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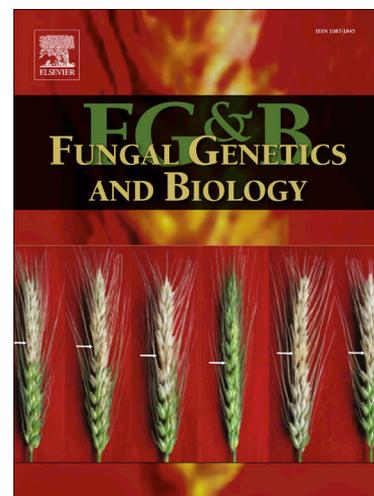
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A SET domain-containing protein involved in cell wall integrity signaling and peroxisome biogenesis is essential for appressorium formation and pathogenicity of *Colletotrichum gloeosporioides*

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ABSTRACT

The chromatin modulator Set5 plays important regulatory roles in both cell growth and stress responses of *Saccharomyces cerevisiae*. However, its function in filamentous fungi remains poorly understood. Here, we report the pathogenicity-related gene *CgSET5* discovered in a T-DNA insertional mutant M285 of *Colletotrichum gloeosporioides*. Bioinformatic analysis revealed that *CgSET5* encodes a SET domain-containing protein that is a homolog of the budding yeast *S. cerevisiae* Set5. *CgSET5* is important for hyphae growth and conidiation and is necessary for appressorium formation and pathogenicity. CgSet5 regulates appressorium formation in a mitogen-activated protein kinase-independent manner. Inactivation of *CgSET5* resulted in a significant reduction in chitin content within the cell wall, indicating CgSet5 plays a vital role in cell wall integrity. CgSet5 is involved in peroxisome biogenesis. We identified CgSet5 as the histone H4 methyltransferase, which methylates the critical H4 lysine residues 5 and 8 in *C. gloeosporioides*. We carried out a yeast two-hybrid screen to find CgSet5 interacting partners. We found CgSet5 putatively interacts with an inorganic pyrophosphatase named CgPpa1, which co-localized in the cytoplasm with CgSet5. Finally, CgPpa1 was found to strongly interact with CgSet5 *in vivo* during appressorium formation by bimolecular fluorescence complementation assays. These data corroborate a complex control function of CgSet5 acting as a core pathogenic regulator, which connects cell wall integrity and peroxisome biogenesis in *C. gloeosporioides*.

Keywords: *Glomerella cingulata*, virulence, conidiogenesis, apple, peroxisome, anthracnose

1. Introduction

Glomerella leaf spot of apple (GLSA), caused by *Colletotrichum gloeosporioides* (anamorph of *Glomerella cingulata*), is a destructive disease during apple cultivation that severely threatens the production of apples worldwide. Under favourable conditions, GLSA can result in 75% defoliation by harvest, weakening trees and reducing yield (Bogo et al., 2012; González et al., 2006; Velho et al., 2015). GLSA can be controlled with the use of fungicides. Although frequent applications are effective, it can also be expensive and poses potential damage to the environment and human health (Moreira et al., 2014). Therefore, there is an urgency to identify new anti-GLSA strategies.

Like most other fungal pathogens, the appressorium plays a central role in the disease cycle of *C. gloeosporioides* by producing penetration pegs that mediate direct penetration of the host epidermal cells (Wang et al., 2018). Many genes involved in appressorium formation in *Colletotrichum* have been identified. Signaling pathways, such as cAMP-dependent protein kinase A (PKA) and mitogen-activated protein kinase (MAPK), have been shown to play pivotal roles in fungal pathogenesis (Kubo and Takano, 2013; Yong et al., 2013). The *CgPKAC* gene encoding a catalytic subunit of PKA is required for normal appressorium formation (Priyatno et al., 2012). The MAPK cascade consisting of CgMek1, CgMk1 and Cgl-SLT2 is known to regulate late stages of appressorium formation (He et al., 2017; Kim et al., 2000; Yong et al., 2013). In addition, *CgRAC1*, which encodes a Rho GTPases, is important for conidial morphogenesis and appressorium maturation (Nesher et al., 2011). Moreover, a laccase gene *LAC1* is involved in appressorium formation. Conidia of the *LAC1* deletion mutant germinate normally, but the end of the germ tube does not differentiate into appressoria (Tsuji et al., 2001). Recent studies have demonstrated two cation stress-responsive transcription factors SltA and CrzA that regulate morphogenetic processes with SltA be essential for appressorium formation (Dubey et al., 2016). Histones are subject to posttranslational modifications with structural and functional consequences (Brosch et al., 2008).

The SET domain proteins are involved in chromatin regulation by catalyzing the methylation of lysine residues of histones (Freitag, 2017; Xiao et al., 2003). The SET domain is a ~ 130 amino acid motif and evolutionarily conserved in a large number of eukaryotic proteins as well as a few bacterial proteins (Zhang et al., 2002). The SET domain proteins were named after three proteins

first identified in *Drosophila melanogaster*: suppressor of variegation 3-9 [Su(var)3-9], enhancer of zeste [E(z)], and trithorax (Trx) (Jenuwein et al., 1998). SET proteins were later found in a diverse group of organisms ranging from yeast to mammals (Jenuwein, 2001). For example, SpSet9 catalyzes H4K20me3 function and is involved in the maintenance of the heterochromatin in metazoans (Hori et al., 2014; Schotta et al., 2004). In *Saccharomyces cerevisiae*, Set3C represses genes in the early/middle phase of the yeast sporulation program, including the key meiotic regulators Ime2 and Ndt80 (Pijnappel et al., 2001). *Candida albicans* Set3C is required for efficient biofilm production and drug resistance (Nobile et al., 2014). *S. cerevisiae* Set5 is evolutionarily conserved and monomethylates lysines 5, 8, and 12 of the histone H4 tail to play a role in several aspects of chromatin function, including transcription and DNA damage responses (Martin et al., 2014). Set5 also appears to play a key role in regulating cell growth and stress responses, particularly in collaboration with Set1 (Green et al., 2012a; Jezek et al., 2017).

Despite the research progress made in SET-domain containing proteins, their functions from plant pathogenic fungi remains obscure. In this study, we have here characterized a SET domain-containing protein, CgSet5, from *C. gloeosporioides* and elucidated its function in the growth, conidiation, appressorium formation and pathogenicity of *C. gloeosporioides*.

2. Materials and methods

2.1. Strains, culture conditions, growth tests, and pathogenicity assays

The fungal strains were cultured on PDA plates at 26°C as described previously (Wu et al., 2016). For transformant selection, hygromycin B or G-418 sulphate was added to PDA with final concentrations of 100 µg/ml or 500 µg/ml, respectively. Aliquots of 1 µl of the conidial suspension were inoculated in the centre of the PDA plate to assess the growth and colony characteristics. The conidiation and appressorium formation were performed as described previously (Zhou et al., 2017). For observing appressorium formation on the leaf, conidial suspensions (1×10^4 /ml) were inoculated onto apple leaves incubated in the center of water agar (12%) plates, and the apple leaves were observed for appressorial formation at 20 hours cultured under a microscope. For infection assays, apple leaves were sprayed with conidial suspensions (1×10^5 conidia/ml). After inoculation, the plants were transferred and incubated in a glasshouse under humid conditions with 70–80%

relative humidity and 28-30°C. The severity of the GLSA on each leaf was estimated with the aid of diagrammatic scale (Bogo et al., 2012). Disease lesions were examined at 4 days post-inoculation. For punch-infection assays, a droplet (2 µl) of freshly harvested conidial suspensions (1×10^5 conidia/ml) was dropped onto the obverse of wounded apple leaves (cv Gold delicious), and inoculated leaves were placed on plates containing water agar (12%). Disease lesions were examined at 3 days post-inoculation. Each test was repeated three times with three repetitions for each time. Cell wall integrity assays were performed on CM plates [$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1.5g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KCl, 0.5 g; KH_2PO_4 , 0.03 g; tryptone, 1 g; yeast extract, 1 g; glucose, 10 g and agar 15 g in 1 l distilled water, pH 7.0] with Congo red (800 µg/ml), CFW (50 mg/ml) or SDS (0.005%). The data were performed by DPS statistical analysis software, using analysis of variance (ANOVA) (Tang and Zhang, 2013).

2.2. Nucleic acid manipulations

Fungal genomic DNA was extracted as described previously (Zhou et al., 2017). Standard procedures for DNA manipulations, such as plasmid DNA isolation, restriction enzyme digestion, and hybridization, were followed. Procedures for both gel electrophoresis and restriction enzyme digestions were performed with standard protocols (Stacey and Isaac, 1994). Southern blot analysis was performed with Amersham ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare, UK) as previously described (Zhang et al., 2014a). Total RNA was isolated from frozen fungal mycelia, spores or inoculated rice leaves with the RNA simple Total RNA Kit (TIANGEN, China) according to the manufacturer's protocol, treated with RNase-free DNase I (TaKaRa Co., Dalian, China) before subsequent RNA manipulations.

2.3. Cloning and identification of the T-DNA tagged gene

The right flanking sequence of T-DNA was identified using a high-efficiency thermal asymmetric interlaced PCR (*ht*TAIL-PCR), as described previously (Liu and Chen, 2007). The PCR product was cloned, sequenced, and then aligned to *C. fructicola* Nara gc5 assembly as a reference (Gan et al., 2013) using the G-InforBIO program (Tanaka et al., 2006). The ORF sequence of gene tagged by T-DNA was amplified using primer pairs of T1 / T2 (Table S1). To assess the absence or presence of introns in *CgSET5*, RNA was extracted from the WT hyphae as described previously (Zhou et al., 2017). The cDNA was synthesized using PrimeScript strand cDNA Synthesis Kit

(TaKaRa Co., Dalian, China) following the supplier's instructions. *CgSET5* transcripts were detected by reverse-transcriptase polymerase chain reaction (RT-PCR) with primers T3 and T4 (Table S1). The protein-coding sequence was identified by aligning between the cDNA sequence and the gene sequence using the Clustal X (Larkin et al., 2007). Conserved Domain was drawn by using InterProScan (Hunter et al., 2012; Mitchell et al., 2015).

2.4. Vector construction and fungal transformation

To generate a *CgSET5* complementation vector, a DNA fragment containing the *CgSET5* and its putative promoter was amplified with primers C1 and C2 (Table S1), cloned into the binary vector pGapneoR10C1. The expression plasmids pGapneoR49 consisting of mCherry-PTS1, under the control of the *gpdA* promoter, was introduced into the WT and the M285 to visualize the localization of the peroxisome. For constructing a CgPpa1-mCherry fluorescent vector, a fusion fragment containing the entire protein-coding sequence with its native promoter was cloned into the binary vector pXcherry2C1. The fungal transformation was performed as described previously (Zhou et al., 2017). The primers used in this study were shown in Table S1.

2.5. Gene expression analysis

One μg of total RNA was used to synthesize cDNA using PrimeScript strand cDNA Synthesis Kit (TaKaRa Co., Dalian, China) following the supplier's instructions. Reverse-transcription PCR (RT-PCR) was performed with the PrimeScript strand cDNA Synthesis Kit (TaKaRa Co., Dalian, China) following the supplier's instructions. Quantitative RT-PCR (qRT-PCR) reactions were performed with the TransStart Tip Green qPCR SuperMix (Trans, China), employing the Real-Time PCR System (BIO-RAD, USA). Quantification of β -tubulin gene (GenBank accession number: U14138) expression was performed as an internal reference. The relative transcript level of treatment versus control was calculated as its transcript ratio using the method $2^{-\Delta\Delta\text{Ct}}$ (Livak and Schmittgen, 2001). For each sample, three independent biological replicates were analyzed to calculate the mean and standard deviation. The primers used in qRT-PCR experiments are shown in Table S1.

2.6. Immunoblotting

Fungal mycelia powder was suspended in $1\times$ TBS buffer [10 mM Tris-HCl (pH 7.5), 600 mM NaCl, 300mM sucrose, 5mM MgCl_2 , 5mM ethylenediaminetetraacetic acid (EDTA) , 1 mmol

phenylmethylsulfonyl fluoride (PMSF)]. The homogenates were centrifuged (16,000 g, 20 min), and supernatants were collected. Proteins in the supernatant were then heat-treated at 80°C for 10 min to precipitate contaminating proteins. The supernatant was recovered by centrifugation and subjected to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF). Blots were probed with the following antibodies: Anti-H4, Anti-H4K5me1 and Anti-H4K8me1 rabbit polyclonal antibodies were raised using peptides as antigen (Abmart). Proteins reacting with the primary antibodies were visualized by appropriate peroxidase (HRP)-conjugated secondary antibodies. Imaging was performed using a BIO-RAD ChemiDoc™ Imaging System.

2.7. CFW staining and measurement of the chitin content

Calcofluor staining, using Calcofluor White M2R (1 g/ml, Sigma-Aldrich, USA), was performed as described previously (Yin et al., 2016). The fungal strains were cultured in liquid CM for 48 h. hyphae were removed and stained with 10 mg/ml CFW for 2 min in the dark, rinsed twice with phosphate-buffered saline (PBS) and viewed under a fluorescence microscope (Leica TCS SP8, Germany). The amount of chitin was estimated by measurement of the glucosamine content. The preliminary analysis allowed us to set up the following optimal conditions for hydrolysis: 25 mg of dried mycelium and 5 ml 6 M HCl were transferred in tubes which were then sealed and heated 6 hours at 100°C. The chitin content (μg of glucosamine/mg of the dry weight of fungal biomass) was measured by as described previously (Kong et al., 2012).

2.8. Construction and screening of the yeast two-hybrid libraries

The yeast two-hybrid libraries were constructed, in collaboration with Shanghai OE Biotech (China). Total RNA was extracted from the hyphae and conidia mixtures using Trizol (Invitrogen, USA), mRNA was isolated using the FastTrack®MAG mRNA Isolation Kit (Invitrogen). Purified cDNA ligated with three different forward adapters, were mixed and transformed into *E. coli* (DH10B) by electroporation. The cDNA library plasmid was recombined with pGADT7-DEST using the Gateway1 LR Clonase™ II enzyme mix (Invitrogen). The *CgSET5* was cloned into the pGBKT7 BD vector to generate pGBKTS1. Screening for the CgSet5-interacting proteins was performed according to the manufacturer's instructions (Clontech). To verify positive interactions, full-length ORF of the *CgPPA1* was cloned into the pGADT7 AD vector and cotransformed with pGBKTS1 into Y2HGold Competent Cells. The Cells were grown at 30°C for 3 days on SD/-Ade/-

His/–Leu/–Trp medium containing 2.5 mM 3-AT (depending on the bait-prey combination).

2.9. Construction of bimolecular fluorescence complementation (BiFC) strains

A BiFC strain expressing CgSet5-YFP¹⁷³⁻²³⁸ and CgPpa1-YFP¹⁻¹⁷³ was generated to study at the interactions between the two proteins *in vivo*. For this purpose, two constructs were generated, one in which *CgSET5* was cloned into pR44H3YFP-9 and tagged C-terminally to YFP¹⁷³⁻²³⁸ under the glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter; whereas the second construct involved cloning *CgPPA1* into pR32YFP-1 and tagged at the C-terminus to YFP¹⁻¹⁷³ under the *gpdA* promoter. The selected strains were confirmed by PCR analysis. The plasmid maps used in this study were shown in Additional File 1.

3. Results

3.1. Identification of *CgSET5* from *C. gloeosporioides*

To identify novel infection-related genes, we screened a *C. gloeosporioides* T-DNA insertional library (Wu et al., 2016). We identified one transformant (M285) that was unable to cause disease lesions on apple leaves, (cv Gold delicious) (Fig. 1A, B). A single T-DNA insertion in the M285 genome was identified by Southern blot analysis (Fig. S1A), using the hygromycin phosphotransferase gene as a probe. The insertion site of the T-DNA within the M285 was located at the upstream site (-82 bp) of a predicted gene *CgSET5* (CGGC5_4508) in the *C. gloeosporioides* genome (Gan et al., 2013) (Fig. S1B). The *CgSET5* gene encodes a 561 amino-acid protein containing a SET domain (265-506 aa) in its C-terminal terminus (Fig. S1C). CgSet5 is highly conserved with other Set5 proteins identified from fungal species including *Verticillium dahliae* (63% identity to VDAG_06514), *Neurospora crassa* (61% identity to NCU00089), *Fusarium graminearum* (61% identity to FGSG_07032), *Magnaporthe oryzae* (57% identity to MGG_10152), *Aspergillus fumigatus* (42% identity to Y699_06971), and *Saccharomyces cerevisiae* (42% identity to Set5). Interestingly, the *CgSET5* gene is not present in *Schizosaccharomyces pombe* (NCBI blast, Cut-off $\geq 10^{-5}$). This finding indicates that CgSet5 homologs are highly conserved in phytopathogenic fungi (Fig. S1D).

As expected, transcripts of *CgSET5* were not detected in M285 by RT-PCR analysis, while the transcripts of *CgSET5* were present in the wild type isolate W16 (WT) (Fig. S1E). Before exploring

the functions of *CgSET5*, we evaluated the transcriptional levels in various growth and infection-related-development stages. The expression of *CgSET5* was higher in the conidium and appressorium compared to mycelium, with the highest level of expression occurring in conidia (Fig. 1C). These observations suggest that *CgSET5* plays an important role in conidiation-related morphogenesis.

3.2. CgSet5 is indispensable for full virulence

To determine the function of *CgSET5* we functionally complemented the M285 mutant with a C-terminal *CgSET5*-GFP fusion under the control of its native promoter. The resulting complemented strain C5 could restore the ability of *C. gloeosporioides* to cause disease, confirming that the observed phenotypes of the M285 mutant are caused by the inactivation of *CgSET5* (Fig. 1A). The M285 mutant failed to proliferate *in planta* even when inoculated onto wounded apple leaves (Fig. 1D), suggesting a requirement for *CgSET5* activity for *in planta* growth. We concluded *CgSET5* is important for pathogenicity. Plant pathogenic fungi including *Colletotrichum* spp. secrete an array of cell wall-degrading enzymes (CWDEs) to depolymerize the polysaccharides of primary cell walls during infection and colonization (Centis et al., 1996; Li and Goodwin, 2002; Shih et al., 2000; Wei et al., 2002; Yakoby et al., 2001; Zhou et al., 2017). We were interested in determining whether *CgSet5* has roles in regulating the expression of endopolygalacturonase *CgPG1*, endopolygalacturonase *CgPG2*, pectin lyase *pnl-1*, pectin lyase *pnl-2*, pectate lyase *pelA*, and pectate lyase *pelB* (Zhou et al., 2017). We examined the expression of six CWDEs (*CgPG1*, *CgPG2*, *pnl-1*, *pnl-2*, *pelA*, and *pelB*) that were continuous in the fungal development and infection (Fig. S2). Compared with the WT, the M285 mutant showed a significant increase in *CgPG1* and *CgPG2* transcripts, however, transcripts of *pnl-1*, *pnl-2*, *pelA*, and *pelB* were not affected (Fig. 1E), suggesting *CgSet5* negatively regulates *CgPG1* and *CgPG2* expression.

3.3. CgSet5 is important for aerial hyphae growth, conidiation, and appressorium formation

A striking characteristic of the M285 mutant was its impaired aerial hyphae growth and colony morphology (Fig. 2A, B and Table S2). The M285 mutant formed smaller conidia (av. $8.16 \times 3.81 \mu\text{m}$) compared with those of the WT (av. $13.13 \times 5.04 \mu\text{m}$) (Table S2). On hydrophobic glass slides, conidia of M285 could germinate to form germ tubes after 4 hours of incubation, indicating that *CgSET5* is dispensable for conidial germination (Table S2). At 8 hours post-incubation, both the

WT and the *CgSET5* complemented strain C5 produced appressoria, whereas M285 did not form appressoria. After prolonged incubation of 24 hours post incubation and even 48 hours post incubation, the M285 mutant still failed to form appressoria (Fig. S3). Consistently, the WT produced normal appressoria on apple leaves, but the M285 mutant did not form appressoria (Fig. 2C), indicating *CgSet5* is essential for appressorium development of *C. gloeosporioides*. The role of cAMP/PKA signaling in stimulating appressorium formation on artificial surfaces is well known (Han et al., 2015; He et al., 2017; Yue et al., 2016; Zhu et al., 2017). To test whether cAMP rescues the defect of M285 in appressorium formation, 5 mM exogenous cAMP was added to conidial suspensions on hydrophobic surfaces but no effect was found (Fig. 2D), suggesting *CgSet5* functions independently of a cAMP/PKA signaling in appressorium formation.

3.4. *CgSet5* is involved in cell wall integrity

The fungal cell wall plays an essential role in maintaining hyphal morphology and adapting to changes in the environment (Qi et al., 2012). We observed hyphal growth was impaired in M285 (Fig. 2A). We investigated the effects of various cell wall perturbing agents on M285, including sodium dodecyl sulfate (SDS), calcofluor white (CFW) and Congo red (Fig. 3A). SDS disrupts the plasma membrane, whereas CFW and CR specifically bind to chitin and β -1, 3-glucan of the cell wall (Elorza et al., 1983; Wood et al., 1983). Mycelium from WT and the M285 mutant were inoculated onto medium containing varying concentrations of CFW, SDS and Congo red and their sensitivity was assessed post-incubation for 6 days. We found the M285 mutant grew significantly slower on CFW medium than the WT. Growth on both CR and SDS medium was unaffected (Fig. 3B). Furthermore, CFW staining was used to visualize the distribution of chitin in the cell wall (Miguel-Rojas and Hera, 2016). Interestingly, the CFW fluorescence of M285 was considerably weaker than the WT (Fig. 3C). Further measurement of the chitin contents in the cell wall, we revealed that the chitin content was reduced to 65% in M285, compared with WT (Fig. 3D). These results collectively indicate that *CgSet5* plays a vital role in cell wall integrity.

3.5. *CgSet5* methylates the histone H4

To determine if the histone H4 is methylated by *CgSet5* in *C. gloeosporioides*, we purified histones from WT and the M285 mutant and probed them with specific antibodies. In the M285 mutant, the signals of H4K5me1 and H4K8me1 were reduced to the undetectable level compared

with WT (Fig. 4). The decreased levels of histone methylation in the M285 mutant were completely recovered in gene complementation strain C5. These results indicated that CgSet5 catalyzes methylation of H4K5 and H4K8 in *C. gloeosporioides*.

3.6. CgSet5 is involved in peroxisome biogenesis

To determine whether CgSet5 mediates import of matrix proteins into peroxisomes, we introduced mCherry-PTS1 (the tripeptide SKL, peroxisomal targeting signal 1) (Titorenko and Rachubinski, 2001) into the WT, M285, and C5 strains. The *WT:mCherry:PTS1* and *C5:mCherry:PTS1* showed a punctate pattern of fluorescent labeling in conidia (Fig. 5A), consistent with peroxisomal localization of the fusion proteins. In contrast, M285:mCherry:PTS1 revealed a strong reduction of peroxisome numbers (Fig. 5B), indicating the biogenesis of peroxisomes is impaired in M285.

3.7. Yeast two-hybrid screen identified CgSet5 specific interactions

To identify CgSet5-interacting proteins, we performed a yeast two-hybrid capture experiment. A total of 10 distinct putative interactors were identified and confirmed by re-transformation into yeast (Table 1 and Fig. S4). CGGC5_9418 is homologous to the yeast NDUFV2 that encodes the 24 kDa subunit of the NADH-ubiquinone oxidoreductase complex (complex I) of the mitochondrial respiratory chain and is involved in electron transfer (Benit et al., 2003; de Coo et al., 1995). CGGC5_10359 is homologous to the yeast SAR1 that plays an essential role during the protein transport from the endoplasmic reticulum to the Golgi apparatus (Jiang et al., 2002). CGGC5_13693 is homologous to the yeast Pdb1 that is involved in the conversion of pyruvate into acetyl-CoA (Miran et al., 1993; Pronk et al., 1996). CGGC5_9977 is homologous to the yeast Ppa1 that catalyzes the hydrolysis of inorganic pyrophosphate (Kolakowski et al., 1988; Lundin et al., 1991). CGGC5_10102 is homologous to the yeast Pex19 that is essential for peroxisomal membrane proteins targeting and import (Gotte et al., 1998; Sacksteder et al., 2000). CGGC5_2753 is homologous to the yeast Ugdh that catalyzes the conversion of UDP-glucose to UDP-glucuronic acid (Ikushiro et al., 2016). CGGC5_5510 is homologous to the *Alternaria alternata* SdhB that possess succinate dehydrogenase activity (Avenot et al., 2008). The captured proteins also included two uncharacterized proteins (CGGC5_1060 and CGGC5_1838) with unclear functions.

3.8. CgSet5 physically interacts with CgPpa1 *in vivo*

The Ppa1 is an inorganic pyrophosphatase, which is essential for growth in *S. cerevisiae* (Lundin et al., 1991). We designated *C. gloeosporioides* CGGC5_9977 as CgPpa1 (Table 1). CgSet5-GFP significantly overlapped with CgPpa1-mCherry in the co-localization analysis (Fig. 6A). To further confirm the CgSet5-CgPpa1 interaction *in vivo*, the CgSet5-YFP¹⁷³⁻²³⁸ and CgPpa1-YFP¹⁻¹⁷³ fusion constructs were generated and transformed into WT for bimolecular fluorescence complementation (BiFC) assays. In the resulting transformant FC1, very weak YFP signals were observed in the cytoplasm of the fungal hyphae (Fig. S5). However, stronger YFP signals were observed in the appressoria (Fig. 6B). These results confirmed that the interaction between CgSet5 and CgPpa1 increases during appressorium formation. To assess the role of CgPpa1 in *C. gloeosporioides*, we tried to generate a CgPpa1 deletion strain by gene replacement, however, we failed to obtain a deletion mutant for CgPpa1, suggesting like yeast, deletion of CgPpa1 may be lethal to *C. gloeosporioides* (Lundin et al., 1991).

4. Discussion

The fungal cell wall primarily acts as a barrier against mechanical stress and is required to maintain cell shape and cell division (Gow et al., 2017; Levin, 2011). In this study, we found that the M285 mutant showed reduced chitin content in hyphae. Thus, the inactivation of *CgSET5* may weaken the strength and tenacity of the fungal cell wall. Previous studies have revealed that most cell wall-defective mutants involved in cell wall integrity exhibit various vegetative hyphal growth defects and are nonpathogenic (Albarouki and Deising, 2013; Li et al., 2017; Oliveira-Garcia and Deising, 2016). Consistent with these studies, the M285 mutant resulted in a significant defect in vegetative growth and lost the ability to colonize the host cell. The polarized synthesis of cell wall components such as chitin is essential for the hyphal tip growth of filamentous fungi (Cabib et al., 2007; Takeshita et al., 2006). Interestingly, the CFW staining showed that the chitin is poor in the hyphal tips of the M285 mutant. Thus, it is reasonable to speculate that the defects of vegetative growth and pathogenicity of the M285 mutant result from a breach in cell wall integrity.

CgSet5 belongs to the SET domain protein family responsible for methylation at histone H4 and is homologous to yeast Set5. In yeast, Set5, as H4 methyltransferase, targets the functionally important lysines 5, 8, and 12 of the histone H4 tail (Green et al., 2012a). Because the $\Delta set5$ mutant showed specific decreases in the H4K5me1 and H4K8me1 signal in yeast (Green et al., 2012a;

Green et al., 2012b), CgSet5 is likely involved in methylation of histone H4 in *C. gloeosporioides*. Consistent with this hypothesis, H4K5me1 and H4K8me1 were reduced to the undetectable level in the M285 mutant, indicating that CgSet5 catalyzes H4K5 and H4K8 methylation in *C. gloeosporioides*. Previous studies illustrated that Set5 interacts with chromatin in cells and maintained transcriptional repression (Martin et al., 2014). Although it is not clear that CgSet5 plays the roles in stabilizing repressive activities at chromatin in *C. gloeosporioides*, we have shown that inactivation of *CgSET5* resulted in the drastic increase of CgPG1 and CgPG2 transcripts. It has been reported previously that PG1 and PG2 disintegrated the endopolygalacturonases of the plant cell walls during infection and colonization (Centis et al., 1996; Li and Goodwin, 2002). This is inconsistent, however, with the M285 mutant failing to colonize in the host cells. Recently, it was shown that the fungal endopolygalacturonases are recognized as microbe-associated molecular patterns by the host and induced systemic resistance of the host (Sarrocco et al., 2017; Zhang et al., 2014b). Thus, CgSet5 maybe plays a role in suppressing defense response of the host, possibly by inhibiting the production of the endopolygalacturonases during the initial infection.

In every living cell, interacting proteins form a regulatory network that controls the biological functions (He et al., 2008). Using the yeast two-hybrid, we identified Pex19 (CGGC5_10102) as an interaction partner of CgSet5. Previous studies reported that Pex19 plays key roles in peroxisomal membrane assembly and maintenance of peroxisomal structures (Li et al., 2014; Peraza-Reyes et al., 2011; Rottensteiner et al., 2004). We observed that the inactivation of *CgSET5* results in a strong reduction of peroxisome numbers. These finding suggested that CgSet5 is involved in peroxisome biogenesis by cooperating with Pex19. Moreover, we found that the inorganic pyrophosphate CgPpa1 interacts with CgSet5 in *C. gloeosporioides*. Interestingly, inorganic pyrophosphatase has a function in peroxisomal redox energy metabolism (Stiebler et al., 2014; Watkins and Ellis, 2012). But the role of CgPpa1 in *C. gloeosporioides* was not uncomprehending, because CgPpa1 may be lethal to *C. gloeosporioides*, consistent that the PPA1 disruption is lethal to yeast. We also found that CgSet5 interacts with a series of proteins, such as mitochondrial respiratory chain, and succinate dehydrogenase, but the exact functions of these proteins will be explored in the future.

Taken together, we have identified an important pathogenic factor, CgSet5, which regulates

appressorium formation in a MAPK-independent manner. Our results indicated that CgSet5 plays an important role in growth, conidiation, cell wall integrity, and peroxisome biogenesis in *C. gloeosporioides*.

Acknowledgments

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Supporting Information legends

Fig. S1. Identification of T-DNA targeted gene *CgSET5*. (A) Southern blots analysis of the mutant M285. Total genomic DNA was digested with *Hind*III. The probe was amplified with primers H-F1 and H-R1 using the plasmid pCamhybgfp1 (used to carry out a mutagenesis population of WT, GenBank accession number: KX223837) as a template. M, λ DNA/*Hind*III digestion as molecular weight marker. (B) Position of the T-DNA insertion in the M285. The arrow (▼) indicates the insertion position of the T-DNA in M285. (C) Prediction of domains in *CgSet5* using the InterProScan program. (D) The phylogenetic tree of *CgSet5* homology proteins from several organisms was viewed using the MEGA 7.0 program by the Maximum Likelihood method with 1000 bootstrap replicates. All the *CgSet5* homology proteins were downloaded from the NCBI database and their accession numbers are as following: *Verticillium dahliae* (XP_009657187.1), *Neurospora crassa* (XP_011393746.1), *Fusarium graminearum* (XP_011326720.1), *Magnaporthe oryzae* (XP_003713790.1), *Aspergillus fumigatus* (KMK57430.1), *Saccharomyces cerevisiae* (Set5, NP_012077.1). (E) Transcript detection of *CgSET5* in WT and the M285 by RT-PCR analysis. M, molecular weight marker. WT, wild type strain. M285, the *CgSET5* mutant.

Fig. S2 Relative expression levels of *CgPG1*, *CgPG2*, *pnl-1*, *pnl-2*, *pelA*, and *pelB* in different development stages of the wild type strain W16. The data were normalized to β -tubulin gene expression. Error bars represent standard deviations. Lowercase letters represent significant differences ($P < 0.01$).

Fig. S3 *CgSet5* is essential for appressorium formation. The conidia suspension from WT, M285 and C5 were cultured on the hydrophobic glass slides at 28°C. WT, wild type strain. M285, the *CgSET5* mutant. C5, the *CgSET5* complementation strain.

Fig. S4 Yeast two-hybrid identification of *CgSet5*-interacting proteins. Yeast transformants expressing the labeled bait and prey constructs were assayed for growth on SD-Leu-Trp-His-Ade plates with 40 μ M X-a-gal. The interaction of pGBKT7-53 and pGADT7-T was used as the positive control while the interaction of pGBKT7-Lam and pGADT7-T served as the negative control.

Fig. S5 Bimolecular fluorescence complementation (BiFC) assay showing weak interaction between CgSet5 and CgPpa1 in hyphae. Bar equal to 2 μm .

Additional File 1 The plasmid maps used in this study.

Figure legends

Fig. 1 CgSet5 is required for pathogenicity. (A) Introduction of CgSet5-eGFP fusion construct into M285 recovered its pathogenicity defect. Photographs were taken 4 days post inoculation (dpi). (B) Disease severity was determined at 4 dpi. At least 10 apple leaves were examined in each replicate. (C) Transcription profiles of *CgSET5* at different stages of fungal development. The phase-specific expression of *CgSET5* was quantified by quantitative real-time polymerase chain reaction (qRT-PCR), with the synthesis of cDNA from the different developmental stages. HY, vegetative hyphae were harvested from 3-day-old PDA cultures. CO, conidia were collected from 10-day-old PDA cultures. AP, appressoria were harvested at 10 hours post incubation. 24 hpi, at 24 hours post inoculation. Lowercase letters represent significant differences ($P < 0.01$). (D) The M285 is nonpathogenic on wounded leaves. Photographs were taken at 3 days post inoculation. (E) Relative expression levels of *CgPG1*, *CgPG2*, *pnl-1*, *pnl-2*, *pelA*, and *pelB* in WT (grey column) and the M285 (white column). The data were normalized to β -tubulin gene expression. Error bars represent standard deviations. Asterisks indicate significant difference at $P = 0.01$. WT, wild type strain. M285, the *CgSET5* mutant. C5, the *CgSET5* complementation strain.

Fig. 2 CgSet5 is important for aerial hyphae growth, conidiation and appressorium formation. (A) Colonies of WT, M285 and C5. (B) Conidia were harvested from PDA and observed using light microscopy. Bars equal to 2 μm . (C) Penetration assay on onion epidermal cells. The WT and C5 can penetrate the epidermal cells and produce invasive hyphae. M285 fails to form appressoria. Photographs were taken at 20 hours post inoculation on onion epidermis. Bars equal to 10 μm . (D) M285 fails to form appressoria in the presence of 5 mM cAMP. Conidia were allowed to germinate on hydrophobic surfaces for 48 h in darkness. Bars equal to 10 μm . WT, wild type strain. M285, the *CgSET5* mutant. C5, the *CgSET5* complementation strain.

Fig. 3 CgSet5 plays an essential role in cell wall integrity. (A) Hyphae growth of M285 under cell

wall perturbing agents. The WT, M285 and C5 were cultured on CM plates with Congo red (800 $\mu\text{g/ml}$), Sodium dodecyl sulfate (SDS) (0.005%) and calcofluor white (CFW) (50 mg/ml) at 26°C for 6 days. (B) Bar chart showing the inhibition rates of the strains under cell wall perturbing agents. The growth inhibition rate is relative to the growth rate of each untreated control [Inhibition rate = (the diameter of the untreated strain - the diameter of the treated strain) / (the diameter of the untreated strain \times 100%)]. Error bars represent the standard deviations. Asterisks indicate significant difference at $P = 0.01$. (C) The distribution of chitin in the cell wall. Hyphae were stained with 10 $\mu\text{g/ml}$ Calcofluor white for 2 min in the dark, washed and images captured by epifluorescence microscopy. Bars equal to 2 μm . (D) Chitin content is significantly reduced in M285 when compared to WT. Asterisks indicate significant differences between the M285 and the W16 at $P = 0.01$. Error bars represent the standard deviations. Data represent three independent experiments. WT, wild type strain. M285, the *CgSET5* mutant. C5, the *CgSET5* complementation strain.

Fig. 4 Immunoblotting analysis of the histone H4 methylation by CgSet5 in *C. gloeosporioides*. Total protein extracted from hyphal cells was subjected to 12% SDS polyacrylamide gel electrophoresis, and probed with antibodies against H4, H4K5me1 and H4K8me1, respectively. WT, wild type strain. M285, the *CgSET5* mutant. C5, the *CgSET5* complementation strain.

Fig. 5 The *CgSET5* mutant is deficient in peroxisome biogenesis. (A) The confocal observation of peroxisomes visualized with mCherry-PTS1 in WT, M285 and C5. Bars equal to 2 μm . (B) Analysis of the peroxisomes occupied the area of cytoplasm in WT, M285 and C5, respectively. WT, wild type strain. M285, the *CgSET5* mutant. C5, *CgSET5* complementation strain.

Fig. 6 Visualization of the CgSet5-CgPpa1 interaction by the BiFC assay. (A) Co-localization of CgSet5-eGFP and CgPpa1-mCherry in the wild type strain W16. (B) Bimolecular fluorescence complementation (BiFC) assay showing the interaction between CgSet5 and CgPpa1 *in vivo*. Bar equal to 2 μm .

Dear Editor,

We thank you for your circumspective comments. We revised the manuscript seriously. We have rewritten the discussion section. We responded to the reviewers by point to point. The following is our answer to questions that you mentioned.

The editor:

I have completed my evaluation of your manuscript. As you can see, both reviewers think that your manuscript is interesting and have suggested some recommendations for improving the ms. Therefore, the decision is major revision. Please, note that both reviewers suggest extensive revision of the discussion section and I do agree. This also covers the connection between CWI and fungal development. The reviewers also mention some further experiments, e.g. with regards to the peroxisomes or the construction of a set5 mutant. I'd like to add that demonstration of the set5 mutant shows defects in H4 methylation would be nice.

Response: We added the histone H4 methylation experiment and revealed methylation on the histone H4 by the CgSet5. You could find the revised traces marked with highlights in the manuscript_R1, and you may notice revised contents as follow:

“Fungal mycelia powder was suspended in 1×TBS buffer [10 mM Tris-HCl (pH 7.5), 600 mM NaCl, 300mM sucrose, 5mM MgCl₂, 5mM ethylenediaminetetraacetic acid (EDTA), 1 mmol phenylmethylsulfonyl fluoride (PMSF)]. The homogenates were centrifuged (16,000 g, 20 min), and supernatants were collected. Proteins in the supernatant were then heat-treated at 80°C for 10 min to precipitate contaminating proteins. The supernatant was recovered by centrifugation and subjected to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF). Blots were probed with the following antibodies: Anti-H4, Anti-H4K5me1 and Anti-H4K8me1 rabbit polyclonal antibodies were raised using peptides as antigen (Abmart). Proteins reacting with the primary antibodies were visualized by appropriate peroxidase (HRP)-conjugated secondary antibodies. Imaging was performed using a BIO-RAD ChemiDoc™ Imaging System.” in 2.6. Immunoblotting of Materials and methods (Lines 133-143).

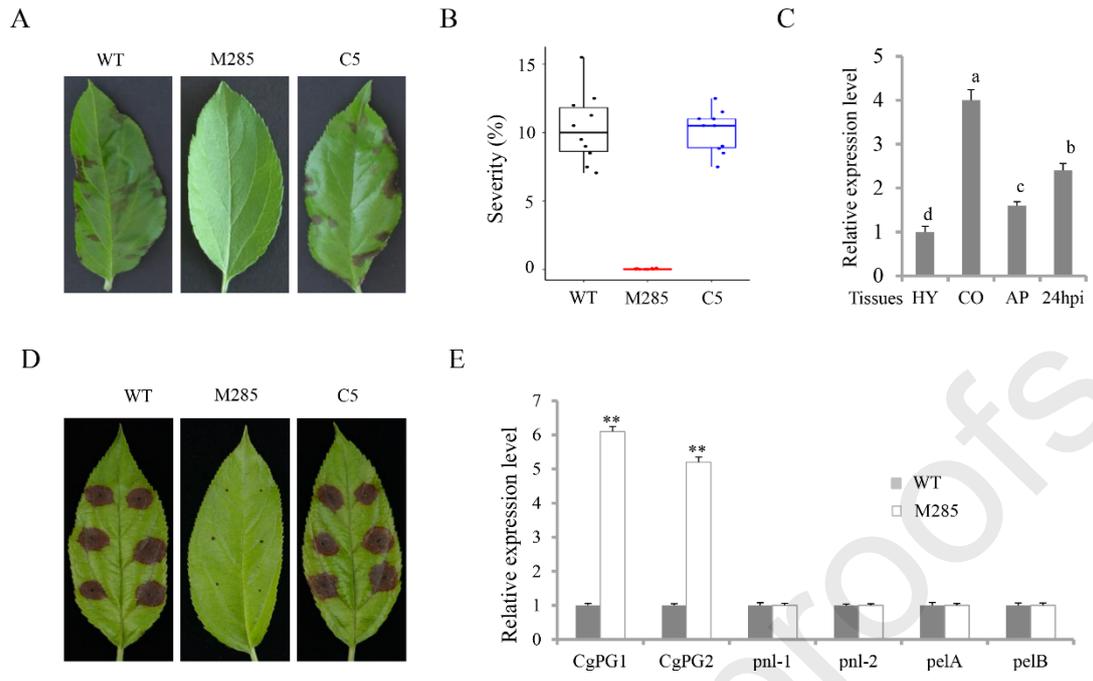
“To determine if the histone H4 is methylated by CgSet5 in *C. gloeosporioides*, we purified histones from WT and the M285 mutant and probed them with specific antibodies. In the M285 mutant, the signals of H4K5me1 and H4K8me1 were reduced to the undetectable level compared with WT (Fig. 4). The decreased levels of histone methylation in the M285 mutant were completely recovered in gene complementation strain C5. These results indicated that CgSet5 catalyzes methylation of H4K5 and H4K8 in *C. gloeosporioides*.” In 3.5. CgSet5 methylates the histone H4 of Results (Lines 246-252).

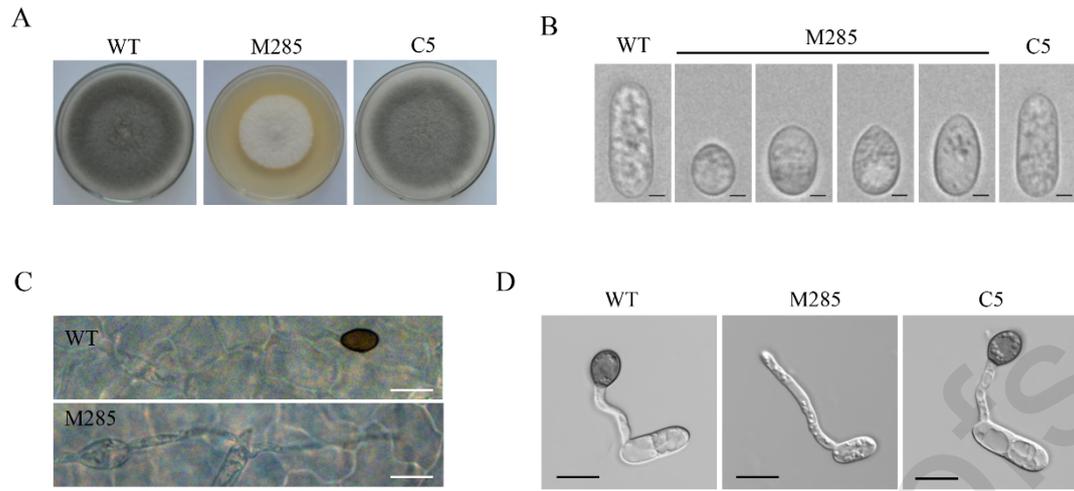
“Fig. 4 Immunoblotting analysis of the histone H4 methylation by CgSet5 in *C. gloeosporioides*. Total protein extracted from hyphal cells was subjected to 12% SDS polyacrylamide gel electrophoresis, and probed with antibodies against H4, H4K5me1 and

H4K8me1, respectively. WT, wild type strain. M285, the *CgSET5* mutant. C5, the *CgSET5* complementation strain.” In lines 607-610 and see Fig. 4

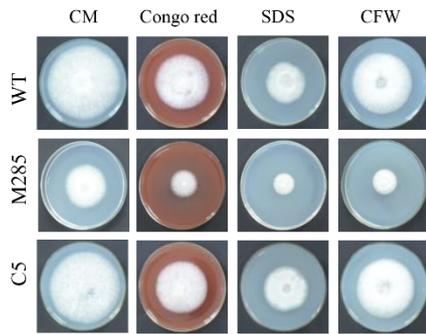
Moreover, we rewrote the discussion section. (Lines 292-334).

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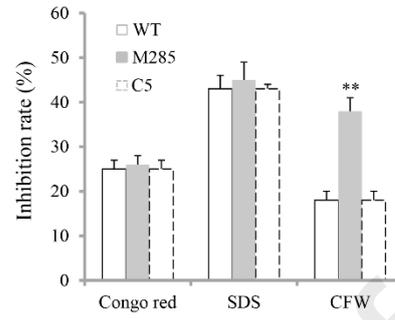




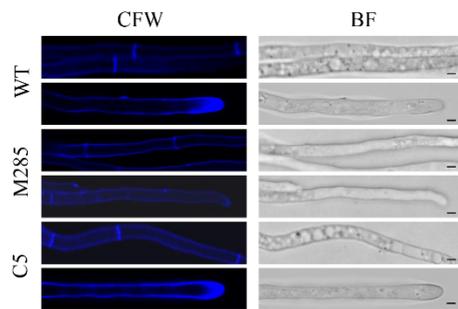
A



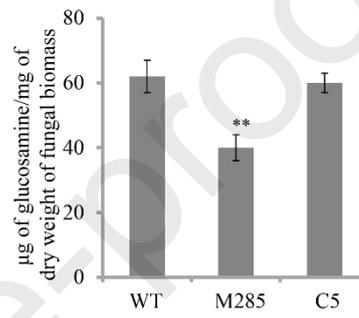
B

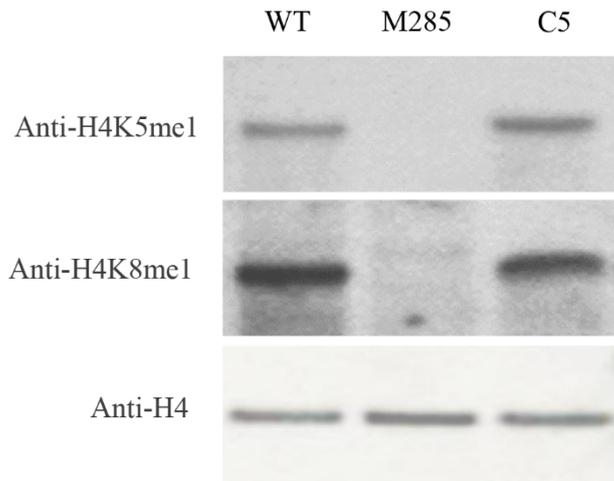


C

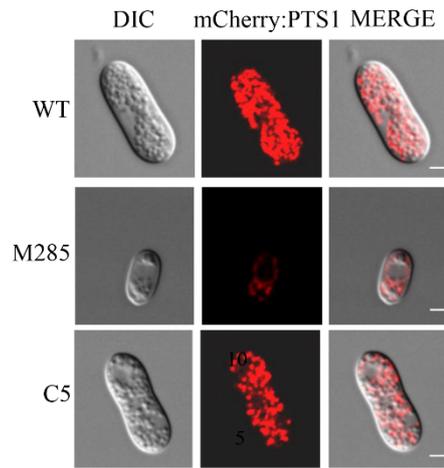
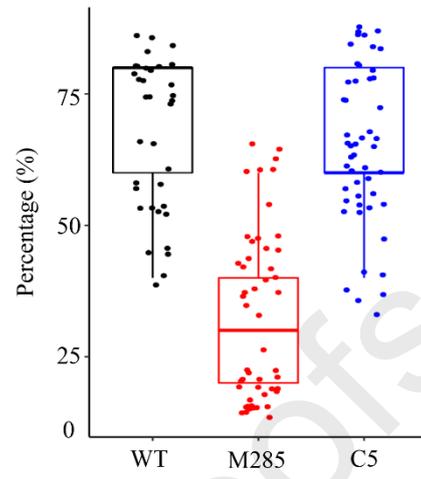


D





Journal Pre-proofs

A**B**

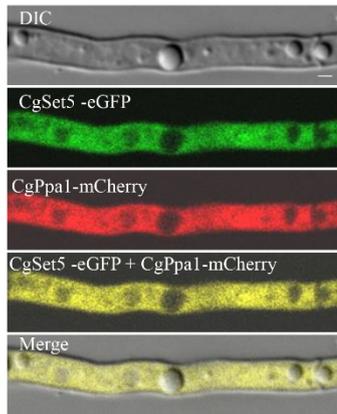
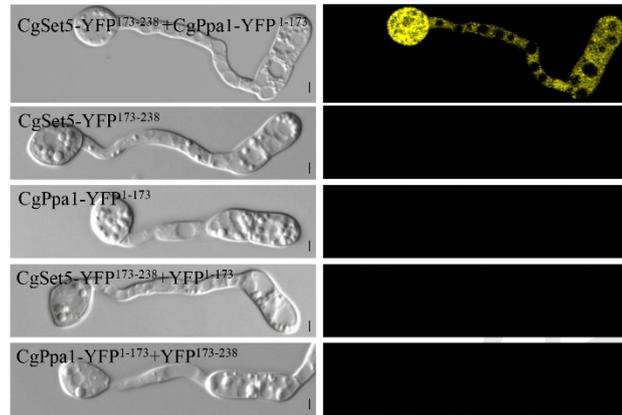
A**B**

Table 1. Identification of CgSet5-interacting proteins by yeast two-hybrid assays

Protein name	Predicted function
CGGC5_9418	NADH-ubiquinone oxidoreductase subunit
CGGC5_10359	small monomeric gtpase
CGGC5_13693	pyruvate dehydrogenase beta subunit
CGGC5_9977	inorganic pyrophosphatase
CGGC5_1060	duf1682 domain protein, hypothetical protein in filamentous fungi
CGGC5_10102	PEX19 family protein
CGGC5_2753	udp-glucose 6-dehydrogenase
CGGC5_8288	TPR domain containing protein
CGGC5_5510	succinate dehydrogenase iron-sulfur protein
CGGC5_1838	pectate lyase

Highlights

CgSet5 is indispensable for full virulence

CgSet5 is important for conidiation and appressorium formation

CgSet5 is involved in cell wall integrity

CgSet5 is involved in peroxisome biogenesis

CgSet5 methylates the lysine residues 5 and 8 of histone H4

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