Identification and Functional Analysis of the Pheromone Response Factor Gene of Sporisorium scitamineum

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The sugarcane smut fungus Sporisorium scitamineum is bipolar and produces sporidia of two different mating types. During infection, haploid cells of opposite mating types can fuse to form dikaryotic hyphae that can colonize plant tissue. Mating and filamentation are therefore essential for S. scitamineum pathogenesis. In this study, we obtained one T-DNA insertion mutant disrupted in the gene encoding the pheromone response factor (Prf1), hereinafter named SsPRF1, of S. scitamineum, via Agrobacterium tumefaciens-mediated transformation (ATMT) mutagenesis. Targeted deletion of SsPRF1 resulted in mutants with phenotypes similar to the T-DNA insertion mutant, including failure to mate with a compatible wild-type partner strain and being non-pathogenic on its host sugarcane. qRT-PCR analyses showed that SsPRF1 was essential for the transcription of pheromone-responsive mating type genes of the a1 locus. These results show that SsPRF1 is involved in mating and pathogenicity and plays a key role in pheromone signaling and filamentous growth in S. scitamineum.

Keywords: Sporisorium scitamineum, pheromone response factor, mating, pathogenicity, fungi

INTRODUCTION

Sugarcane smut caused by Sporisorium scitamineum is a devastating disease in sugarcane worldwide. Plants infected with the pathogen are severely stunted with thin stalks, producing no millable canes. The most recognizable characteristic of this disease is a black or gray growth from the top of plant cane that is referred to as a “smut whip” that is composed of a central core of pithy plant tissue surrounded by the fruiting structures of the fungus and the brown to black teliospores (Croft and Braithwaite, 2006; Sundar et al., 2012). S. scitamineum is bipolar and produces sporidia of two opposite mating types, MAT-1 and MAT-2 (Yan et al., 2016b). Sporidia of different mating types can fuse to form pathogenic dikaryotic hyphae to infect sugarcane buds and the hyphae grow within the meristematic tissue, eventually producing whip-like fruiting structure and teliospores in the infected plants. The diploid teliospores germinate and undergo meiosis to yield haploid sporidia, which need to mate again to infect the plant and to initiate a next round of infection (Albert and Schenck, 1996; Croft and Braithwaite, 2006; Yan et al., 2016b). Thus, mating plays a central role in the life cycle of smut pathogens, as it initiates parasitism by a morphological and
physiological transition from saprotrophic yeast cells to pathogenic filaments (Hartmann et al., 1996; Bakkeren et al., 2008; Kellner et al., 2011).

The conserved MAPK and cAMP/PKA signaling pathways regulate important aspects of fungal virulence in various pathogenic fungi such as Magnaporthe oryzae (Marroquin-Guzman and Wilson, 2015), U. maydis (Mayorga and Gold, 1999), and Setosphaeria turcica (Li et al., 2016) etc. The life cycle of S. scitamineum is similar to that of the well-studied model fungus Ustilago maydis that causes corn smut disease. In U. maydis, mating is regulated by two loci, a and b. The biallelic a locus (a1 and a2) encodes pheromone precursors mfa1 and mfa2, respectively, and receptors pra1 and pra2, respectively. The pheromone-receptor system is required for recognition and fusion of haploid sporidia, while the multiallelic b locus encodes bE and bW, subunits of a heterodimeric transcription factor that regulates filamentation, dikaryon maintenance and pathogenicity. The expression of the genes in the a and b loci is induced upon pheromone stimulation (Hartmann et al., 1996). Basal as well as pheromone-induced transcription of these mating type genes is regulated by the pheromone responsive factor Prf1, which binds specifically to the pheromone response elements (PREs) located in the vicinity of all pheromone-inducible genes at the a and b loci (Hartmann et al., 1996; Urban et al., 1996). The activity of Prf1 is, in turn, regulated by the cyclic-AMP (cAMP) signaling pathway and the mitogen-activated protein (MAP) kinase module (Regenfelder et al., 1997; Krüger et al., 1998; Müller et al., 1999, 2003; Andrews et al., 2000; Kaffarnik et al., 2003). Prf1 is also regulated by other regulators such as rop1, hap2 and med1 (Brefort et al., 2005; Zarnack et al., 2008; Mendoza-Mendoza et al., 2009; Chacko and Gold, 2012).

In recent years, the whole genome sequences of S. scitamineum strains from China, Brazil, Australia and South Africa have been reported (Que et al., 2014; Taniguti et al., 2015; Dutheil et al., 2016). The availability of genome sequences facilitates the investigation of the pathogenicity mechanism of S. scitamineum. To understand the molecular basis of sexual mating and pathogenic development in S. scitamineum, we developed an efficient Agrobacterium tumefaciens-mediated transformation (ATMT) system (Sun et al., 2014) and identified the b locus as essential for sexual mating and filamentous growth in S. scitamineum (Yan et al., 2016b). During the screening of an ATMT transformant library, we found a T-DNA insertion mutant, 248E3, that was unable to mate or form filamentous hyphae. The disrupted gene was identified as an ortholog of the U. maydis Prf1 gene, designated as SsPRF1. Functional characterization of SsPRF1 revealed that it functions as a pheromone response regulator, plays an essential role in the regulation of a locus gene expression, and is required for S. scitamineum pathogenicity.

MATERIALS AND METHODS

Strains and Growth Conditions

The compatible haploid strains JG35 (MAT-2) and JG36 (MAT-1) of S. scitamineum were isolated from germinated teliospores collected from the sugarcane smut in Guangxi, China (Lu et al., 2017). Cultures were grown in YePS broth medium (1.0% yeast extract, 2.0% peptone, 2.0% sucrose) on a rotary shaker at 200 rpm at 28°C or on solid YePS agar at 28°C.

Plant Materials

Sugarcane seedlings of the highly susceptible variety, ROC22, were cultivated in B. Chen’s experimental field in Guangxi University, and used for pathogenicity assay.

Molecular Manipulations

Plasmid DNA was isolated with the SanPrep plasmid mini kit (Sangon, B518191) and S. scitamineum genomic DNA was extracted using the method described previously (Yan et al., 2016b). For Southern blot analysis, DNA samples (3–5 μg) digested with appropriate restriction enzymes were separated by electrophoresis and blotted to Hybond N+ membrane. Standard hybridization and detection protocols were performed using the method of DIG DNA labeling and detection kit (Roche, 11093657910). Total RNA was isolated with TRNzol-A+ (Tiangen, DP421) and first-strand cDNA was synthesized using the Revert AidFirst Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1621).

ATMT Mutagenesis and Identification of T-DNA Insertion Site

Agrobacterium tumefaciens-mediated transformation (ATMT) was used to generate mutants of S. scitamineum haploid strains JG35 and JG36 using the A. tumefaciens strain AGL1 (Sun et al., 2014). The transformation procedure was adapted and modified from the methods developed for U. maydis (Ji et al., 2010). T-DNA left-border flanking sequence was amplified by high-efficiency TAIL-PCR (hiTAIL-PCR; Bölker et al., 1992; Liu and Chen, 2007), using genomic DNA of T-DNA insertion mutants as templates. Two rounds of amplifications were performed. Specific primers (LB1, LB2, and LB3) in combination with arbitrary degenerate primer (LAD1-4) and AC1 were used. The primer pair LB1/LAD1-4 was used in the pre-amplification step, while the primer pairs AC1/LB2 and AC1/LB3 were used in the primary and secondary-amplification, respectively. Primer sequences are listed in Table 1. The primary TAIL PCR product of about 850 bp was purified using the PCR-Clean kit (Sangon, SK8142), cloned into pMD 18-T Vector (TaKaRa, 6011), and sequenced using M13 forward or reverse primer.

Plasmid Construction for SsPRF1 Deletion and Complementation

Binary vectors pEX1-GAP-eGFP-pnGR1, and pEX2, all derived from the binary T-DNA vector pZP200 (Hajdukiewicz et al., 1994) and with T-DNA, were kindly provided by Dr. Lianghui Ji from National University of Singapore (Ji et al., 2010). SsPRF1 deletion construct was made according to protocol previously described (Ji et al., 2010). Briefly, a 1577 bp upstream fragment (HS1) and a 841 bp downstream fragment (HS2) of the SsPRF1 gene were amplified with primer pairs prf1-P1/prf1-P2 and
### Table 1 | Primers used in this study.

| Name   | Sequence                  |
|--------|---------------------------|
| LAD1-4 | 5'ACGGATGAGCTCCAGAGGCGGCCGC(G/C/T)(G/A/T)N(G/C/T)NNNCGGT |
| AC1    | 5'ACGGATGAGCTCCAGAG         |
| LB1    | 5'TGACGCGAGGTTATCATGAGTCAAGC |
| LB2    | 5'GGACGAGGTTATCATGAGTCAAGC |
| LB3    | 5'CGATGCAAGGTTATCATGAGTCAAGC |
| M13-R  | 5'TCACACAGGAAACAGCTATAGGAC |
| M13-F  | 5'CAGGTTTTCATCAGTCAAGC     |
| hpH1-F | 5'GGTCAAGAAGCAATACGGCAAGC |
| hpH1-R | 5'GGTCAAGAAGCAATACGGCAAGC |
| prf1-P1| 5'AAAGTTTAAACTGCTCTGTGCCACGCCTTGA |
| prf1-P2| 5'AAACTGCTCTGTGCACGCTCTGTTCAAGC |
| prf1-P3| 5'AAACTGCTCTGTGCACGCTCTGTTCAAGC |
| prf1-P4| 5'AAAGTTTAAACTGCTCTGTGCCACGCCTTGA |
| prf1-P5| 5'CAGGTTTTCATCAGTCAAGC |
| prf1-P6| 5'GGTCAAGAAGCAATACGGCAAGC |
| prf1-P7| 5'TGTGAGGAATGTCGCTGCTGAGGAG |
| prf1-P8| 5'GGTCAAGAAGCAATACGGCAAGC |
| 248E3-F| 5'ATGCCGAGGACCAATGACATCAC |
| 248E3-TR| 5'CGTAAATGGGACCATCTTTTG |
| prf1-2R| 5'AAAGTTTAAACTGCTCTGTGCCACGCCTTGA |
| Nat-F  | 5'CAGGTTTTCATCAGTCAAGC |
| Nat-R  | 5'CAGGTTTTCATCAGTCAAGC |
| 248E3-qF| 5'CGTAAATGGGACCATCTTTTG |
| 248E3-qR| 5'CGTAAATGGGACCATCTTTTG |
| pra1-F | 5'CGAAGATGTTCTGAGGAGATGTAG |
| pra1-R | 5'TGTGAGGAATGTCGCTGCTGAGGAG |
| mfa1-qF| 5'TGTGAGGAATGTCGCTGCTGAGGAG |
| mfa1-qR| 5'TGTGAGGAATGTCGCTGCTGAGGAG |
| be1-qF | 5'TGAAGATACTGTCATGCAAGC   |
| be1-qR | 5'TGAAGATACTGTCATGCAAGC   |
| bV1-qF | 5'CAAGCTGCTGCTGCTGCTGCTGCTG |
| bV1-qR | 5'CAAGCTGCTGCTGCTGCTGCTGCTG |
| 18S-qF| 5'GACACCTGACATGCAAGCAGAAAAC |
| 18S-qR| 5'GACACCTGACATGCAAGCAGAAAAC |
| 248E3-F3| 5'GAGAAGGCTAGCAGACGAGTT    |
| 248E3-R| 5'GAGAAGGCTAGCAGACGAGTT    |
| mfa1-P1| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| mfa1-P2| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| mfa1-P3| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| mfa1-P4| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| mfa1-P5| 5'TGAAGATACTGTCATGCAAGC   |
| mfa1-P6| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| pra1-P1| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| pra1-P2| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| pra1-P3| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| pra1-P4| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| pra1-P6| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| pra1-P8| 5'AAAGTATACACCTGCTGTGTTGAGAGGAG |
| pra1-F | 5'ATGCTTGAGCAGATGACCTAC    |
| pra1-R | 5'ACTGCTTGAGCAGATGACCTAC    |

The **prf1-P3/prf1-P4** fragment (Table 1) using JG36 genomic DNA as template. The HS2 fragment was digested with *Spe*I and *Bam*HI and ligated into pEX2 to produce pEX2-HS2. The HS1 fragment was digested with *Pme*I and *Pst*I and then ligated into pEX2-HS2 at the corresponding sites to yield the gene knock out construct pEX2-Δ**prf1**. The recombinant plasmid pEX2-Δ**prf1** was introduced into *Agrobacterium* strain AGL1 and used for transformation of JG35 and JG36 sporidia (Sun et al., 2014). Transforms were screened by PCR using primer pairs **prf1-P5/prf1-P6**, **prf1-P7/prf1-P8**, and 248E3-F/248E3-TR (Table 1), respectively. Locations of the primers were illustrated in Figure 3B. Gene knockout and absence of ectopic copy were further confirmed by Southern blot analysis as described.

For functional complementation assays, the primer pairs **prf1-P1/prf1-P2-R** (Table 1) were used to amplify a 4627 bp fragment carrying the entire **spa** gene from JG36 genomic DNA. The PCR product was digested with *Pme*I and *Bam*HI and cloned into the Nourseothrin-resistance vector pNGR1 at the corresponding sites to yield the complementatory plasmid pCP**prf1**. The plasmid pCP**prf1** was introduced into *Agrobacterium* strain AGL1 and used for transformation of the T-DNA insertion mutant 248E3.

### Mating Assays

Mating assays were performed as previously described (Yan et al., 2016b). The *S. scitamineum* transformants were picked from the selection plates and inoculated in YePS medium (supplemented with 300 µg/ml Cefotaxime) for 2 days at 28°C on a rotary shaker. The 2 days cultures of wild-type opposite mating type strains (JG35, JG36, or mutants) were mixed with equal amounts of cells and a small drop was spotted on YePS-agar plate and incubated at 28°C for observation of colony morphology.

### Pathogenicity Assay

Pathogenicity assay was performed using the method described by Yan et al. (2016b). Fungal strains or mutants were grown in YePS broth for 2 days in a shaking incubator at 28°C and then collected by centrifugation to remove the medium. Fungal cells were resuspended in sterilized double-distilled water adjusted to 1 × 10⁶ cells/ml before mixing with the opposite mating type for inoculation. Sugarcane seedlings of the highly susceptible variety, ROC22, at 5–6 leaf stage grown in greenhouse were inoculated by injecting the stem near the growing point with approximately 200 µl of the mixed culture per plant. The control plants were injected with sterilized double-distilled water. Three biological repeats were applied in the inoculation and each repeat contained 5 plants grown in a pot of 30 cm in diameter. Pathogenicity was examined and documented till 120 days post-inoculation when the characteristic symptoms of a “smut whip” could be fully observed on the sugarcane plants inoculated with the positive control of JG36/JG35.

For fungal biomass assessment in the infected sugarcane seedlings, the sugarcane stem tissue infected by *S. scitamineum* sporidia (compatible mating types mixing) was collected at 72 hpi. Quantification of relative fungal biomass in infected sugarcane stem tissue was performed using the fungal *ACTIN* gene as reference, and sugarcane glyceraldehyde dehydrogenase (GAPDH) gene as reference for normalization, following the established protocol (Sun et al., 2019).
Transcriptional Profiling

Strains or mutants pre-grown in YePS broth for 2 days at 28°C with shaking were diluted to 0.1 OD_{500} with YePS. Equal amounts of the diluted cells of JG35 were mixed with those of JG36, JG36Δprf1, and 248E3, respectively and 50 ml of the mixtures or the haploid strains were incubated for 2 days at 28°C on a rotary shaker. Cultures were harvested by centrifugation and total RNA was isolated from haploid strains or mixture of strains. Two µg of purified total RNA of each sample was used as a template for first-strand cDNA synthesis using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1621). For real-time quantitative polymerase chain reaction (qRT-PCR) assay, the cDNA samples were diluted 20-fold with ddH_2O and used as qRT-PCR templates. The specific primer pairs of 248E3-qF/248E3-qR, mfa1-qF/mfa1-qR, pra1-qF/pra1-qR, and 18S-rRNA were used to amplify the target genes, SsPRF1, SsMFA1, SsPRA1, and 18S rRNA, respectively. 18S rRNA was used as the reference gene for expression normalization of the target genes and wild-type strain JG36 was used as the calibrator. qRT-PCR was performed in a LightCycler® 480 Real-time PCR system (Roche). Amplification reaction contained 10 µl of 2 × SYBR Green I Master Mix (Roche, 4707516001), 1.0 µl of each primer (10 µM), 1.0 µl of template cDNA and nuclease-free water was added to a final volume of 20 µl. The cycling parameters were: 95°C for 10 min, followed by 50 cycles of 95°C for 10 s and 60°C for 60 s. Then, the PCR products were heated to 95°C with 4.4°C/s, cooled to 65°C with 2.2°C/s, and heated to 97°C with 0.11°C/s. Negative controls were reactions without template or transcriptase and were included in each experiment set. qRT-PCR reactions were performed for three technical replicates and three biological replicates for each sample. For comparison of gene expression, the average threshold cycle (Ct) values for target genes and the house-keeping gene 18s rRNA were first calculated and then relative quantification was calculated using the comparative 2^{-ΔΔCt} method (Livak and Schmittgen, 2001). Data were normalized with endogenous 18s rRNA level from the same samples.

RESULTS

Characterization of the T-DNA Insertion Mutant 248E3

To identify genes essential for S. scitamineum mating/filamentation, we constructed an ATMT mutant library with two compatible mating-type strains of S. scitamineum, JG36 (MAT-1), and JG35 (MAT-2). A total of 25056 transformants with hygromycin resistance marker were obtained, in which 15873 transformants were derived from JG36 and 9183 from JG35. All the T-DNA insertion mutants exhibited bright green fluorescence under epifluorescent microscope (Figure 1A), confirming the presence of a T-DNA cassette carrying an eGFP in the S. scitamineum genome (Supplementary Figure S1A).

We then screened these transformants for mating defects. Mixing of the wild-type strain JG36 (MAT-1) with the compatible wild-type mating partner JG35 (MAT-2) gave rise to a fluffy colony, producing visible thin and white aerial filaments at 1–2 days post-spotting on YePS-agar plate (Figure 1B), indicating successful mating and formation of dikaryotic hyphae. Six isolates showing glossy appearance of mating mixture, indicative of mating defects, were selected and subjected to a second round of mating test. Among these was the mutant 248E3 from JG36 background, which was unable to produce dikaryotic hyphae when co-spotted with JG35 (Figure 1B), confirming that it was defective in mating. Apart from mating defect, 248E3 appeared indistinguishable from the wild type strain JG36, in colony and basidiospore (sporidium) morphology (Figures 1B,C) and the growth rate in YePS liquid medium (Figure 1D; p > 0.05).

Identification of the S. scitamineum PRF1 Homolog

Southern blotting analysis showed that mutant 248E3 contains a single copy of T-DNA inserted into its chromosome (Supplementary Figure S1B). By high-efficiency TAIL-PCR with the primers LAD1-4, AC1 paired with LB1, LB2 and LB3 (Table 1 and Supplementary Figure S1C), a fragment of about 850 bp was obtained using 248E3 genomic DNA as template (Supplementary Figure S1D). Sequence analysis showed that this fragment contained a 348 bp stretch of the genome sequence. Using this 348 bp fragment to screen the genome sequence of S. scitamineum in the NCBI database, we identified a putative gene (ID: SSCI14340.1), in which the T-DNA was inserted inside its coding sequence at coordinate 1033 bp from the translation start codon (Supplementary Figure S1C). The open reading frame (ORF) of SSCI14340.1 was 2166 bp, encoding a predicted polypeptide of 722 amino acids (GenBank: CDW96669.1). This protein shows 78% identity to the probable pheromone response factor Prf1 of S. reilianum (GenBank: CBQ73103.1), 57% to Prf1 of U. maydis (AAC32736.1) and 56% to a protein related to pheromone response factor Prf1 of U. hordei (CCF52951.1), respectively (Figure 2A). All these fungal Prf1 orthologs contain a highly conserved domain of HMG box superfamily at the N-terminus (Figure 2B). Therefore we named this gene SsPRF1. By sequencing the RT-PCR products using total RNA from JG35 (MAT-2), we found that SsPRF1 was also present in the strain with the opposite mating type. Southern blotting analysis confirmed that this gene was present in both JG35 and JG36 with a single copy in their genomes (Supplementary Figure S1B).

SsPRF1 Is Essential for Sexual Matting in S. scitamineum

To further demonstrate the functionality of SsPRF1, a 4.6 kb fragment containing the entire SsPRF1 gene of S. scitamineum MAT-1 (JG36) strain was cloned into the vector pNGR1 to result in the complementation plasmid pCPPrf1. This plasmid was introduced into the T-DNA insertion mutant 248E3 to generate the complementation transformant C248E3-34. Sporidia of the complementation strain C248E3-34 were able to form white fluffy colonies when co-spotted with a compatible wild-type mating partner JG35 (MAT-2), similar to the sexual mating between the two wild-type mating-type strains (Figure 3A).
FIGURE 1 | Characterization of T-DNA insertion mutant 248E3. (A) Fluorescence imaging of the mutant haploid sporidia. Photographs were taken at day 3 after inoculation onto the YePS plates. Scale bar = 20 µm. (B) Mating behavior of mutant 248E3. The mutant was co-spotted with compatible partner JG35 on YePS plate and incubated at 28°C for 3 days. (C) Differential interference contrast (DIC) images of sporidia: mutant 248E3 and JG36 (wild type). Scale bar = 10 µm. (D) Growth curves of mutant 248E3 and JG36 in YePS liquid medium. Cultures were kept at 28°C in a rotary incubator at 200 rpm. The initial OD₆₀₀ value was 0.2 and was measured once every 3 h. Data give averages ± SE of three technical replicates conducted at the same time.
**FIGURE 2** | Analysis of fungal PRF1 orthologs. (A) Phylogenetic analysis of four Prf1 orthologs in fungi was conducted in MEGA7 (Kumar et al., 2016) using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.99597394 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jones-Taylor-Thornton (JTT) matrix-based method (Jones et al., 1992) and are in the units of the number of amino acid substitutions per site. SrPrf1: CBQ73103.1; UmPfr1: AAC32736.1; UhPrf1: CCF52951.1. (B) Sequence alignments of the above mentioned fungal SsPrf1 proteins was performed using Boxshade server (https://embnet.vital-it.ch/software/BOX_form.html), with the aligned sequence generated by Clustal Omega (www.ebi.ac.uk/Tools/services/web/toolresult.elti?jobId=clustalo-I20180725-032536-0068-98009260-p2m&showColors=true&tool=clustalo). A conserved domain (MATA_HMG-box, class I member of the HMG-box superfamily of DNA-binding proteins) is underlined.
We further generated the SsPRF1 null mutants in both JG35 and JG36 background by homologous recombination (Supplementary Figure S2A). After antibiotic selection and two rounds of successive screening by mating tests, the SsPRF1 null mutants JG35Δprf1 from JG35 and JG36Δprf1 from JG36 were verified by PCR amplification (Supplementary Figures S2B,C) and confirmed by Southern blotting (Supplementary Figure S2D). In agreement with the phenotype of the T-DNA insertion mutant 248E3, the morphology of these SsPRF1 deletion mutants appeared indistinguishable from the wild-type strain (Figure 3B), except that they both failed to form fluffy colonies when co-spotted with compatible wild-type mating partners (Figure 3A). The growth rates of the ssprf1D mutants in YePS liquid medium were also comparable to that in their wild-type strains (Figure 3C; p > 0.05).

**SsPRF1 Is Required for S. scitamineum Pathogenicity**

To determine if SsPRF1 is involved in pathogenicity, we inoculated the sugarcane seedlings of variety ROC22 (highly susceptible to S. scitamineum) with the mixed sporidial cells of S. scitamineum in combinations of 248E3(MAT-1)/JG35(MAT-2), C248E3-34(MAT-1)/JG35(MAT-2), and JG36Δprf1(MAT-1)/JG35(MAT-2), with wild-type strains JG35(MAT-2)/JG36(MAT-1) as positive control. It was found
that sugarcane seedlings inoculated with either the T-DNA disrupted \textit{SsPRF1} mutant (248E3) or the \textit{SsPRF1} null mutant (JG36\textit{Δprf1}) remained healthy throughout the observed period of 120 days. In contrast, plants infected with wild-type combination JG36/JG36 displayed severe stunting, spindly stalks and upright and narrower leaves after 60 days, and whips emerged from the shoots at 90–120 days post-inoculation (Figure 4). In agreement with the restoration of mating, the complementation strain C248E3-34 incited the characteristic symptoms of "smut whip" when mixed with wild-type strain JG35 (MAT-2), comparable to those by the wild-type strains (Figure 4; \( p > 0.05 \)).

We further quantified the relative fungal biomass in infected sugarcane stem tissue, at 3 days post-infection (dpi), and our result showed that the relative fungal biomass in the sugarcane seedlings infected by JG36\textit{Δprf1}(MAT-1)/JG35(MAT-2) was 139.65 ± 72.12% of that in the ones infected by the wild-type JG35(MAT-2)/JG36(MAT-1) mixture. Similarly, the relative fungal biomass from the 248E3(MAT-1)/JG35(MAT-2) infected seedlings was 118.21 ± 24.21% of that of WT. Difference between WT infection and mutant infection was not significant (\( p > 0.05 \)). This result indicates that colonization of the plant tissue by WT or mutant mixed sporida at early stage were comparable, however, we could not differentiate the \textit{in planta} fungal biomass detected by this assay was in sporidial or hyphal form. This result at least rules out the possibility that failure of developing disease symptom in the mutant infected sugarcane seedlings was due to insufficient inoculum at the beginning.

**\textit{SsPrf1} Regulates the Expression of Pheromone-Responsive Genes**

Recently, the whole genome sequences of \textit{S. scitamineum} strains from China, Brazil, Australia and South Africa have been reported (Que et al., 2014; Taniguti et al., 2015; Dutheil et al., 2016). The presence of pheromone receptor gene \textit{SsPRA1} was first reported by Que et al. (2014) and the whole coding region was identified by RT-PCR and located to the \textit{a1} locus (Yan et al., 2016b). During our BLAST search against \textit{S. scitamineum} genome, the pheromone precursor gene \textit{SsMFA1} with high identity to the pheromone precursor gene \textit{mfa1.2} in \textit{S. reilianum} was also identified and verified by RACE in the \textit{a1} locus of \textit{S. scitamineum} (GenBank: CP010914.1, 857085-857204).

To further dissect the function of \textit{SsPRF1} in pheromone signaling and pathogenic development, transcription of the JG36-specific genes \textit{SsMFA1} and \textit{SsPRA1}, as well as the gene \textit{SsPRF1} that is present in both JG35 and JG36, were assessed in the haploid strains of JG35, JG36, JG36\textit{Δprf1}, and 248E3, with or without pheromone induction from the opposite mating type strain. As shown in Figure 5A, \textit{SsPRF1} was detected at comparable levels in JG36 and JG35, while undetectable in JG36\textit{Δprf1}, as expected. \textit{SsMFA1} and \textit{SsPRA1} expression was undetectable in JG35 (MAT-2) and was barely detectable in JG36\textit{Δprf1} or 248E3 (Figure 5A). However, it was interesting that the \textit{SsPRF1} transcript level was threefold up-regulated in 248E3, suggesting that (1) the truncated \textit{SsPRF1} was transcriptionally active, and (2) the mutated \textit{SsPRF1} showed enhanced mRNA transcription.

To investigate the induction effect of the opposite mating type strain on mating type-related gene expression, JG36, JG36\textit{Δprf1}, and 248E3 were co-cultured with their compatible wild-type mating partner JG35 (MAT-2), which was assumed to provide \textit{a2} pheromone and should stimulate the transcription of pheromone-inducible genes. As shown in Figure 5B, \textit{SsMFA1} expression was significantly elevated (10.7-fold) in JG35/JG36 combination but not in JG35/JG36\textit{Δprf1} or JG35/248E3 combinations. \textit{SsPRA1} expression was up-regulated by 4.3-fold in the JG35/JG36 combination but no increase in JG35/JG36\textit{Δprf1} or JG35/248E3 combinations could be detected, suggesting that \textit{SsPRF1} in JG35 could not complement the \textit{SsPRF1} defect in JG36\textit{Δprf1} and 248E3. It was interesting that \textit{SsPRF1} expression was stimulated in JG35/JG36 combination (2.4-fold up-regulated) and in JG35/248E3 (1.9-fold up-regulated), implying that \textit{SsPRF1} expression is regulated by signal(s) released during mating by the opposite mating type strains. In contrast, no stimulation in \textit{SsPRF1} transcription was seen in JG35/JG36\textit{Δprf1}, whose transcript level reached about 50% of the wild-type level for \textit{SsPrf1} mRNA. This reduced transcript level may likely be caused by the dilution of the \textit{SsPRF1}-transcribing JG35 with \textit{SsPRF1}-non-transcribing JG36\textit{Δprf1}.

We also assessed transcriptional regulation of the \textit{b} locus genes \textit{bE} and \textit{bw} by qRT-PCR. Our result showed that transcription of \textit{bE} or \textit{bw} genes was slightly reduced in the sporidia of \textit{SsPRF1} disruption or deletion mutants (Figure 5A), but the difference was not significant (\( p > 0.05 \)). Under pheromone-induction condition (JG35/JG36), the \textit{b} locus genes were upregulated (\textit{bE1}: 1.90-folds; \textit{bW1}: 2.54-folds; \( p < 0.05 \)) while loss or disruption of \textit{SsPRF1} in JG36 background made it unable to induce \textit{b} locus gene transcription when mixed with wild-type \textit{JG35} sporidia (Figure 5B; \( p < 0.05 \)). Overall, we conclude that \textit{SsPrf1} is responsible for transcriptional induction of \textit{a} and \textit{b} locus genes under mating condition.

**\textit{a} Locus Genes Are Essential for \textit{S. scitamineum} Mating/Filamentation**

To further confirm that \textit{S. scitamineum} \textit{a1} locus genes are functional in mating/filamentation, we next deleted \textit{SsMFA1} and \textit{SsPRA1} respectively, in JG36 (MAT-1) background. PCR amplification of the gene of interest (\textit{SsMFA1} or \textit{SsPRA1}) or the flanking sequences, using the primer pairs as indicated in Supplementary Figure S3A and listed in Table 1, confirmed successful gene deletion (Supplementary Figure S3B). We also performed Southern blot analysis to confirm deletion of \textit{SsPRA1} gene (Supplementary Figure S3C).

Sporidia of the wild-type JG36 (MAT-1), JG36\textit{Δmfa1}, or JG36\textit{Δpra1} were respectively mixed and co-spotted with a compatible wild-type mating partner JG35 (MAT-2), to test their ability of mating/filamentation. We found that similar to the sexual mating between the \textit{Δprf1} mutant and wild-type mating-type strain, deletion of \textit{SsMFA1} or \textit{SsPRA1} led to failure of filamentation (Figure 6A). This confirms that \textit{S. scitamineum}
a locus gene SsMFA1 and SsPRA1 are functional in regulating fungal dimorphic switch, and likely acting at downstream of SsPrf1. This result is also consistent with a recent report on SsMFA1 characterization (Sun et al., 2019).

**SsPRF1 Is Independent/Downstream of cAMP-PKA Signaling Mediated ROS Regulation**

Chang et al. (2018) recently reported a cAMP-PKA signaling pathway regulating *S. scitamineum* intracellular redox homeostasis, to promote mating/filamentation and host infection. As in the corn smut fungus *U. maydis*, Prf1 was shown to act at downstream of both PKA and MAPK pathway and not involved in redox regulation (Kaffarnik et al., 2003), we here assessed the epistatic interaction between SsPrf1 and the cAMP-PKA pathway, and the role (if any) of SsPrf1 in redox regulation.

As the mating/filamentation defect of the cAMP-PKA mutants could be fully or partially restored by exogenous addition of cAMP (Chang et al., 2018), we first tested the effect of cAMP on Δprf1 mutant. The Δprf1 mutant remained un-mating or un-filamentous when mixed with the compatible wild-type JG35 (MAT-2) sporidia (Figure 6B). This indicates that SsPrf1 may act at downstream of cAMP-PKA signaling pathway, as consistent with what has been reported in *U. maydis*. Alternatively, SsPrf1 may act in parallel with cAMP-PKA signaling.

We also tested tolerance to the oxidative stress caused by 1 mM H₂O₂, in comparison between the wild-type and the Δprf1 sporidia, and found no obvious difference (Supplementary Figure S4A). We next measured the intracellular H₂O₂ of the Δprf1 sporidia, and found that it was comparable to that of the wild-type sporidia (Supplementary Figure S4B; *p* > 0.05). Finally, exogenous addition of low concentration (0.1 mM) of H₂O₂ could not restore the mating/filamentation of the Δprf1 mixed with the wild-type sporidia (Supplementary Figure S4C), as it does to the cAMP-PKA mutants (Chang et al., 2018). These results suggest that SsPrf1 may not be involved in redox regulation in *S. scitamineum*, which is at downstream of cAMP-PKA pathway. Therefore the role of SsPrf1 in *S. scitamineum* mating/filamentation is more likely due to transcriptional regulation of the mating-type genes.
FIGURE 5 | qRT-PCR analysis for expression of pheromone-inducible genes in SsPRF1 mutants. The expression of SsMFA1, SsPRA1, bE, bW, and SsPRF1 was analyzed in haploid strains JG35, JG36, JG36 Δprf1, and 248E3, cultivated alone (A) or together with JG35 sporidia (B). Total RNA was isolated from haploid strains and mixtures of strains incubated for 2 days at 28°C on a rotary shaker. The gene-specific primer pairs 248E3-qF/248E3-qR, mfa1-qF/mfa1-qR, pra1-qF/pra1-qR, bE1-qF/bE1-qR, bW1-qF/bW1-qR, and 18S-qF/18S-qR (Table 1) were used to amplify the respective target genes. The S. scitamineum 18S rRNA gene was used as an endogenous control, and wild-type strain JG36 as the calibrator. The error bars represent standard deviations, derived from three biological repeats, each with three technical repeats.

DISCUSSION

The morphological switch from yeast-like growth to filamentous growth occurs during the life style switch from the saprophytic to the biotrophic stage, and is critical for virulence of several animal- and plant-pathogenic fungi (Hartmann et al., 1996; Urban et al., 1996; Nadal et al., 2008; Elías-Villalobos et al., 2015). The human fungal pathogen Candida albicans can switch from a unicellular yeast form into pseudohyphae or hyphae, and such transition is important for virulence (Lo et al., 1997; Wartenberg et al., 2014). In the fungal plant pathogen U. maydis, morphological switching from yeast-like sporidia to dikaryotic hyphae occurs after sexual mating between two cells of opposite mating-types, a process under regulation of biallelic a and multiallelic b loci ( Bölker et al., 1992; Gillissen et al., 1992; Spellig et al., 1994). Sporisorium reilianum, a smut fungus closely related to U. maydis, possesses three a alleles containing two active pheromone genes each, and at least five alleles for the b locus, that govern its ability of sexual mating and dimorphic switching essential for virulence ( Schirawski et al., 2005). S. scitamineum is also a dimorphic pathogen with two different life styles, a saprophytic stage growing by budding as unicellular sporidia, and a pathogenic stage growing as dikaryotic hyphae. The morphological switch in S. scitamineum also depends on sexual mating (Que et al., 2014), but its regulatory mechanism is still not fully understood. The conserved b locus genes have also been functionally investigated, with the gene sequence encoding the pheromone receptors, PRA1 and PRA2, annotated (Yan et al., 2016a,b). The MFA1 and MFA2 gene, respectively encoding the pheromone precursors in MAT-1 and MAT-2 mating-type strain, have both been annotated and characterized by reverse genetics (Lu et al., 2017; Sun et al., 2019). However, the pheromone response factor Prf1 that governs pheromone-induced transcription of a and b loci in U. maydis, has not yet been identified or characterized in S. scitamineum. Neither was it characterized in the biological function of the pheromone receptor encoding genes, SsPRA1 and SsPRA2. As reported in this study, we identified a Prf1 ortholog in S. scitamineum and found that it was responsible for both basal and pheromone-induced expression of mating type genes of the a1 locus. We further showed that deletion of the a locus gene SsMFA1 or SsPRA1 resulted in similar phenotype (Figure 6A) as the T-DNA insertion mutation or deletion mutant of the SsPRF1 gene (Figure 3A), as well as the reported MFA1 deletion phenotype (Sun et al., 2019). Our results provide new insight into the mechanism of pathogenicity of this important sugarcane pathogen, by confirming the biological function of the a1 locus gene in S. scitamineum mating/filamentation, and indicating that SsPrf1 plays a key role in pheromone signaling and filamentous growth in S. scitamineum through transcriptional induction of the mating locus.

Under pheromone-induced conditions, expression of SsPRF1 in JG35/JG36 culture was about 2.4-fold up-regulated compared
This is consistent with what has been reported in SsPrf1 acts at downstream of cAMP-PKA signaling pathway (Chang et al., 2018), indicating that mating/filamentation of the S. scitamineum found that exogenous addition of cAMP could not restore through regulation of intracellular redox homeostasis. We have shown that Prf1 undergoes post-translational modification (phosphorylation) for activation (Kaffarnik et al., 2003). We found no obvious difference between the wild-type and the Δprf1 mutant in aspects of oxidative stress tolerance or intracellular H2O2 level (Supplementary Figures S4A,B). Also, addition of low concentration (0.1 mM) of H2O2 could not promote mating/filamentation in the Δprf1 mutant (under mating condition with the opposite mating-type sporidia, Supplementary Figure S4C), as it does to the wild-type strain or the cAMP-PKA mutants (Chang et al., 2018). These results confirmed that the SsPrf1 function is not relevant to intracellular redox homeostasis but may be solely on regulation of mating locus genes.

Overall, our present study, together with previous published functional study of a and b locus, completes the regulation network of S. scitamineum mating/filamentation at downstream of the cAMP-PKA signaling pathway, and in parallel of redox signaling.

**DATA AVAILABILITY**

All datasets generated for this study are included in the manuscript and/or the Supplementary Files.

**AUTHOR CONTRIBUTIONS**

GZ, YD, EC, MY, GC, ZW, CZ, and BZ performed the experiments. PX, BC, CC, and ZJ conceived and designed the experiments. GZ, BC, CC, and ZJ contributed reagents, materials, and analysis tools. GZ, YD, and ZJ wrote the manuscript. All authors read and approved the final manuscript.

**FUNDING**

This work was supported in part by the National Basic Research Program of China (973 Program, Grant No. 2015CB150600), National Natural Science Foundation of China (Grant No. 31660500), and Guangxi Key Laboratory of Biology for Crop Diseases and Insect Pests Project (Grant No. 15-140-45-ST-1). The funders had no role in the design of the study, collection, analysis, and interpretation of data, and in the writing of the manuscript.

**ACKNOWLEDGMENTS**

We thank Dr. L. H. Ji and Dr. L. H. Sun for sharing the vectors pEX1-GAP-eGFP, pNGR1, and pEX2.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02115/full#supplementary-material.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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