A Dual-Color Imaging System for Sugarcane Smut Fungus *Sporisorium scitamineum*

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Abstract

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The life cycle of the sugarcane smut fungus *Sporisorium scitamineum* is a multistep process. Haploid sporidia of compatible (*MAT-1* versus *MAT-2*) mating types fuse to generate pathogenic dikaryotic hyphae to infect the host. Within the host tissues, diploid teliospores are formed and induce a characteristic sorus that looks like a black whip. The diploid teliospores germinate to form haploid sporidia by meiosis. In order to monitor fungal development throughout the whole life cycle, we expressed the green fluorescent protein (GFP) and red fluorescent protein (RFP) in *S. scitamineum* *MAT-1* and *MAT-2* sporidia, respectively. Observation by epifluorescence microscope showed that conjugation

tube formation and sporidia fusion occurred at 4 to 8 h, and formation of dikaryotic filaments was detected at 12 h after mating. The resultant teliospores, with diffused GFP and RFP, underwent meiosis as demonstrated by septated hypha with single fluorescent signal. We demonstrated that GFP- and RFP-tagged strains can be used to study the life cycle development of the fungal pathogen *S. scitamineum*, including the sexual mating and meiosis events. This dual-color imaging system would be a valuable tool for investigation of biotic and abiotic factors that might affect the fungal life cycle development and pathogenesis.

The basidiomycete fungus *Sporisorium scitamineum* is the causal agent of sugarcane smut disease, which is a serious threat in global sugarcane production. The main symptom of the disease is a characteristic whip-like sorus produced from the top of the cane or side shoots. The life cycle of *S. scitamineum* is similar to the corn smut fungus *Ustilago maydis*, and consists of three cell types (Albert and Schenck 1996; Kronstad and Leong 1990). Diploid teliospores are formed from the host tissues and are disseminated in the field mainly by wind or rain splashes, but the disease also spreads through the use of infected planting materials (Croft and Braithwaite 2006). The haploid sporidia emerge by teliospores germination and meiosis. Sporidia grow by budding, and compatible (*MAT-1* versus *MAT-2*) sporidia fuse to develop into dikaryotic pathogenic hyphae, which infect plant canes and result in formation of diploid teliospores within the host tissues to complete the life cycle (Albert and Schenck 1996; Croft and Braithwaite 2006).

Regulation of sexual mating and filamentous growth in *S. scitamineum* is similar to what has been revealed in *U. maydis*, which relies on two mating-type loci, *a* locus and *b* locus (Fedler et al. 2009; Vollmeister et al. 2012; Wahl et al. 2010). The *a* locus encodes components of the pheromone-receptor system, allowing recognition of sporidia of opposite mating type, and conjugation of mating tubes (Bölker et al. 1995; Gillissen et al. 1992). The resulting dikaryotic filament formation and activation of virulence is then controlled by the *b* locus, which encodes two transcription factors, bE and bW (Kronstad and Leong 1990; Schlesinger et al. 1997; Schulz et al. 1990). The conserved mating type genes *a* locus and *b* locus have been detected in *S. scitamineum* (Taniguti et al. 2015), and the function of *b* locus were characterized (Yan et al. 2016).

The GFP gene (Prasher et al. 1992) and its derivatives have been widely used to generate reporter systems to visualize the subcellular organelles as well as microbes, including many plant pathogens (Maor et al. 1998). In filamentous fungi, GFP has been successfully expressed in *U. maydis* (Spellig et al. 1996), *Aspergillus nidulans* (Fernández-Ábalos et al. 1998), and *Magnaporthe oryzae* (Kershaw et al. 1998). Utilization of GFP has enormously facilitated investigation on pathogen invasion of host plant tissue by *Fusarium graminearum* (Jansen et al. 2005), *M. oryzae* (Kankanala et al. 2007), *Colletotrichum graminicola* (Oliveira-Garcia and Deising 2013), and *Zymoseptoria tritici* (Kilaru et al. 2015b), and nuclear movement and division during spore germination in *M. oryzae* (Veneault-Fourrey et al. 2006), *Colletotrichum gloeosporioides* (Nesher et al. 2008), *Fusarium oxysporum* (Ruiz-Roldan et al. 2010), *U. maydis* (Fink and Steinberg 2006), and *Zymoseptoria tritici* (Kilaru et al. 2015a). GFP-tagged protein was also routinely used to monitor

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translocation of regulatory proteins in pathogens *Colletotrichum trifolii* (Chen and Dickman 2002), *Mycosphaerella graminicola* (Rohel et al. 2001), and *U. maydis* (Doehlemann et al. 2009; Valinluck et al. 2014). Similarly, RFP derived from the wild type protein DsRed (Shaner et al. 2004) is also an ideal reporter protein for live-cell imaging and can be used for multiple labeling together with GFP for tracking two different organelles or proteins in the same living cell (Su et al. 2004). The first true monomeric red fluorescent protein named mRFP was developed by genetic modification of DsRed protein (Campbell et al. 2002) and was used to investigate microtubule dynamics in *U. maydis* (Straube et al. 2006), subcellular localization of UDP-galactopyranose mutase in *A. nidulans* (El-Ganiny et al. 2010), and effector secretion in *M. oryzae* (Ribot et al. 2013). Given that GFP and RFP were widely used in filamentous/pathogenic fungi as convenient tools for visualization of cell, cellular proteins, or subcellular organelles, cytosolic GFP or RFP respectively expressed in compatible sporidia of *S. scitamineum* are potentially a simple but versatile approach for studying fungal mating and interaction with host.

A previous study reported the expression of eGFP in *S. scitamineum* haploid spores (Sun et al. 2014). Aiming to develop useful visualizable model strains for further investigations on *S. scitamineum* mating and development under both in vitro and in vivo conditions, we expressed GFP and RFP in the opposite mating type sporidia of *S. scitamineum* for direct microscopic observation on critical differentiation steps during the life cycle of *S. scitamineum*. The results showed that expression of these fluorescent proteins in fungal cells enabled

visualization and determination of timing of the key events of fungal life cycle, such as sporidia fusion and meiosis.

Materials and Methods

Bacterial and fungal cultures. *Escherichia coli* strain *Trans 5α* (TransGen Biotech, CD201) was used for cloning purposes. Growth conditions and media for the cultivation of *E. coli* followed the protocols described by manufacturer (TransGen Biotech, CD201). Growth conditions as well as media for the mating type strains *MAT-1* (Ss17) and *MAT-2* (Ss18) of *S. scitamineum* have been described previously (Yan et al. 2016).

Mating and pathogenicity assays. *S. scitamineum* mating and pathogenicity assays were as described (Yan et al. 2016). Whip-like sorus samples were collected from an infected plant at 3 to 4 months after inoculation. Genetic transformation of *S. scitamineum* strains was as described (Sun et al. 2014). Transformants were selected on YePSA supplemented with hygromycin B (200 µg/ml) and cefotaxime (300 µg/ml), and purified on the same medium.

Plasmids used in this study. Plasmid pEX1GPD-EGFP was constructed previously (Sun et al. 2014). A 1.2-kb fragment containing mRFP and promoter *GPD* was digested with restriction enzymes *SpeI* and *PacI* and ligated into vector pEX2 to formed vector pEX2-RFP. Both *eGFP* and *mRFP* were driven by promoter *GPD* derived from *U. maydis*, which was as described (Sun et al. 2014). These constructs were transformed into *Agrobacterium tumefaciens* strain *AGL1* through electroporation for genetic transformation of *S. scitamineum*.

DNA techniques. Genomic DNA from transformants and the parental wild type strains (*MAT-1* and *MAT-2*) was isolated according to the established protocol (Lee and Taylor 1990), and was then digested by *HindIII*, fractionated on 1.0% agarose gel, and transferred to Nylon membranes (Roche, Cat. No. 11 417 240 001). The membrane was probed with about 500 bp DIG-labeled PCR-amplified fragment using Hygromycin resistance gene *hph* as template. Probe-hybridization was performed with PCR DIG Probe Synthesis Kit (Roche, Cat. No. 11 636 090 910), and detection by DIG Nucleic Acid Detection Kit (Roche, Cat. No. 11 175 041 910). Three nested primers were designed based on T-DNA sequences of vector pEX2, which is LB1: GGGTCGATGCGACGCAATCGT, LB2: ACGATG GACTCCAGAGTCCGGCCGGACCGATGGCTGTGTAGAAAGT, LB3: CGATCGACAAGCTCGAGTTTCTCC, respectively. Genomic fragments were amplified using the hiTAIL-PCR combined with degenerate primers randomly, followed the protocol as described (Liu and Chen 2007). These PCR-amplified fragments were sequenced by Invitrogen Trading Co., Ltd (Shanghai).

Microscopy. For microscopic analyses, wild-type and transformed *S. scitamineum* cells were cultured in YePS liquid medium for 24 h. Cells were then placed on top of a 2% agar cushion placed on glass slide, and observed and imaged using an Axioimager Z1 microscope equipped with an AxioCam MRm camera (Carl Zeiss). Standard filter sets for GFP, RFP, and DIC were used.

Results

Detection of stable epifluorescent signal in transformants. Genetic transformation of *MAT-1* and *MAT-2* of *S. scitamineum* with constructs pEX1GPD-EGFP and pEX2-RFP, respectively, yielded hygromycin-resistant fungal transformants that appeared yeast-like, with no visible anomalies. To verify T-DNA insertion and estimate the number of integrations in the individual transformants, a number of GFP and RFP transformants were chosen for Southern blot analysis. Results showed that most GFP transformants have one copy of hygromycin gene cassette (Supplementary Fig. S1, Lane 1-5 and 7-8) except for one transformant with two copies of insertion (Lane 6). Similar results were obtained with RFP transformants, in which eight out of nine transformants have one copy of hygromycin gene cassette (Lane 1 and 3-9) and the remaining one has two copies of ectopic insertion (Lane 2). To test the possibility that epifluorescent signal might be affected by T-DNA integration site, genomic sequences flanking T-DNA of GFP and RFP transformants were isolated by hiTail-PCR (Liu and Chen 2007) and sequenced. The sequencing

Fig. 1. Expression of GFP and RFP in *Sporisorium scitamineum* colonies. Wild-type *MAT-1* (A) or *MAT-2* (C) colonies and colonies of GFP (B) or RFP (D) transformants were analyzed by DIC optics. Autofluorescence was dim and negligible in the wild type (E, H). Two GFP (F, G) and two RFP (I, J) colonies were imaged with respective filter set. Scale bar: 500 µm.

results revealed that T-DNA was randomly inserted in coding or non-coding region of *S. scitamineum* genome (Supplementary Table S1).

Significant GFP signal was observed in the yeast-like colonies (Fig. 1F and G) and cells (Fig. 2I) by epifluorescent microscopy, while the colonies of wild-type untransformed *MAT-1* and *MAT-2* showed very weak autofluorescence (Fig. 1E). The level of epifluorescence varied among GFP transformants (Fig. 1F and G). Similarly, different levels of epifluorescence were also observed in RFP transformants (Fig. 1I and J) and cells (Fig. 2I), while autofluorescence in the wild type was weak and negligible (Fig. 1H). Next we used these two GFP- and RFP-expressed *MAT-1* and *MAT-2* haploid cell lines to monitor morphologic transition in mating and the following stages in *S. scitamineum* life cycle.

Expression of GFP and RFP during sexual mating. To examine whether GFP and RFP are suitable reporters for the infectious filament formation, we first test mating ability of transformants. *MAT-1* GFP transformant was mixed with *MAT-2* RFP transformant, and *vice versa*. All the mating mixtures, along with mixed wild-type *MAT-1* and *MAT-2* as control, were allowed to grow on YePSA plates in 28°C for 2 days. The mating results showed that all mixed transformants exhibited growth of white fluffy colonies and thus were able to mate sexually (Supplementary Fig. S2). We chose the *MAT-1* strain with the strongest GFP signal, namely *MAT-1G*, and the *MAT-2* with the brightest RFP signal, *MAT-2R*, for the following study.

Under epifluorescence microscope, the fungal cells were cigar-like with cytosolic GFP or RFP at the early stage of mating (Fig. 2A and I). Conjugation tubes were produced at 4 h post mating (Fig. 2B and C, arrows), also with cytosolic GFP or RFP (Fig. 2J and K, arrows). Fusion of sporidia was observed at 4 to 8 h and two such events were documented under bright field (Fig. 2D and E and Fig. 2F and G) as well as under epifluorescence microscopy (Fig. 2L and M and Fig. 2N and O). Before fusion, GFP and RFP were separated in respective sporidia (Fig. 2D and L and Fig. 2F and N, arrows), and successful mating were indicated by merging of GFP and RFP fluorescence in cytoplasm of fused sporidia (Fig. 2E and M and Fig. 2G and O, arrows). Formation of dikaryotic filaments was detected at 12 h after mating (Fig. 2H and P, arrows).

Expression of GFP and RFP in teliospores formation and germination. To analyze whether GFP and RFP can be expressed in teliospores stage, a number of mixture of GFP and RFP transformants of compatible mating types, including *MAT-1G* and *MAT-2R*, were inoculated to sugarcane seedlings by injection, with mixture of untransformed *MAT-1* and *MAT-2* as control. After 3 months, infection symptom as black whips appeared and the induction rate of black whips, a good indicator of *S. scitamineum* pathogenicity, caused by the wild-type infection ($n = 16$) was approximately 63%, while it varied from 22 to 63% for the mixed GFP- and

Fig. 2. Expression of GFP and RFP in *Sporisorium scitamineum* sexual mating. Mixture of *MAT-1G* and *MAT-2R* was inoculated on 2% agar plates. Cells were observed and imaged at 0 h (A, I), 4 h (B, C, D, F, J, K, L, N), 8 h (E, G, M, O), and 12 h (H, P), postinoculation. (A-H) DIC images; (I-P) epifluorescent images. Scale bar: 10 μ m.

RFP-transformed strains ($n = 16$). This demonstrates that *S. scitamineum* teliospore development and pathogenicity were not detrimentally affected by GFP or RFP expression (three repeats for each instance). The epifluorescent microscopy with teliospores formed by mating *MAT-1G* and *MAT-2R* showed strong GFP and RFP signal (Fig. 3A-D). Different levels of GFP and RFP were detected in teliospores sorus, and RFP signal was brighter than GFP and displayed longer duration (Fig. 3B-D). Next we followed the process of teliospore germination with this strain. At 0 h, GFP and RFP were evenly distributed in the teliospores (Fig. 3B-D). Promycelium that consists of a short septated hypha and haploid cigar-like conidia was observed at 6 h (Fig. 3E), when GFP and RFP were found to coexist in same cells or distribute to different cells (Fig. 3F-H), likely due to the meiosis taking place.

Discussion

In this work, we have demonstrated the use of GFP and RFP as convenient tools for investigation on *S. scitamineum* life cycle development, which are critical for the fungal pathogenesis. The genomic locations of the reporter gene inserted were examined, and the effect of transgene integration on fungal growth and life cycle development were monitored to select best GFP- and RFP-tagged *MAT* strains as a live-cell imaging system for further investigations on the life cycle development of this important fungal pathogen. The various levels of epifluorescent signals were likely due to site effect rather than

the number of integration, as the transformants harboring two copies of GFP/RFP-expression cassettes showed no stronger epifluorescence signal than those with one copy (data not shown).

Several studies reported that GFP expression leads to reduced virulence in transformed oomycetes (Dunn et al. 2013; Riedel et al. 2009) or bacteria (Sexton and Howlett 2001), likely due to the influences of insertion disruption of vital genes or the extra metabolic burden of GFP overexpression. However, our results showed that the selected transformants exhibited comparable mating ability and pathogenicity as wild-type *S. scitamineum*. This indicates that GFP and RFP expression might not necessarily interfere with the mating or virulence of *S. scitamineum*. The finding is in good agreement with the analysis of GFP effect on pathogenicity in *U. maydis* (Spellig et al. 1996) and *Z. tritici* (Kankanala et al. 2007; Schuster et al. 2015).

We were able to monitor the mating process of *S. scitamineum* with GFP and RFP reporter strains. Production of a long tube toward sporidia of opposite mating type was clearly seen under microscopy. Tube conjugation and fusion could be judged by diffusion of GFP and RFP signal into cytosol of opposite cells. Our results showed that using GFP and its derivative to tag compatible *MAT* strains could provide valuable information on critical process of fungal life cycle development. For example, by examining the relative locations of GFP and RFP fluorescence, we found that sporidia fusion, which is a good indicator of successful mating, occurred at 4 to 8 h post mating under the experimental conditions used in this study.

Fig. 3. Expression of GFP and RFP in *Sporisorium scitamineum* teliospores development. Teliospores harvested from sugar cane infected with *MAT-1G* × *MAT-2R* were incubate on 2% agar plates and imaged at 0 h (A-D) and 6 h (E-H), postinoculation. (A, E) DIC; (B, F) GFP; (D, H) RFP; (C, G) merged images of GFP and RFP. Scale bar: 10 μ m.

A critical comparison of the entire infection cycle using this dual-color imaging system, including in planta stages, in comparison with the wild type strains would certainly benefit mycologists and plant pathologists who are working on sugarcane smut by understanding the part of the life cycle of *S. scitamineum*. However, our repeated attempts to visualize invasion of host plant tissues by transformants with GFP or RFP signal failed, due to strong autofluorescence of sugarcane tissues (data not shown). We are currently working on conditions to minimize the autofluorescence of the sugarcane tissues to facilitate observation of the fluorescence tagged fungal cells. We are also looking for alternatives, artificial surface/materials that support dikaryotic filament growth and could be used for epifluorescent microscopy observation beyond 12 h post inoculation. Nevertheless, in addition to observation of fungal life cycle events, this dual-color imaging systems could also be useful for other applications, such as characterization of environmental factors that might affect the fungal life cycle and screening of inhibitors that could interfere with mating and meiosis.

In conclusion, we successfully expressed GFP and RFP in the phytopathogenic fungus *S. scitamineum*, and developed a dual-color imaging system to monitor its pathogenic life cycle. We visualized detailed structure/morphology transition and cellular fusion/division during sexual mating and teliospore development with this system. Our results illustrate the application of such reporter system for better understanding of *S. scitamineum* life cycle development and pathogenicity. This dual-color system would be a valuable tool for further investigation of biotic and abiotic factors that might affect the pathogen life cycle and virulence.

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