth inhibition of each strain under each treatment were estimated with data from three biological replicates. Different letters indicate significant differences based on ANOVA analysis followed by Tukey's HSD test ($P = 0.01$). D. Flowering wheat heads inoculated with the labeled strains were photographed 14 day-post-inoculation (dpi). Black dots mark the inoculated spikelet. *E. Perithecia*, ascus and ascospore on carrot agar cultures of the labeled strains were examined after 8 days post-fertilization. Bar = 20 µm

nucleus was confirmed by staining with 4, 6-diamidino-2-phenylindole (DAPI). Nuclear localization of FgSrp2 was consistent with its likely functions as a SR protein involved in RNA processing.

Table 2 Defects of strains in growth, conidiation, pathogenicity, and DON production

Strain	Growth rate[a] (mm/d)	Conidiation[b] ($\times 10^6$/ml)	Disease index[c]	DON[d] (ppm)
PH-1	12.5 ± 0.9^{A}	1.1 ± 0.1^{A}	11.3 ± 2.8^{A}	650.9 ± 99.2^{A}
M5 (*Fgsrp1*)	9.8 ± 1.0^{C}	Rare	0.6 ± 0.3^{B}	17.0 ± 4.2^{B}
M4 (*Fgsrp2*)	11.1 ± 1.7^{B}	1.2 ± 0.1^{A}	11.5 ± 1.9^{A}	654.8 ± 78.7^{A}
C1 (*Fgsrp2*/*FgSRP2*-GFP)	12.5 ± 0.3^{A}	1.2 ± 0.1^{A}	11.5 ± 1.3^{A}	738.7 ± 115.1^{A}
DK2 (*Fgsrp1srp2*)	8.7 ± 1.5^{D}	Rare	0.5 ± 0.7^{B}	13.4 ± 1.5^{B}

Data from three biological replicates were analyzed with the protected Fisher's least significant difference (LSD) test. The same letter indicated that there was no significant difference. Different letters were used to mark statistically significant difference ($P < 0.05$)

[a]Growth rate was assayed by measuring colony diameters on Φ9 cm PDA cultures

[b]Conidiation in 5-day-old CMC cultures

[c]Disease was rated by the number of symptomatic spikelets 14 days after inoculation. Mean and standard deviation were calculated with results from three independent repeats. At least 10 wheat heads were examined in each repeat

[d]DON production was measured with infected rice grain cultures

Both N- and C-terminal regions of FgSrp1 are important for its localization to the nucleus

We then generated the *FgSRP2*$^{\Delta N}$-GFP by deletion of the N-terminus (aa 1–174, containing the two RRM domains) and transformed it into the *Fgsrp2* deletion mutant. The resulting *Fgsrp2/FgSRP2*$^{\Delta N}$ transformant had normal growth and sexual reproduction as wild type (Fig. S2), suggesting that the N-terminus of FgSrp2 is dispensable for its function. However, when we generated the *FgSRP2*$^{\Delta C}$-GFP by deletion of the C-terminus (aa 175–312, containing the R-rich region) and transformed it into the *Fgsrp2* deletion mutant, we failed to identify real *Fgsrp2/FgSRP2*$^{\Delta C}$ transformant after screening over 60 transformants from three independent transformation experiments. It seems likely that expressing only the N-terminal region of FgSrp2 is lethal for *F. graminearum*. To assay its subcellular localization, we transformed the *FgSRP2*$^{\Delta C}$-GFP into wild-type PH-1 strain. The resulting WT/*FgSRP2*$^{\Delta N}$ transformant had normal growth and sexual reproduction as wild type (Fig. S2). When 6-h germlings were examined by epifluorescence microscopy, GFP signals were observed throughout the cell in the *Fgsrp2/FgSRP2*$^{\Delta N}$-GFP transformant, although they were significantly enriched in the nucleus (Fig. 3b). In the WT/*FgSRP2*$^{\Delta C}$-GFP transformant, however, GFP signals were evenly distributed in the nucleus and cytoplasm (Fig. 3b). These results indicate that both N- and C-terminal regions of FgSrp2 are important for its localization to the nucleus, and the R-rich C-terminal region plays a more important role for the nuclear localization.

FgSrp2 has one putative nuclear localization signal (NLS) (residues 186–193) in the R-rich region (Fig. S1), predicted by NLS tradamus (Nguyen Ba et al. 2009). To determine its function, the *FgSRP2*$^{\Delta NLS}$-GFP fusion construct deleted of residues 186–193 was generated and transformed into the *Fgsrp2* deletion mutant. The resulting *Fgsrp2/ FgSRP2*$^{\Delta NLS}$ transformant was normal in growth but only generated a few smaller perithecia without asci and ascospores (Fig. S2). Different from the *Fgsrp2*/*FgSRP2*-GFP transformant, weak GFP signals were also observed in the cytoplasm in the *Fgsrp1*/*FgSRP2*$^{\Delta NLS}$-GFP transformant (Fig. 3c). Therefore, although not essential, deletion of this predicted NLS in FgSrp2 affects the efficiency of its localization to the nucleus.

Deletion of Srk1 and FgPrp4 kinases does not affect the subcellular localization of FgSrp2-GFP protein

In *S. pombe*, Dsk1 is an SR protein-specific kinase that phosphorylates SR protein Srp2 and determines the subcellular localization (Tang et al. 2007; Wang et al. 2018). *PRP4* encodes the only protein kinase among all the spliceosome components and is an essential gene in eukaryotic organisms reported except *F. graminearum* (Gao et al. 2016). Out previous studies showed that Srk1, the ortholog of *S. pombe* Dsk1 interacts with the FgSrp1, and FgPrp4 interacts with FgSrp1 in *F. graminearum* (Wang et al. 2018; Zhang et al. 2017). To investigate whether the FgPrp4 and Srk1 determine the subcellular localization of FgSrp2, we transformed *FgSRP2*-GFP fusion construct into the *Fgprp4* and *srk1* deletion mutants, respectively. When examined by epifluorescence microscopy, GFP signals were observed only in the nucleus in 6-h germlings of both *Fgprp4* and *srk1* deletion mutants (Fig. 4). These results indicate that the subcellular localization of *FgSRP2* is not affected by FgPrp4 and Srk1 kinases in *F. graminearum*.

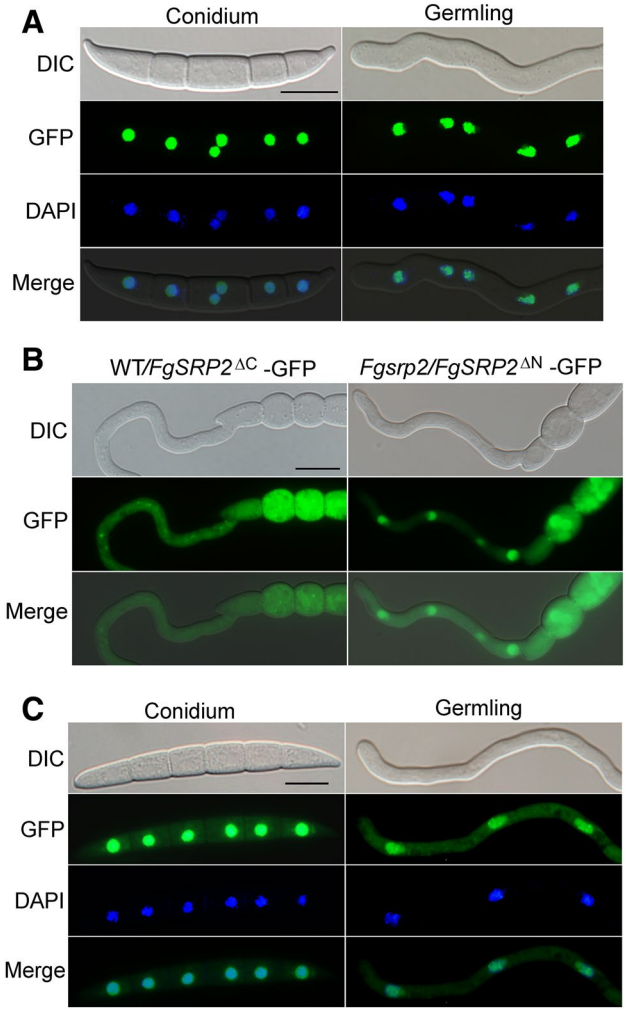

Fig. 3 Subcellular localization of the *FgSRP2*-, *FgSRP2*$^{\Delta C}$-, *FgSRP2*$^{\Delta N}$-, and *FgSRP2*$^{\Delta NLS}$-GFP fusion proteins. **a** Conidia and 6-h germlings of the *Fgsrp2*/*FgSRP2*-GFP transformant were stained with DAPI and examined by differential interference contrast (DIC) and epifluorescence microscopy. Bar = 10 μm. **b** Six-hour germlings of *FgSRP2*$^{\Delta C}$- and *FgSRP2*$^{\Delta N}$-GFP transformant were examined by DIC and epifluorescence microscopy. Bar = 10 μm. **c** Conidia and 6-h germlings of the *FgSRP2*$^{\Delta NLS}$-GFP transformant were stained with DAPI and examined by DIC and epifluorescence microscopy. Bar = 10 μm

FgSrp2 interacts with FgSrp1 to form a complex

To determine the co-localization of FgSrp2 and FgSrp1, we generated *FgSRP1*-mCherry and *FgSRP2*-GFP fusion constructs and co-transformed them into the wild-type PH-1. The resulting transformants expressed FgSrp1-mCherry and FgSrp2-GFP fusion proteins simultaneously. When examined by epifluorescence microscopy, both GFP and mCherry signals were observed in the nucleus in 6-h germlings (Fig. 5a). These results indicate that FgSrp1 and FgSrp2 co-localize to the nucleus.

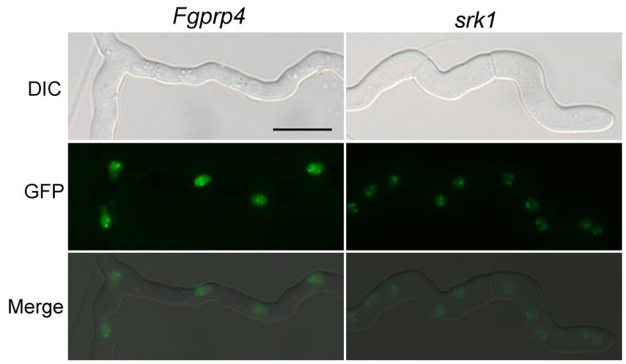

Fig. 4 Subcellular localization of the *FgSRP2*-GFP fusion proteins in the *Fgprp4* and *srk1* deletion mutants, respectively. Six-hour hyphae of *Fgprp4*/*FgSRP2*-GFP and *Fgdsk1*/*FgSRP2*-GFP transformants were examined by DIC and epifluorescence microscopy. Bar = 10 μm

In *S. pombe*, Srp1 and Srp2 interact with each other to form a complex (Tang et al. 2002). To determine the relationship between FgSrp1 and FgSrp2, we generated the *FgSRP1*-YFPN and *FgSRP2*-YFPC fusion constructs and co-transformed them into the wild-type strain PH-1. In the resulting transformants (Table 1), YFP signals were observed in the nucleus of conidia and 6-h germlings (Fig. 5b), suggesting that they interact with each other in vivo. To further verify their interactions, we generated the *FgSRP1*-3xFLAG and *FgSRP2*-GFP fusion constructs and co-transformed them into the wild-type strain PH-1. In the resulting transformants (Table 1), Srp1-3xFLAG proteins were detected with an anti-FLAG antibody in total proteins and in proteins eluted from anti-GFP beads (Fig. 5c). These results indicate that FgSrp1 and FgSrp2 interact with each other to form a complex in vivo.

To further determine whether the expression level of *FgSRP1* or *FgSRP2* was affected by deletion of each other, RNA samples of *Fgsrp1* and *Fgsrp2* deletion mutants were isolated from 12-h germlings in YEPD cultures. Compared with PH-1, the expression levels of *FgSRP1* in the *Fgsrp2* deletion mutant and *FgSRP2* in the *Fgsrp1* deletion mutant were not detectably different (Fig. S3). These results indicate that the transcription of *FgSRP1* and *FgSRP2* is independent.

FgSRP1 and *FgSRP2* have overlapping functions in vegetative growth and sexual reproduction

To determine whether there is functional overlap between *FgSRP1* and *FgSRP2*, we deleted *FgSRP2* in the *Fgsrp1* deletion mutant. The resulting *Fgsrp1srp2* double deletion mutant had more serious phenotypic defects in vegetative growth and sexual reproduction compared with either of the two single mutants (Fig. 1). Similar to the *Fgsrp1* deletion mutant, the *Fgsrp1srp2* deletion mutant rarely produced

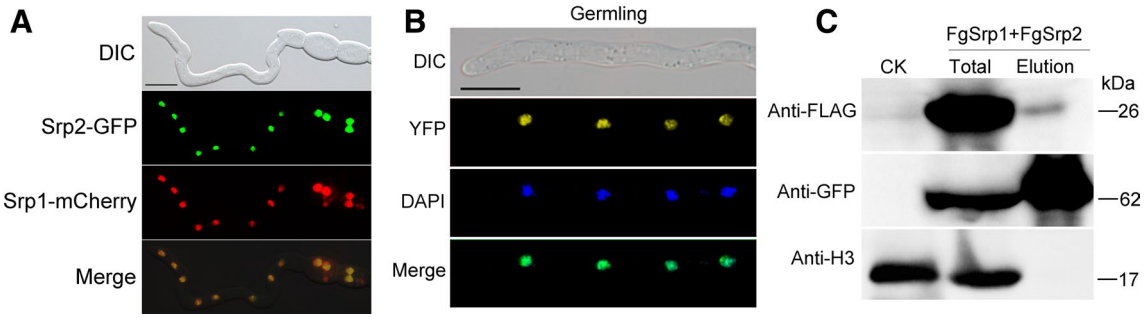

Fig. 5 Bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (co-IP) assays for the interaction of FgSrp1 and FgSrp2. **a** Six-hour germlings of transformants expressing *FgSRP1*-mCherry and *FgSRP2*-GFP were examined by DIC and epifluorescence microscopy. Bar = 10 μm. **b** Six-hour germlings of transformants expressing the FgSrp1-YFPN and FgSrp2- YFPC fusion constructs were stained with DAPI and examined by DIC and epifluorescence microscopy. Bar = 10 μm. **c** Co-IP assays. Immunoblots of total proteins (Total) and proteins eluted from anti-GFP beads (Elution) from the transformant expressing the *FgSRP1*-3xFLAG and *FgSRP2*-GFP fusion constructs. Western blots were detected with anti-FLAG, anti-GFP or anti-H3 antibody. Total proteins isolated from the wild-type strain PH-1 were included as the control

conidia in 5-day-old CMC cultures and caused only limited symptoms on the inoculated florets (Fig. 1; Table 2). On PDA plates, however, the growth rate of the *Fgsrp1srp2* deletion mutant was significantly lower than that of the *Fgsrp1* deletion mutant (Table 2). Although not completely melanized, the perithecia formed by the *Fgsrp1srp2* mutant appeared to be fewer and smaller in comparison with either of the two single mutants after 8 days post-perithecial induction (Fig. 1d). These results indicate that *FgSRP1* and *FgSRP2* have overlapping functions in vegetative growth and sexual reproduction.

FgSRP2 regulates transcription and RNA splicing of a subset of genes together with *FgSRP1*

To determine the effects on gene transcription and RNA splicing by deletion of *FgSRP2* and double deletion of *FgSRP2* and *FgSRP1*, we performed strand-specific RNA-seq analysis with RNAs isolated from vegetative hyphae of PH-1, *Fgsrp2*, and *Fgsrp1srp2* collected from 16-h YEPD cultures. In comparison with PH-1, only 130 significantly differential AS events were detected in the *Fgsrp2* mutant, but 363 were found in the *Fgsrp1srp2* mutant (FDR < 0.05) (Fig. 6a; Tables S2 and S3), accounting for 8.0% of total AS events detected. These results suggest that the FgSrp2–FgSrp1 complex regulates RNA splicing of a subset of genes. Among them, intron retention (IR) is the vast majority of AS events detected (Fig. 6a), accounting for approximately 86% of total AS events. Further analysis revealed that the number of IR events with increased and reduced RNA splicing efficiency were comparable in both of the *Fgsrp2* and *Fgsrp1srp2* mutants (Fig. 6b, c), suggesting that *FgSRP2* and *FgSRP1* act as both positive and negative regulators in RNA splicing.

Compared with PH-1, only 93 differentially expressed genes (54 up-regulated and 39 down-regulated) were detected in the *Fgsrp2* mutant (Fig. 6d; Table S4). In the *Fgsrp1srp2* mutant, however, 1308 (765 up-regulated and 543 down-regulated) genes were detected (Fig. 6d; Table S5), accounting for 12.3% of total expressed genes. These results indicate that the FgSrp2–FgSrp1 complex is important for regulating gene transcription. Interestingly, the numbers of up-regulated genes were slightly higher than that of down-regulated genes in both of the *Fgsrp2* and *Fgsrp1srp2* mutants, implying that *FgSRP2* and *FgSRP1* play a more important role in suppression of gene transcription.

Discussion

In eukaryotic organisms, SR proteins have been shown to play wide-ranging roles in gene expression, including constitutive pre-mRNA splicing, alternative splicing, mRNA nuclear export, nonsense-mediated mRNA decay and mRNA translation (Long and Caceres 2009). To study how gene expression influences hyphal development and plant infection in *F. graminearum*, we identified and characterized two SR proteins: FgSrp1 in our previous study (Zhang et al. 2017) and FgSrp2 in this study. Both FgSrp1 and FgSrp2 have the structural components typical of SR proteins. The FgSrp2 has two conserved RRM domains at the N-terminus but FgSrp1 has only one. *FgSRP1* is orthologous to *S. pombe srp1* while *FgSRP2* is orthologous to *S. pombe srp2* and *S. cerevisiae NPL3*. An apparent ortholog of *FgSRP1* is missing in *S. cerevisiae*. In *S. pombe*, an *srp1* deletion mutant has only a mild cold-sensitive phenotype but *srp2* is essential for growth (Gross et al. 1998; Lutzelberger et al. 1999). In *S. cerevisiae, NPL3* is critical for growth; cells lacking *NPL3* are temperature

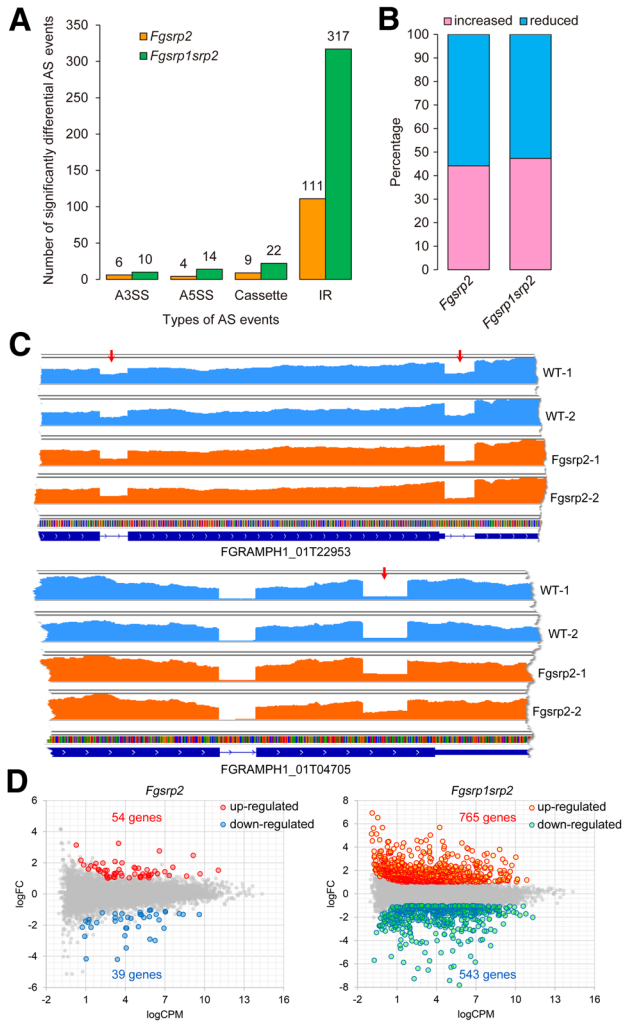

Fig. 6 Differential alternative splicing (AS) and transcription in *Fgsrp2* and *Fgsrp1srp2* mutants. **a** The number of significantly differential AS events in *Fgsrp2* and *Fgsrp1srp2* mutants relative to wild-type PH-1. Types of AS events includes A5SS (Alternative 5′ splice site), A3SS (Alternative 3′ splice site), Cassette (cassette exon) and IR (intron retention). **b** Percentage of IR events with increased or reduced splicing efficiency in *Fgsrp2* and *Fgsrp1srp2* mutants. **c** Examples of IR events with increased (upper panel) or reduced ((lower panel) splicing efficiency in *Fgsrp2* mutant. RNA-seq read coverages of two biological replicates are shown for the *Fgsrp2* mutant and wild type (WT). Red arrows indicate the introns with significantly differential splicing efficiency in the *Fgsrp2* mutant. **d** MA-plot showing the $\log_2$ fold change (logFC) of individual genes plotted with the average expression strength (logCPM) in *Fgsrp2* (left panel) and *Fgsrp1srp2* (right panel) mutants compared with wild type. The numbers of up-regulated and down-regulated genes (logFC inoculated with the labeled strains were photographed $z \geqq 1$, FDR < 0.05) are indicated deviation were calculated with data from three biological replicates.

sensitive (Bossie et al. 1992). In *F. graminearum*, however, deletion of either *FgSRP1* or *FgSRP2* resulted in growth and sexual reproduction defects, but the defects were relatively minor in the *Fgsrp2* deletion mutant. Furthermore, although *FgSRP1* plays a critical role in conidiation, plant infection and DON production, *FgSRP2* is dispensable for those functions. These results suggest that *FgSRP1* is more important than *FgSRP2* in *F. graminearum*. Similar results were also reported in *Candida albicans*, in which deletion of *CaNPL3* resulted in few phenotypic changes, whereas deletion of *SLR1*, the ortholog of *FgSRP1,* caused defects in growth, filamentation, host cell interactions, and virulence (Ariyachet et al. 2013). These results imply that, although their structural components are conserved, the biological functions of SR proteins vary in different fungi.

The R-rich region affects the subcellular localization of SR proteins by acting as an NLS (Long and Caceres 2009). In this study, FgSrp2-GFP fusion protein was found to localize only in the nucleus, whereas the *FgSRP2*$^{1-174}$-GFP that lacks the R-rich C-terminal region localized in both the nucleus and cytoplasm, confirming the important role of R-rich region for localization of FgSrp2 to the nucleus. Interestingly, the N-terminus, which contains the two RRM domains, also influences the subcellular localization of FgSrp2, because the *FgSRP2*$^{175-312}$-GFP signals were observed in both the nucleus and cytoplasm although they were stronger in the nucleus.

Phosphorylation also affects the proper localization of SR proteins in the cell (Jeong 2017). In fission yeast, the cellular localization of Srp2 is regulated by SR protein-specific kinase Dsk1 (Tang et al. 2007). Our previous study revealed that Srk1, an ortholog of Dsk1, physically interacts with both FgSrp1 and FgSrp2 in *F. graminearum* (Wang et al. 2018). In this study, however, we found that the subcellular localization of FgSrp2-GFP did not change in the *srk1* mutant, indicating that the Srk1 does not affect the subcellular localization of FgSrp2 in *F. graminearum*. Additionally, we found that deletion of FgPrp4, the only kinase in the spliceosome, also did not alter the subcellular localization of FgSrp2 in *F. graminearum.* It seems likely that the subcellular localization of Srp2 is not regulated by phosphorylation in *F. graminearum*.

Srp1 and Srp2 interact with each other to form a complex in *S. pombe* (Tang et al. 2002). In *F. graminearum*, the FgSrp2 was also found to interact with FgSrp1 by BiFC and co-IP assays. Therefore, the FgSrp2 and FgSrp1 are likely to function as a complex in *F. graminearum.* However, the transcription of *FgSRP1* and *FgSRP2* is independent. We did not observe that the expression levels of *FgSRP1* in the *Fgsrp2* mutant and *FgSRP2* in the *Fgsrp1* mutant were obviously altered. Furthermore, we found that *FgSRP1* and *FgSRP2* may have overlapping functions in vegetative growth and sexual reproduction. The growth rate of the *Fgsrp1srp2* double deletion mutant was significantly lower than that of either single mutant. Whereas the amount and size of perithecia formed by the

two single mutants were normal, the perithecia of *Fgsrp-1srp2* mutant appeared to be fewer and smaller.

The roles of FgSrp2 and FgSrp2-FgSrp1 complex in gene transcription and RNA splicing were also revealed by strand-specific RNA-seq analysis. By comparison with the wild type, only 130 significantly differential AS events and 93 differentially expressed genes were detected in vegetative hyphae of the *Fgsrp2* mutant, which is consistent with its minor growth defect. However, 363 significantly differential AS events and 1308 differentially expressed genes were detected in *Fgsrp1srp2* double deletion mutant, accounting for 8.0% of total AS events and 12.3% of total expressed genes, respectively. These results suggest that although deletion of *FgSRP2* alone had only minor effects on gene transcription and RNA splicing, deletion of both *FgSRP1* and *FgSRP2* resulted in significant changes in gene transcription and RNA splicing. Moreover, although SR proteins generally function as splicing activators (Busch and Hertel 2012; Zhou and Fu 2013), SRSF1 and SRSF2, two classic SR proteins in human, were recently reported to promote both exon-inclusion and exon-skipping in vivo (Pandit et al. 2013). Our results revealed that *FgSRP2* and *FgSRP1* are capable of being both splicing activators and splicing suppressors, because a comparable number of introns with increased and reduced splicing efficiency were observed in both of the *Fgsrp2* and *Fgsrp1srp2* mutants in comparison with the wild type. Taken together, our studies revealed that FgSrp2 plays roles in vegetative growth, sexual reproduction and pre-mRNA processing by interacting with FgSrp1.

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