Fungal Genetics and Biology 46 (2009) 42–54

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

A screening for suppressor mutants reveals components involved in the blue light-inhibited sexual filamentation in *Cryptococcus neoformans*

Yu-Ling Yeh [1], Yu-Sheng Lin [1], Bei-Jia Su, Wei-Chiang Shen *

Department of Plant Pathology and Microbiology, National Taiwan University, Room 216, Building Number 1, No. 1 Roosevelt Road, Sec. 4, 10617 Taipei, Taiwan

ARTICLE INFO

Article history:
Received 17 June 2008
Accepted 20 October 2008
Available online 25 October 2008

Keywords:
Cryptococcus neoformans
Photoresponse
Cwc1
Cwc2
Agrobacterium insertional mutagenesis
Suppressor

ABSTRACT

Blue light regulates diverse physiological and developmental processes in fungi. Our prior studies demonstrated that the evolutionally conserved Cwc1 and Cwc2 proteins mediate the blue light-inhibited sexual filamentation in *Cryptococcus neoformans*. To characterize the putative domains of the Cwc1 and Cwc2 proteins, we generated partially deleted versions of these genes under the *GPD1* promoter and examined their effects. The results confirmed that LOV and PAS domains are essential for the function of the Cwc1 protein, and the PAS domain and zinc finger DNA-binding motif are also crucial for the Cwc2 protein. To further understand how light inhibits filamentous growth, a genome wide mutant screening was conducted to identify genes important for this process. Mutants which suppressed the light-dependent *CWC1* overexpression phenotype and restored mating filamentation were identified. In the one with fully restored filamentation, the T-DNA was found to disrupt the expression of the *CWC2* gene. Additionally, a mediator component, the *SSN8* gene, known to involve in transcriptional regulation was also identified. Our results demonstrate that Cwc1 and Cwc2 are two central regulators of the *C. neoformans* photoresponses and the roles of other components identified in the screen are under investigation.

1. Introduction

Ambient environmental cues such as light are crucial for diverse organisms including filamentous fungi. Growth and development of many fungal species are intricately regulated by light (Carlile, 1965; Purschwitz et al., 2006; Tan, 1978). Fungal responsiveness to different wavelengths of light has been well documented, and blue light/UV and red/far-red light are two types of photoresponses primarily observed (Griffith et al., 1994; Mooney and Yager, 1990; Purschwitz et al., 2006). Although photoreceptors for different action spectra are present in individual fungal species, only one type of photoresponses was predominantly seen (Blumenstein et al., 2005; Froehlich et al., 2002,2005; He et al., 2002; Purschwitz et al., 2008). As such, blue light photoresponses have been most widely observed in fungal kingdom and were subjected to intensive studies in recent years (Herrera-Estrella and Horwitz, 2007; Purschwitz et al., 2006).

In fungi, *Neurospora crassa* has been the primary model for studying fungal blue light photobiology (Linden et al., 1997). Two central regulators, *wc-1* and *wc-2*, were identified as mutants with altered carotenoid pigment production in response to light, and other light responsive behaviors in these mutants were also defective (Ballario et al., 1996; Linden and Macino, 1997). The WC-1 protein functions as the FAD-binding photoreceptor (Froehlich et al., 2002; He et al., 2002) and transcription factor to interact with WC-2 to coordinately regulate genes involved in the pigmentation, differentiation, and circadian rhythm (Linden et al., 1997; Liu et al., 2003). Recent studies revealed that post-translational modification of WC-1 and WC-2 is critical for their regulation. WC proteins are phosphorylated in the dark and also in a light-dependent manner. The degree of their phosphorylation status is important for their binding activity (Dunlap and Loros, 2006). Light triggers the binding of L-WCC (Light WC-1/WC-2 complexes) to the LREs (light response elements) of immediate early light responsive genes such as *frq*, *al-3*, and *vvd* and activates their expression (He and Liu, 2005). Binding of L-WCC to the LREs can only be transient and hyperphosphorylation of WCC inhibits WC activity, resulting in the repression of *frq* transcription (He et al., 2006). Phosphorylation has been shown to occur sequentially in WC-1 phosphorylation sites. The cAMP-dependent protein kinase A first acts as a FRQ-independent priming kinase (Huang et al., 2007) and then FRQ-dependent hyperphosphorylation mediated by casein kinases CKII and CK-1a in the light eventually leads to the binding inhibition of the L-WCC to the LREs (He et al., 2006; Huang et al., 2007). Additionally, acetylation of histone H3 lysine 14 associated with the promoter of *al-3* was also demonstrated to be required for the WC-1-dependent blue light response (Grimaldi et al., 2006). These studies provide the detailed molecular mechanisms from light-induced transcription to photoadaptation in *N. crassa*.

Since the initial finding and detailed characterization in *N. crassa*, homologues of *wc-1* and *wc-2* were also identified by bioinformatic and genetic approaches in fungi across different phyla

* Corresponding author. Fax: +886 2 23636490.
E-mail address: wcshen@ntu.edu.tw (W.-C. Shen).
[1] These authors contributed equally to this work.

1087-1845/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2008.10.007

(Corrochano, 2007). Examples of the *wc-1* orthologues in the Zygomycetes include the *madA* and *wcoA* genes of *Phycomyces blakesleeanus* (Idnurm et al., 2006), the *mcwc-1a*, *mcwc-1b*, and *mcwc-1c* genes of *Mucor circinelloides* (Silva et al., 2006). Orthologues of *wc-1* in several Ascomycetes include the *blr-1* gene of *Trichoderma atroviride* (Casas-Flores et al., 2004), the *Tbwc-1* gene of *Tuber borchii* (Ambra et al., 2004), the *mgwc-1* gene of *Magnaporthe grisea* (Lee et al., 2006), the *BLR1* gene of *Bipolaris oryzae* (Kihara et al., 2007), and the *wcoA* gene of *Fusarium fujikuroi* (Estrada and Avalos, 2008). In the Basidiomycetes, the *dst1* gene of *Coprinus cinereus* (Terashima et al., 2005), and the *BWC1/CWC1* gene of *Cryptococcus neoformans* (Idnurm and Heitman, 2005; Lu et al., 2005) have also been characterized.

Cryptococcus neoformans is an important human fungal pathogen with a well-defined life cycle (Casadevall and Perfect, 1998; Lin and Heitman, 2006). This fungus has become a model for studying fungal virulence, physiology, and differentiation due to its well-established molecular and genomic resources (Hull and Heitman, 2002; Loftus et al., 2005). *C. neoformans* grows vegetatively as yeasts under normal growth conditions, and filamentous hyphae develop in the sexual differentiation process. *C. neoformans* is a heterothallic basidiomycete with *MAT*α and *MAT***a** mating types. Two types of sexual development, mating and monokaryotic fruiting, have been described (Kwon-Chung, 1976; Lin et al., 2005). Mating in *C. neoformans* is a sexual process involved two opposite mating type cells as typically seen in the heterothallic fungi. Fusion of the conjugated structures produced by the haploid yeast cells leads to the production of dikaryotic hyphae, which give rise to the robustly filamentous appearance of the mating colony. Nuclear fusion and meiosis occur at the tips of hyphae to subsequently generate four chains of sessile basidiospores (Kwon-Chung, 1976). In contrast, monokaryotic fruiting is a differentiation process primarily associated with the *MAT*α cells (Wickes et al., 1996). Under nitrogen starvation and desiccation conditions, rare cell fusion or endoduplication events have been speculated to occur to form the diploid cells. Diploid cells further undergo filamentation process to generate the monokaryotic filaments, which show sparse filamentation around the colony (Lin et al., 2005). Components such as pheromones and pheromone receptors required for α and **a** cell fusion in mating were also shown to function before cell or nuclear fusion during monokaryotic fruiting process (Lin et al., 2005; Shen et al., 2002).

Reports from our laboratory and others showed that filamentation associated with mating and monokaryotic fruiting is inhibited specifically by blue light (Idnurm and Heitman, 2005; Lu et al., 2005). *C. neoformans* orthologues of *N. crassa* WC-1 and WC-2 were identified and named independently as Cwc1/Bwc1 and Cwc2/Bwc2 (Cwc, *Cryptococcus* white collar; Bwc, *B*asidiomycete white collar). Conserved LOV (for *L*ight–*O*xygen–*V*oltage), PAS (for *P*er-*A*rnt-*S*im), and GATA-type zinc finger DNA-binding domains are found among these proteins (Lu et al., 2005). Deletion studies showed that both of these genes are crucial for mediating light-inhibition of mating and monokaryotic filamentation (Idnurm and Heitman, 2005; Lu et al., 2005). Overexpression of these genes further inhibits filamentation upon light treatment (Lu et al., 2005). Interestingly, these genes also contribute to UV resistance and full virulence of *C. neoformans* (Idnurm and Heitman, 2005).

The goals of our study are to dissect the protein regions important for the functions of Cwc1 and Cwc2 and to understand how light regulates filamentous growth. In this report, we describe the characterization of putative domains identified in the Cwc1 and Cwc2 proteins. Using genetic approach, we also demonstrate that the presence of functional copies of both *CWC1* and *CWC2* genes is required for proper light response. To address the issue of how light inhibits filamentation via the Cwc proteins, we have conducted a screen for suppressors of the *CWC1* overexpression strain using the *Agrobacterium*-mediated insertional mutagenesis technique. In this study, we report the identification of the *CWC2* and *SSN8* genes whose mutation suppressed the light-dependent *CWC1* overexpression phenotype.

2. Materials and methods

2.1. Strains, media, and growth conditions

Cryptococcus neoformans strains used in this study are listed in Table 1. Congenic serotype D strains JEC20 (*MAT***a**) and JEC21 (*MAT*α and their auxotrophic derivatives were used throughout the study (Edman and Kwon-Chung, 1990; Moore and Edman, 1993). All strains were handled using standard techniques and media as previously described (Alspaugh et al., 1997; Guthrie and Fink, 1991). Yeast extract–peptone–dextrose (YPD) medium, synthetic (SD) medium, V8 agar, and filament agar were prepared as described (Alspaugh et al., 1997; Wickes et al., 1996). *C. neoformans* strains were routinely maintained on YPD at 30 °C, and mating was conducted on V8 agar at 26 °C.

2.2. Construction of the truncated Cwc1 proteins

To construct the truncated versions of the *C. neoformans* Cwc1 proteins, overlapping PCR with the primers flanking the deleted region was conducted and the PCR product was cloned into pYKL8 under the control of *GPD1* (glyceraldehyde-3-phosphate dehydrogenase gene 1) promoter (Lu et al., 2005). To make the LOV domain-deleted Cwc1 protein, two sets of PCR primers flanking the LOV domain were designed and PCR amplification was conducted with the previously reported *CWC1* overexpression plasmid pYKL9 as the DNA template (Lu et al., 2005). All the oligonucleotide primers used in this study are listed in Table 2. The 5′-flanking 1.76 kb and 3′-flanking 1.93 kb fragments of the LOV domain region were amplified with WC241/WC346 and WC347/

Table 1
C. neoformans strains used in this study.

Strain	Description	Reference
JEC20	*MAT***a**	Kwon-Chung et al. (1992)
JEC21	*MAT*α	Kwon-Chung et al. (1992)
JEC34	*MAT***a** *ura5*	Moore and Edman (1993)
JEC43	*MAT*α *ura5*	Moore and Edman (1993)
H99	*MAT*α	Wang et al. (2000)
BJC2	*MAT*α *pGPD1::CWC1*$_{(568-680\triangle)}$*-URA5 ura5*	This study
BJC5	*MAT*α *cwc1* + *pGPD1::CWC1-URA5 ura5*	This study
BJC6	*MAT*α *cwc1* + *pGPD1::CWC1*$_{(568-680\triangle)}$*-URA5 ura5*	This study
BJC7	*MAT*α *pGPD1::CWC1*$_{(767-929\triangle)}$*-URA5 ura5*	This study
BJC8	*MAT*α *cwc1* + *pGPD1::CWC1*$_{(767-929\triangle)}$*-URA5 ura5*	This study
KHC2	*MAT*α *cwc2::URA5 ura5*	This study
KHC4	*MAT*α *cwc2 ura5* (5-FOA^r)	This study
KHC6	*MAT*α *pGPD1::CWC2-URA5 ura5*	Lu et al. (2005)
KHC21	*MAT*α *cwc1* + *pGPD1::CWC2-URA5 ura5*	This study
YKC7	*MAT*α *cwc1::URA5 ura5*	Lu et al. (2005)
YKC25	*MAT*α *ura5 cwc1* (5-FOA^r)	Lu et al. (2005)
YKC38	*MAT*α *pGPD1::CWC1-URA5 ura5*	Lu et al. (2005)
YLC2	*MAT*α *cwc2* + *pGPD1::CWC1-URA5 ura5*	This study
YLC3	*MAT*α *cwc2* + *pGPD1::CWC2-URA5 ura5*	This study
YLC5	*MAT*α *cwc1 cwc2*	This study
YLC9	*MAT*α *pGPD1::CWC2*$_{(72-140\triangle)}$*-URA5 ura5*	This study
YLC12	*MAT*α *cwc2* + *pGPD1::CWC2*$_{(72-140\triangle)}$*-URA5 ura5*	This study
YLC18	*MAT*α *pGPD1::CWC2*$_{(349-373\triangle)}$*-URA5 ura5*	This study
YLC19	*MAT*α *cwc2* + *pGPD1::CWC2*$_{(349-373\triangle)}$*-URA5 ura5*	This study
YSC1	*MAT*α *ssn8* + *pGPD1::CWC1-URA5 ura5*	This study
YSC13-1∼9	Cross progeny of AY18 and JEC20	This study

Table 2
PCR primers used in this study.

Name	Sequence (5′–3′)
WC53	CCAGAGCGCATCGTGAGCATT
WC54	CTCACCCACTACGATCGTCGT
WC64	CGTGTGTTCTTTGGAATGGCT
WC65	GTCTCCGAGCCGAATTGTTCA
WC241	CGGGATCCATGTCAACAAACCTCACTTCT
WC325	CGGGATCCAATGTCCCTCCTCGCCGAGTCT
WC326	TCCCCCGGGAAGGCCGAAATGAAATAAGCGT
WC346	ACTACAACTCAGGTCGACAGGTCC
WC347	TGGACCTGTCGACCTGAGTTGTAGTGTTGAACAGCCAAACAAAATCCTCCGT
WC355	TCCCCCGGGTAGTTTAGAACCTGTTCCCT
WC373	GTGCCCTGTCGCTTGCGTCATCGT
WC402	GAGTGATAAG GCATGTATGAAGTCT
WC403	AGACTTCATACATGCCTTATCACTCCCGTCTGGCGAACATCTCATTAATA
WC406	GATTGCGTTCCCGCCTTCA
WC407	CGTCCGCAATGTGTTATTAAG
WC408	GTCTCTGAAACCAGGAAGCTA
WC409	GCATGTTATGGAGTCCTC
WC419	TGGCGCACGATTTGTCGGAAAGGAA
WC433	ACTCCAAGCTTCGCCGTTCTGATA
WC434	TATCAGAACGGCGAAGCTTGGAGTCGGACAACATACATCAGGATGCTCT
WC435	GCAAACATGCATTGTCTCTCCCTCT
WC436	AGAGGGAGAGACAATGCATGTTTGCGGACTAAGATGGGCAAAACGAAAT
WC445	CGGGATCCATGTCTTCCAACTTCTATACCTCCT
WC446	TCCCCCGGGCCACTGCCAGATACTTCTCCGAA
WC466	CCAGCTCTCAACGCATTTACAAGCA
WC467	CGGGATCCTCTTTGGTCAATTGGGCAGATGG
WC468	CGGGATCCATCGCATGCGTATATGTACACT
WC469	CGTTACTGGTCGAGACGTTCAGTT
WC496	CATAATGTCGACTGTTGGACAG
WC498	GGGGTACCAATGGTGCACTATGGTTGGT

WC355, respectively. The PCR products were purified and at least 40 ng of each PCR product was mixed in equal molar ratio and further amplified with WC241/WC335 to obtain the final 3.69 kb PCR product. The product was then purified, restriction digested with BamHI and SmaI, and ligated into pYKL8 to obtain pBJS12. The construct was sequenced to verify no additional changes. The LOV domain truncated version of the *CWC1* construct pBJS12 was then biolistically transformed into the *MAT*α *ura5* strain JEC43 and the *MAT*α *ura5 cwc1* mutant strain YKC25 (Lu et al., 2005). Uracil prototrophic transformants were picked and screened by PCR. Since the construct was not targeted to a specific locus and integration might occur ectopically at different sites among transformants. We further verified the expression level of the transgene by Northern blot analysis and transformants with high and similar transcript level were selected for phenotypic characterization.

To make the PAS domains-deleted Cwc1 protein, two sets of PCR primers flanking the PAS domains were also designed and PCR amplification was conducted with the plasmid pYKL9 (Lu et al., 2005). The 5′-flanking 2.46 kb and 3′-flanking 1.13 kb fragments of the PAS domains region were amplified with WC241/WC402 and WC403/WC355, respectively. An equal molar ratio of at least 40 ng purified PCR products were further used to amplify with WC241/WC335 to obtain the final 3.59 kb PCR product. The product was similarly cloned into pYKL8 to obtain pBJS13 and no other mutation was also confirmed by sequencing. The PAS domains truncated version of the *CWC1* construct pBJS13 was transformed into the *MAT*α *ura5* strain JEC43 and the *MAT*α *ura5 cwc1* mutant strain YKC25. Uracil prototrophic transformants were picked and screened by PCR and the expression level was also verified by Northern blot analysis.

2.3. Construction of the truncated Cwc2 proteins

To make the truncated versions of the *C. neoformans* Cwc2 proteins, we also conducted overlapping PCR and similarly expressed the products under the *GPD1* promoter. To make the PAS domain-deleted Cwc2 protein, two sets of PCR primers flanking the PAS domain were designed and PCR amplification was conducted with the plasmid pKHS1 (Lu et al., 2005). The 5′-flanking 0.3 kb and 3′-flanking 1.6 kb fragments of the PAS domain region were amplified with WC325/WC433 and WC434/WC326, respectively. The PCR products were purified, mixed, and used as a template for further amplification with WC325/WC326 to obtain the final 1.9 kb PCR product. The product was further purified, digested with BamHI and SmaI, and ligated with pYKL8 to obtain pYLY1. The PAS domain truncated version of the *CWC2* construct pYLY1 was verified by sequencing and biolistically transformed into the *MAT*α *ura5* strain JEC43 and the *MAT*α *ura5 cwc2* mutant strain KHC4. Uracil prototrophic transformants were picked, screened by PCR, and verified by Northern blot analysis.

To make the *CWC2* gene without the zinc finger DNA-binding domain, PCR primers WC325/WC435 and WC326/WC436 were designed to amplify the 5′- and 3′-flanking sequences, respectively. The amplified 1.25 and 0.77 kb PCR fragments were purified and mixed for overlapping PCR with primers WC325 and WC326 to obtain the final PCR product. The product was cloned into pYKL8 and the sequence of the resulting pYLY6 was confirmed no additional mutation and transformed into the *MAT*α *ura5* strain JEC43 and the *MAT*α *ura5 cwc2* mutant strain KHC4. Uracil prototrophic transformants were picked, screened by PCR, and verified by Northern blot analysis.

2.4. Overexpression of the CWC1 gene in the cwc2 mutant background and overexpression of the CWC2 gene in the cwc1 mutant background

To overexpress the *CWC1* gene in the *cwc2* mutant, pYKL9 was utilized to transform the *cwc2* 5-FOA selected strain KHC4. The resulting uracil prototrophic transformants were picked, screened by PCR, and verified by Northern blot analysis. To overexpress the *CWC2* gene in the *cwc1* mutant, pKHS1 was used to transform the *cwc1* 5-FOA selected strain YKC25. The resulting uracil prototrophic transformants were similarly screened and verified by Northern blot analysis.

2.5. Generation of the cwc1 cwc2 double mutants

To generate the *cwc1 cwc2* double mutant strains, the *cwc1::URA5* disruption allele (Lu et al., 2005) was introduced into the *MAT*α *cwc2 ura5* (5-FOA^r) strain KHC4 by biolistic transformation. The resulting uracil prototrophic transformants were selected, screened by PCR, and verified by Southern blot analysis.

2.6. Agrobacterium-mediated insertional mutagenesis and screening for suppressor mutants

To conduct the insertional mutagenesis, *Agrobacterium tumefaciens* strain LBA4404 bearing the plasmid pPZP-201BK with *NAT* cassette was utilized for transformation (Idnurm et al., 2004). *C. neoformans CWC1* overexpression strain YKC38 was targeted for mutant screening (Lu et al., 2005). In brief, *A. tumefaciens* strain was first grown in Luria–Bertani liquid medium supplemented with 50 μg/ml of kanamycin for 2 days at 28 °C. Cells was then collected, washed with sterile water, and resuspended in induction medium with 100 μM acetosyringone to an optical density of 0.15 at 660 nm. The culture was further incubated for 6 h at 28 °C, and the optical density was expected to reach 0.6. *C. neoformans* strain YKC38 was grown in YPD liquid medium for overnight, washed with sterile water, and resuspended with induction medium to 10^6–10^7 cells per ml. Each of *A. tumefaciens* and *C. neoformans* (200 μl) cells were mixed and co-incubated on induction agar medium with 100 μM acetosyringone for 48 h, and cells were

harvested and selected onto YPD agar medium with nourseothricin and cefotaxime each at the concentration of 100 μg/ml.

C. neoformans transformants resistant to nourseothricin were streaked onto 0.1× YPD agar medium (with 2% glucose) for single colony isolation. Single colonies were picked and reselected on YPD medium with 100 μg/ml of nourseothricin. Strains maintaining the resistance were examined for mating filamentation by crossing with the *MAT***a** wild-type strain JEC20 on V8 medium at 26 °C in the presence of constant white light. Most strains were expected to display complete inhibition of sexual filamentation in the light as the original *CWC1* overexpression strain. Strains restored for filament formation were categorized based on the level of filamentation and subjected to further characterization.

2.7. Nucleic acid manipulations

Agrobacterium T-DNA integration was confirmed by Southern blot analysis. Genomic DNA used for Southern hybridization was prepared by a large-scale isolation protocol described previously (Perfect et al., 1993). Hybridization probe *NAT* was prepared from pCH233 digested with EcoRI and KpnI and labeled by Prime-It® II Random Primer Labeling Kit (Stratagene) with [α-^{32}P]dCTP (NEN Life Science Products). To identify the T-DNA integration site, restriction enzymes which do not cut or cut once in the T-DNA vector were chosen to digest the genomic DNA prepared from the strain of interest. DNA was then purified, diluted, and self-ligated. Inverse PCR was subsequently conducted with primers WC406, WC407, WC408, and WC409, which reside in the T-DNA region, to recover the flanking sequences. PCR products with expected sizes based on Southern analysis were further purified, sequenced, and searched against the JEC21 genome and NCBI database to define the site of insertion and the gene of interest. All nucleic acid manipulations were performed according to standard procedures (Sambrook and Russell, 2001).

2.8. Isolation and characterization of the progeny from the cross between the AY18 insertional mutant and MAT***a*** wild-type strain

To obtain the progeny from the cross of AY18 and *MAT***a** wild-type strains, we first grew them on YPD at 30 °C for 1 day. Equal amount of cells for both strains were separately resuspended in sterile water and mixed together. Before spotting the mating mixture, a metal ring of 1 cm in diameter and height was sterilized by flame, and pressed down on the surface of V8 agar to create an isolated circle area with 0.1 cm gap. Then the mixture was spotted onto the center of circle area and incubated in the dark for 10–14 days. When filaments crossed the gap and developed fruiting structures, the agar outside the gap was excised, put into 1.5 ml of sterile water in a microfuge tube and vortexed to disperse the basidiospores. The suspension was spread onto YPD agar medium containing 100 μg/ml nourseothricin. The resistant transformants were streaked onto 0.1× YPD agar medium for single colony isolation. Colonies were picked and reselected on YPD medium with 100 μg/ml of nourseothricin. Stably resistant strains were subjected to further phenotypic and genotypic characterization.

To verify the genotypes of the progeny, genomic DNA was extracted and subjected to PCR and Southern blot hybridization. To confirm mating type, two sets of mating type specific PCR primers WC53/WC54 and WC64/WC65 were designed for the *STE12*α and *STE12***a** genes, respectively. The presence of intact P_{GPD1}-*CWC1* expression cassette was confirmed by primer WC373 and WC419. T-DNA integration at the *SSN8* locus in the AY18 mutant was confirmed by PCR primer WC407 and WC466. Uracil auxotrophy or prototrophy was determined by the growth on SD medium lacking or containing uracil.

2.9. Disruption of the C. neoformans SSN8 gene in the CWC1 overexpression strain

To disrupt the *C. neoformans SSN8* gene in the *CWC1* overexpression strain, a *ssn8::NAT* disruption allele was constructed for homologous recombination. Primer pairs of WC458/WC467 and WC468/WC469 were designed to amplify the flanking sequences of the *SSN8* genomic region. The 1.2 kb 5′- and 3′-flanking PCR products were, respectively, digested with SacI/BamHI and BamHI/XhoI and subcloned into pBluescript SK(+). Then four fragments ligation was conducted with the digested 5′- and 3′-flanking fragments, BamHI digested *NAT* selectable marker, and SacI/XhoI digested pBluescript. The resulting *ssn8::NAT* disruption allele was introduced into the YKC38 strain by biolistic transformation (Toffaletti et al., 1993). Transformants were selected on YPD medium with 100 μg/ml nourseothricin and verified by PCR and Southern blot analysis.

2.10. RNA isolation and Northern blot analysis

RNA used in Northern analysis was isolated from yeast cells by using the TRIzol total RNA isolation reagent according to the manufacturer's instructions (Invitrogen). Twenty micrograms of total RNA from each sample was separated by electrophoresis in a 1.2% agarose–formaldehyde gel. RNA was transferred by capillary action to nylon membrane (Immobilon™-Ny+, Millipore) and hybridized in hybridization buffer (0.12 M Na_2HPO_4 [pH 7.2], 0.25 M NaCl, 1 mM EDTA [pH 8], 7% SDS, and 50% formamide). All probes were labeled as described for Southern blot analysis.

2.11. Mating assay

Strains for mating assay were first grown on YPD at 30 °C for 1 day. Mating reactions were conducted by co-incubating cells with desired partners on V8 agar medium at 26 °C under white light or dark conditions for 1–5 days. JEC20 (*MAT***a**) and JEC21 (*MAT*α strains were used as mating testers. Dikaryotic filaments at the periphery of mating spot were evaluated under Olympus BX41 microscope. Pictures were taken 16–48 h post-incubation at 100× magnification.

2.12. Monokaryotic fruiting assay

Strains for monokaryotic fruiting assay were first grown on YPD at 30 °C for 1 day. Yeast cells of each strain were suspended in sterile water and then 5 μl was spotted on FA plate and incubated at 26 °C under white light or dark conditions for 3–14 days. Filaments at the periphery of the spot were evaluated under Olympus BX41 microscope. Pictures were taken 5–14 days post-incubation at 100× magnification.

3. Results

3.1. LOV and PAS domains of the Cwc1 protein are essential for blue light responses in C. neoformans

Based on the predicted structural organization, a putative LOV domain, two putative PAS domains, and a putative nuclear localization sequence were identified in the *C. neoformans* Cwc1 protein. LOV domain was known as a specialized PAS domain responsible for chromophore binding and light sensing. Cwc1 has been proposed to function as a blue light photoreceptor through its putative FAD-binding LOV domain and light treatment was shown to be required for its activation (Lu et al., 2005). Cwc1 forms complex with Cwc2 possibly via the PAS domains and in turn binds to target gene promoter through the zinc finger DNA-binding domain of Cwc2

and negatively regulates filamentous growth (Idnurm and Heitman, 2005; Lu et al., 2005).

To confirm the functions of the putative LOV and PAS domains, we generated truncated versions of the *CWC1* gene under the *GPD1* promoter (Fig. 1A). The constructs were sequenced to confirm no additional mutation and were transformed into the *C. neoformans* wild-type and *cwc1* mutant strains. Using the mutant as the recipient strains, the overexpression effect of the truncated gene can be purely scored and the importance of deleted region can thus be determined. When using the wild-type strain, the phenotype may imply if the truncated protein still retains the interaction ability. If deleted the region important for protein interaction, the phenotype of the transformant will be similar to the wild-type strain since the functional complex can be formed by the endogenous wild-type proteins. The transformants with elevated levels of the truncated *CWC1* transcripts were verified by Northern blot analysis (data not shown) and subjected to phenotypic characterization. If the LOV domain is essential for the function of Cwc1, overexpression of the *CWC1* gene lacking the LOV domain should fail to inhibit sexual filamentation upon light activation. As expected, overexpression of the $CWC1_{(568–680\triangle)}$ gene in the *cwc1* mutant background (Fig. 1Bf) produced strains that were insensitive to light and filament production in these transformants were similar to the original *cwc1* mutant (Fig. 1Bb). Overexpression in the wild-type also yielded light insensitive transformants, indicating that overexpression of the $CWC1_{(568–680\triangle)}$ gene had a dominant negative effect on light regulation (Fig. 1Be). In contrast, filament production in the wild-type cross was inhibited by light (Fig. 1Ba) and overexpression of the wild-type *CWC1* gene in the wild-type strain (Fig. 1Bc) and *cwc1* mutant (Fig. 1Bd) both completely inhibited filamentation under light conditions.

The importance of the PAS domains in Cwc1 was also confirmed by overexpression of the $CWC1_{(767–929\triangle)}$ gene that lacked the PAS domains (Fig. 1A). The *cwc1* mutant overexpressing $CWC1_{(767–929\triangle)}$ resembled the *cwc1* mutant (Fig. 1Bh and Bb); whereas, overexpression of the $CWC1_{(767–929\triangle)}$ construct in the wild-type background was similar to the wild-type strain (Fig. 1Bg and Ba). Our results demonstrated that the LOV and PAS domains are essential for the function of *C. neoformans* Cwc1 protein.

3.2. The PAS domain and GATA-type zinc finger motif of the Cwc2 protein are important for the light regulation in C. neoformans

Based on the predicted structural feature, *C. neoformans* Cwc2 protein contains a putative PAS domain and GATA-type zinc finger DNA-binding motif (Lu et al., 2005). The PAS domain is involved in protein–protein interaction and the zinc finger DNA-binding motif is a feature of transcription factor. To confirm their importance, we similarly generated truncated versions of the *CWC2* gene under control of the *GPD1* promoter and no sequence change during amplification was also confirmed (Fig. 2A). The expression level of truncated transcripts was also verified by Northern blot analysis (data not shown). Overexpression of the $CWC2_{(72–140\triangle)}$ gene, which lacked the PAS domain, in the wild-type background (Fig. 2Be) displayed the same mating phenotype as the wild-type strain (Fig. 2Ba), while in the *cwc2* mutant background (Fig. 2Bf) the strain displayed the phenotype as the *cwc2* mutant (Fig. 2Bb). Furthermore, overexpression of the $CWC2_{(349–373\triangle)}$ gene, which was deleted for the zinc finger motif, in the *cwc2* mutant background (Fig. 2Bh) produced filaments similar to the *cwc2* mutant (Fig. 2Bb). In the wild-type background (Fig. 2Bg), the level of filamentation was intermediate between the wild-type (Fig. 2Ba) and *cwc2* mutant strains (Fig. 2Bb), suggesting a complex poisoning or semi-dominant negative effect by the mutation version of Cwc2. These results demonstrate that PAS domain and GATA-type zinc finger motif of the Cwc2 protein are also crucial for blue light responses in *C. neoformans*.

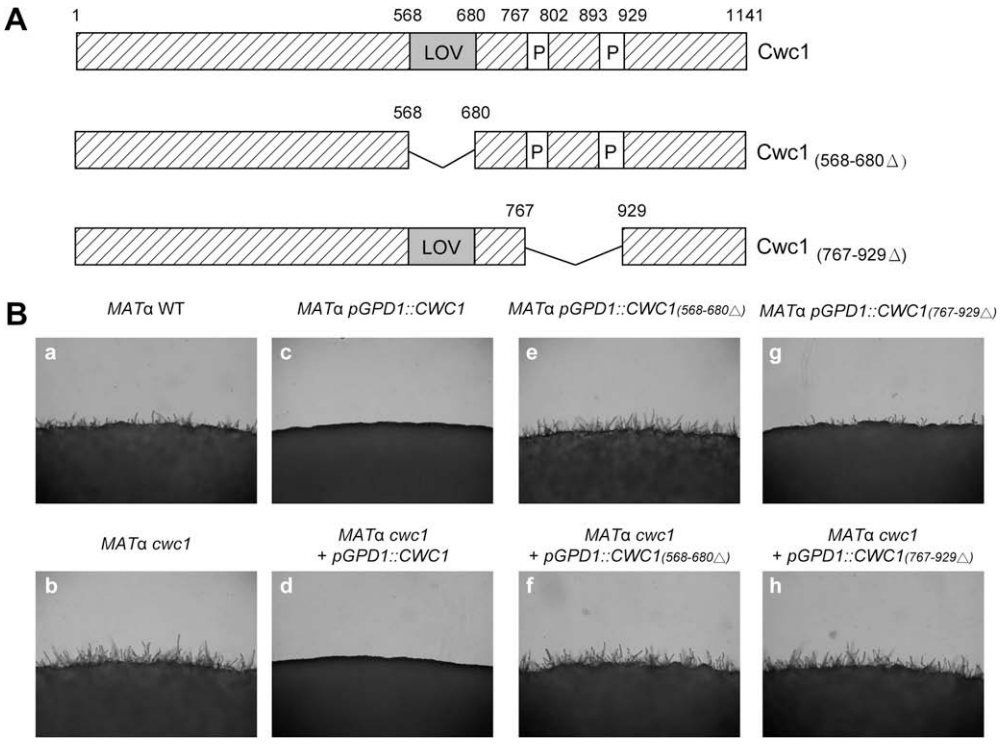

Fig. 1. LOV and PAS domains are essential for the function of the Cwc1 protein. (A) Schematic illustration of the constructs of the partially deleted Cwc1 proteins. LOV: the *L*ight–*O*xygen–*V*oltage domain; P: the *P*ER-ARNT-SIM (PAS) domain. (B) Mating filamentation between the *C. neoformans MAT*α partially deleted *CWC1* transformants and *MAT***a** wild-type strain (JEC20) on V8 agar medium at 26 °C for 18 h under light condition. The edges of mating mixtures were photographed at a magnification of 100×.

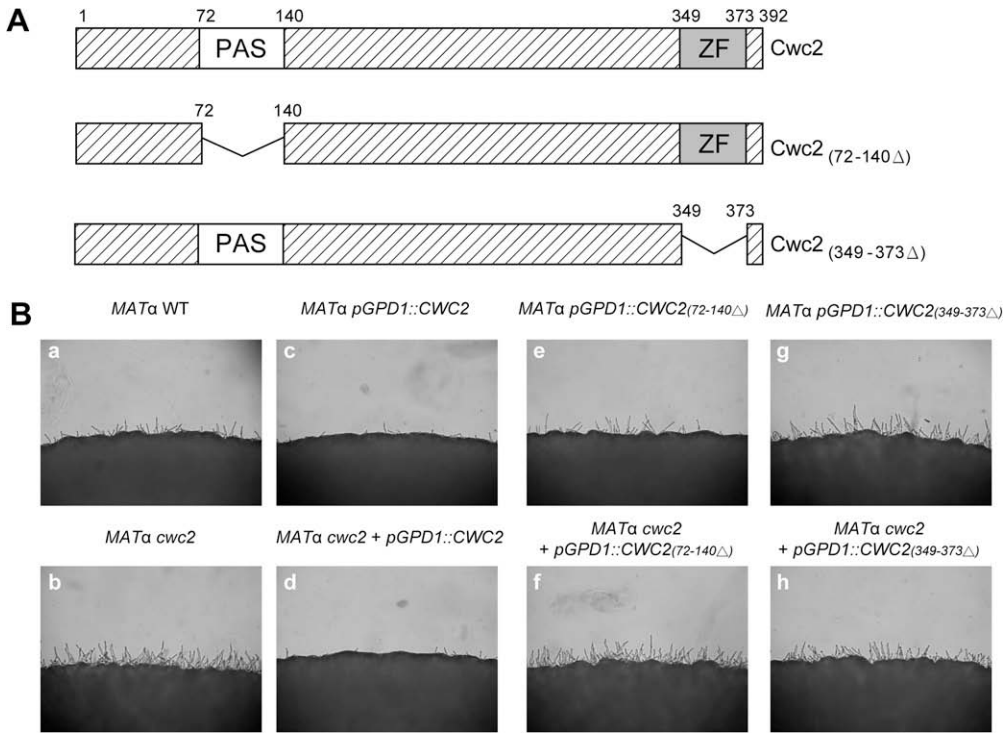

Fig. 2. PAS domain and GATA-type zinc finger motif are important for the light regulation of the Cwc2 protein. (A) Schematic illustration of the constructs of the partially deleted Cwc2 proteins. PAS: the *P*ER-*A*RNT-SIM domain; ZF: the GATA-type zinc finger DNA-binding motif. (B) Mating filamentation between the *C. neoformans MAT*α partially deleted *CWC2* transformants and *MAT***a** wild-type strain (JEC20) on V8 agar medium at 26 °C under light condition. The edges of mating spots were photographed after 18 h incubation at a magnification of 100×.

3.3. *Cryptococcus neoformans Cwc1 and Cwc2 proteins function interdependently to regulate light response*

Based on the conservation of the PAS domains in the Cwc1 and Cwc2 proteins and also similar phenotypes observed in the *CWC1* and *CWC2* gene deletion or overexpression strains, *C. neoformans* Cwc1 and Cwc2 may form a complex to regulate downstream related genes. To test their relationship genetically, we created the *CWC2* overexpression strains in the *cwc1* mutant background and also the *CWC1* overexpression strains in the *cwc2* mutant background. The elevated expression of the *CWC2* and *CWC1* transcripts in these strains was verified by Northern blot analysis (data not shown). The confirmed strains were examined for their mating phenotypes under light illumination. As predicted, overexpression in the mutant background in either case (Fig. 3G and H) failed to inhibit sexual filamentation upon light induction, and their phenotypes all resembled to the *cwc1* and *cwc2* single or double mutants (Fig. 3B–D). These findings demonstrate that *C. neoformans* Cwc1 and Cwc2 proteins function interdependently to regulate photoresponses, which is consistent with a biochemical study shown by the yeast two-hybrid assay that the two proteins physically interact (Idnurm and Heitman, 2005).

3.4. *Dissecting the C. neoformans blue light-inhibited filamentation pathway by screening for suppressor mutants*

In order to identify genes interacting with or acting downstream of the Cwc complex in the blue light-inhibited filamenta-

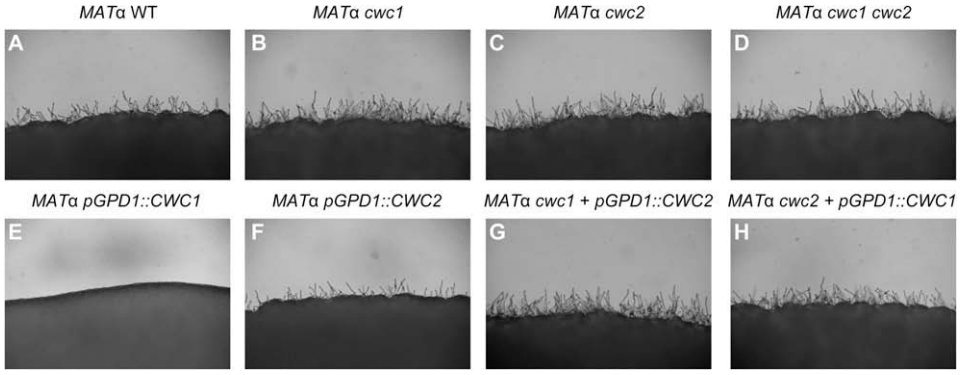

Fig. 3. Cwc1 and Cwc2 function interdependently to regulate the blue light-mediated inhibition of sexual filamentation in *C. neoformans*. The *MAT*α wild-type (JEC21), *cwc1* mutant strain (YKC7), *cwc2* mutant strain (KHC2), *cwc1 cwc2* mutant strain (YLC5), P_{GPD1}-*CWC1* overexpression strain (YKC38), P_{GPD1}-*CWC2* overexpression strain (KHC6), *cwc1* P_{GPD1}-*CWC2* overexpression strain (KHC21), and *cwc2* P_{GPD1}-*CWC1* overexpression strain (YLC2) were individually crossed with the *MAT***a** wild-type strain (JEC20) as indicated. Mating was conducted on V8 agar medium at 26 °C under light condition, and photos were taken after 20 h incubation at 100× magnification.

tion pathway in *C. neoformans*, a screening mediated by *Agrobacterium* insertional mutagenesis was conducted to isolate T-DNA mutants which suppressed the light-dependent inhibition of mating filamentation phenotype in the *CWC1* overexpression strain. Overexpression of the *CWC1* gene was proposed to up-regulate genes with negative roles in the pathway and lead to complete inhibition of sexual filamentation upon light induction (Lu et al., 2005). Therefore, identification of mutants restoring the filamentation will reveal *C. neoformans* genes important for negative regulation of sexual filamentation in response to light. The filamentation phenotype we screened is a consequence of cell fusion between two haploid parents. Since one wild-type copy derived from the *MAT***a** wild-type parent is present in the dikaryotic hyphae, our screen will not detect the mutation with recessive nature.

To conduct *Agrobacterium* insertional mutagenesis, the *CWC1* overexpression strain was co-incubated with *A. tumefaciens* cells containing the *NAT* delivery plasmid for 2 days and spread onto selection media containing nourseothricin and cefotaxime. After single colony isolation, 10,341 nourseothricin-resistant transformants were obtained and mating assays were conducted with the *MAT***a** wild-type strain. Of these 10,341 strains, 134 transformants which restored sexual filamentation under light illumination were verified after repeating three round of mating assays. The ratio of filament-producing suppressors in our screen was about 1.3%. We classified suppressor strains into five categories according to the amount of filamentation produced in the mating assay under light irradiation. Mutants producing a filamentation level similar to the wild-type were designated as level 5 and AZ5 mutant was an example (Fig. 4C). Intermediate levels of filamentation were classified from level 4 to 1 (Fig. 4D–G). We obtained six mutants classified as level 5, 16 mutants as level 4, and 28, 30, and 54 mutants as level 3, 2, and 1, respectively.

The copy number and fate of transforming T-DNA in the filamentous suppressors were further determined by Southern analysis with *NAT* selectable marker. So far, the copy number of T-DNA integration has been determined among 66 transformants, of which 47 were one-copy, 15 were 2 copies, and 4 were 3 copies. T-DNA integration sites of selected strains have been recovered by inverse PCR. Products of expected sizes were sequenced, and blast analyses were conducted to identify the disrupted genes. Currently, 17 T-DNA integrated sites have been identified and their filamentation phenotypes associated with mating and monokaryotic fruiting are listed in Table 3. Since background mutation may occur in the transformants along the procedure, the linkage of T-DNA

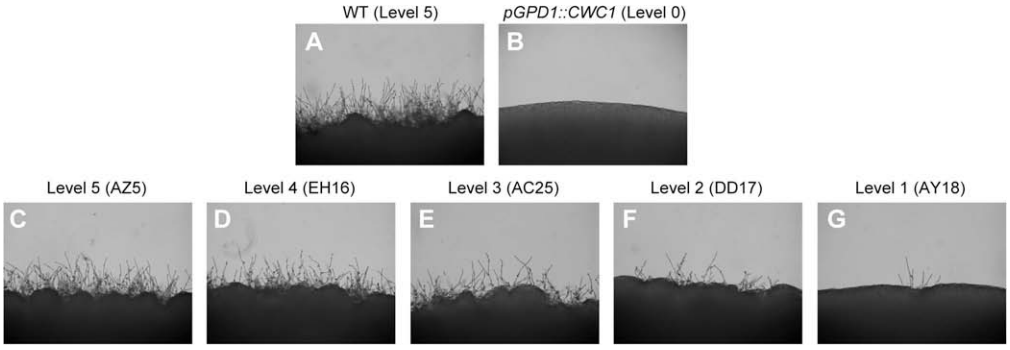

Fig. 4. Different levels of mating filamentation are restored among the *Agrobacterium* T-DNA suppressors. *Agrobacterium* T-DNA insertional mutants AZ5, EH16, AC25, DD17, and AY18 (C–G) exhibited mating filamentation from level 5 to level 1. The *MAT*α wild-type (A) and *CWC1* overexpression strain (B) were used to categorize the filamentation level. Mating was conducted with the *MAT***a** wild-type strain (JEC20) on V8 agar at 26 °C under light condition, and photos were taken after 48 h incubation at 100× magnification.

Table 3
ATMT transformants in this study.

Strains	Related gene	Locus No.	Mating[a]	Fruiting[b]	Linkage[c]
AZ5	*CWC2* gene	CNE01220	5	0	NA
AY18	General RNA polymerase II transcription factor	CNA04230	1	5	+
AC25	DNA mismatch repair protein Pms1	CNC02500	3	0	NA
AS31	Histone–lysine *N*-methyltransferase	CNG03810	2	0	+
BU26	Structural maintenance of chromosomes (SMC) protein	CND06390	3	0	NA
CZ31	*UXS1* gene	CNG02560	1	0	NA
DD17	Nicotinamide mononucleotide permase	CNA05810	2	0	NA
DI6	Hypothetical protein	CNE02520	1	0	NA
DJ22	Cdk-related kinase 1: *CRK1*	CNM01920	1	0	+
DT21	Hypothetical protein	CNF04850	3–4	0	NA
EG30	Bud site selection protein	CNL04250	4–5	0	+
EH16	Amino acid transporter	CNB04970	4	0	NA
ET21	Conserved hypothetical protein	CNC02650	1	0	NA
GC17	Intergene (between two hypothetical proteins)	CNL04560 CNL04570	4	–	NA
GJ5	Vacuole protein	CNI02790	1	–	NA
GK17	Intergene (between pre-mRNA splicing factor: *PRP1* and hypothetical protein)	CNA00700 CNA00710	2	–	NA
NO7	Expressed protein	CNI03950	1	0	NA

[a] Mating assay was conducted on V8 agar at 26 °C under light condition for 48 h. No filamentation designated as level 0; filamentation level of the AZ5 and JEC21 strain designated as level 5.

[b] Monokaryotic fruiting was conducted on FA agar at 26 °C under light condition for 5 days. The level of YKC38 and JEC21 designated as level 0 and 3, respectively; the level of AY18 designated as level 5.

[c] +, the linkage of T-DNA insertion and observed phenotype was established by complementation or progeny segregation analyses. NA, not available.

insertion and observed phenotype established by complementation experiments in few strains was also indicated in Table 3.

3.5. T-DNA integration in the AZ5 strain, which restored the highest filamentous phenotype, was found at the CWC2 locus

To find the *C. neoformans* genes important for the photoresponse, we chose suppressor mutants from level 5 as initial targets for characterization. Their high and similar filamentation level in the light and dark indicates they may be "blind" and the genes disrupted in these strains might function with Cwc complex or at the early steps of the photoregulatory pathway. One of these mutant strains, AZ5, suppressed the light-dependent inhibition of filamentation by the *CWC1* overexpression and restored abundant filamentation under light and dark conditions (Figs. 4C and 5A; data not shown). One copy of T-DNA was found by Southern blot analysis to integrate in the AZ5 strain (Fig. 5B). Sequencing the recovered PCR product revealed that T-DNA with the *NAT* selectable marker was inserted 67 bp upstream of the *CWC2* start codon (Fig. 5C and D). Integration at the *CWC2* locus was further verified by Southern hybridization with the *CWC2* probe (Fig. 5B). Furthermore, based on Northern analysis, we confirmed that the *CWC2* gene was not expressed in the AZ5 strain due to the T-DNA insertion (data not shown). The mating phenotype of AZ5 was similar to that of the *CWC1* gene overexpression in the *cwc2* mutant background (Fig. 5A). This finding confirmed that Cwc2 is an important component of the blue light-inhibited filamentation pathway in *C. neoformans* and also validated our screening approach.

3.6. AY18 suppressor restored limited amount of mating filaments but showed dramatic derepression of monokaryotic filamentation

Among the 134 filamentous suppressors identified by mating assay, 17 strains have been subjected to monokaryotic filamentation assay. Two suppressor mutants, AY18 and DN16, were found capable of producing monokaryotic fruiting filaments in the *CWC1* overexpression background. The mating filamentation levels for AY18 and DN16 were categorized as level 1 and 3, respectively (Fig. 4 and Table 3; data not shown). The extent of monokaryotic filamentation was examined in few mutants and also briefly cate-

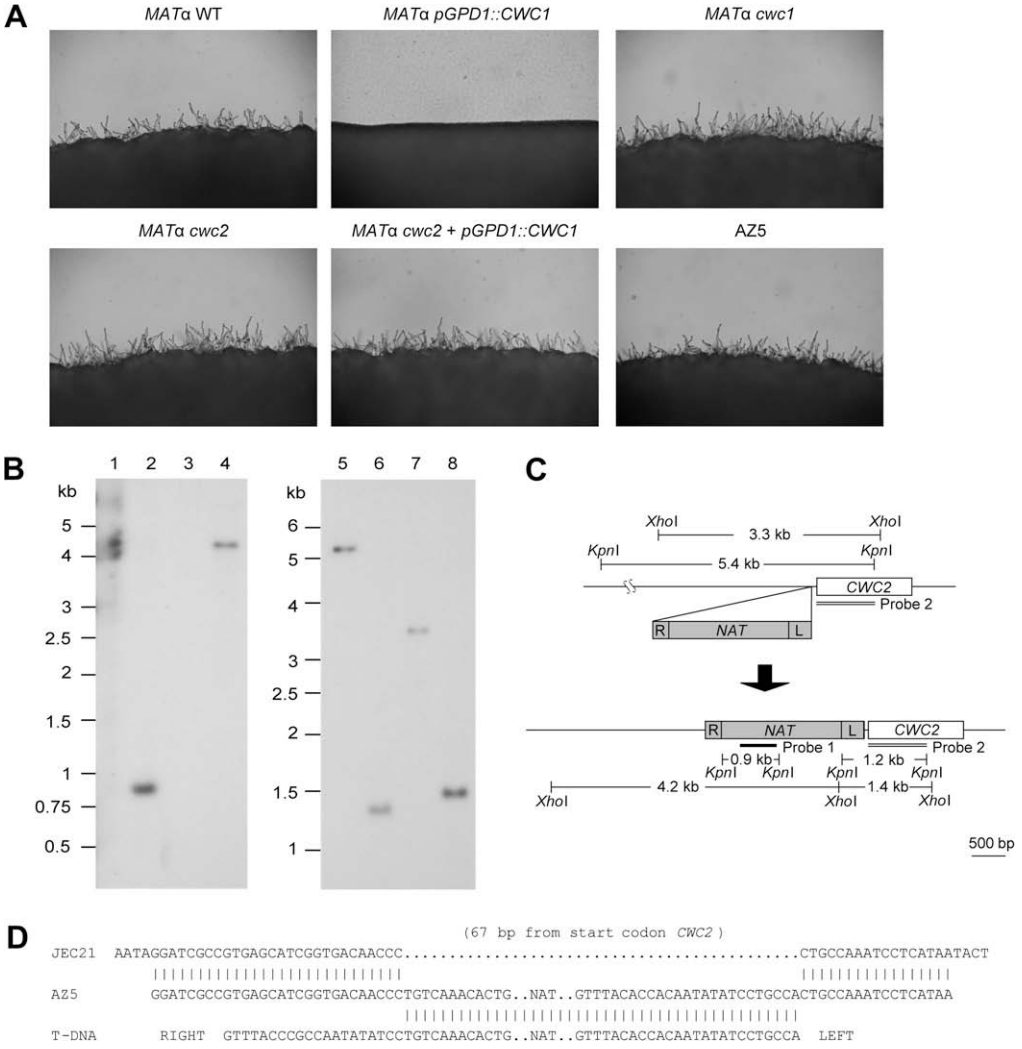

Fig. 5. Insertion of T-DNA at the *CWC2* locus was found to restore the highest level of mating filamentation among the *Agrobacterium* suppressors. (A) AZ5 strain restored mating filamentation of the *CWC1* overexpression strain in the light. Mating was conducted with the *MAT***a** wild-type strain (JEC20) on V8 agar at 26 °C under light condition, and photos were taken after 20 h incubation at 100× magnification. (B) Southern hybridization analysis of the *MATα* wild-type and AZ5 strains. Lanes 1 and 5, the wild-type strain genomic DNA digested with KpnI; lanes 2 and 6, AZ5 strain genomic DNA digested with KpnI; lanes 3 and 7, the wild-type strain genomic DNA digested with XhoI; lanes 4 and 8: AZ5 strain genomic DNA digested with XhoI. Lanes 1–4 was hybridized with probe 1 and lanes 5–8 was hybridized with probe 2 as indicated in (C). (C) Schematic diagram showing the T-DNA integration at the promoter of the *CWC2* gene in AZ5 strain. (D) Sequence analysis of the T-DNA insertional site in AZ5 strain. Sequences of the wild-type JEC21, AZ5 strain, and the right and left borders of T-DNA were aligned.

gorized. For the *CWC1* overexpression strain and most of the suppressor mutants listed in Table 3, no filamentation was observed under light illumination (level 0). The filamentation level normally seen in the *MAT*α wild-type strain was designated as level 3 and DN16 was similar to the *MAT*α wild-type strain. AY18 interestingly showed more dramatic production of monokaryotic filaments when compared to the *MAT*α wild-type strain and was categorized as level 5 (Fig. 6A). Due to its interesting phenotype in the monokaryotic filamentation, AY18 strain was selected for further characterization.

3.7. Progeny segregation analysis of the AY18 suppressor

To determine the genetic linkage between the AY18 phenotypes and the *NAT* integration, we first verified the copy number of T-DNA integration in the AY18 strain. The results of Southern hybridization indicated that 3 copies of T-DNA were present in the AY18 genome (Fig. 6B). Segregation analysis was then conducted on the mating progeny of the AY18 and *MAT***a** wild-type strain. To prevent parental yeast cell contamination, we developed a technique which restricts the parental yeast cells by making a gapped circular area around the mating colony and allows isolating the basidiospores from the mating filaments crossing the circular gap. Progeny were selected on nourseothricin containing YPD plates. Among the 96 resistant strains, 29 strains were uracil auxotrophic and not subjected to further analyses (data not shown). Using the *STE12*α/*STE12***a** genes as the PCR targets for mating type determination (Fig. 7Aa and Ab), 28 *MAT*α and 16 *MAT***a** strains were confirmed, suggesting that the *MAT* locus represented an independently segregating genetic marker. Among these 44 strains, 29 strains contained the intact P_{GPD1}-*CWC1* expression cassette (Fig. 7Ac), and 43 strains retained the T-DNA integration pattern at the *SSN8* locus as revealed by PCR analyses (Fig. 7Ad; described in Section 3.8). Nine selected strains were further verified by Southern hybridization and all three original copies of inserted T-DNA were found to be present in these strains (Fig. 7B). Based on the genotypic analyses among the nourseothricin-resistant progeny, we conclude that the integration of three copies of T-DNA was linked in the AY18 strain and the integration site responsible for the AY18 phenotypes remained unclear.

3.8. T-DNA integration at a mediator gene, SSN8, resulted in the restoration of mating filamentation and dramatic derepression of monokaryotic filamentation in the AY18 suppressor

Since all three T-DNAs appeared to be tightly linked in the AY18 strain, we failed to separate the insertion sites by sexual recombination. However, due to its interesting phenotypes, we decided to define all three integration sites. Digestion with HindIII yielded 2.5, 8.2, and 11 kb fragments using the *NAT* gene as the probe (Fig. 6B). Inverse PCR was first attempted to recover the insertion site around the 2.5 kb fragment and a product of the expected size from one flanking region was successfully obtained and sequenced (Fig. 6C). Blast searches against the GenBank and *C. neoformans* genome databases revealed that T-DNA was inserted at the N-terminus of an *SSN8* homologue of *Saccharomyces cerevisiae* (Fig. 6C and D). *S. cerevisiae SSN8* gene is a cyclin-like component of the RNA polymerase II and is involved in a wide array of cellular processes.

To determine if the phenotypes of AY18 was due to the disruption of the *SSN8* gene, we made the *SSN8* gene deletion in the *CWC1* overexpression background. The *SSN8* gene was replaced with the

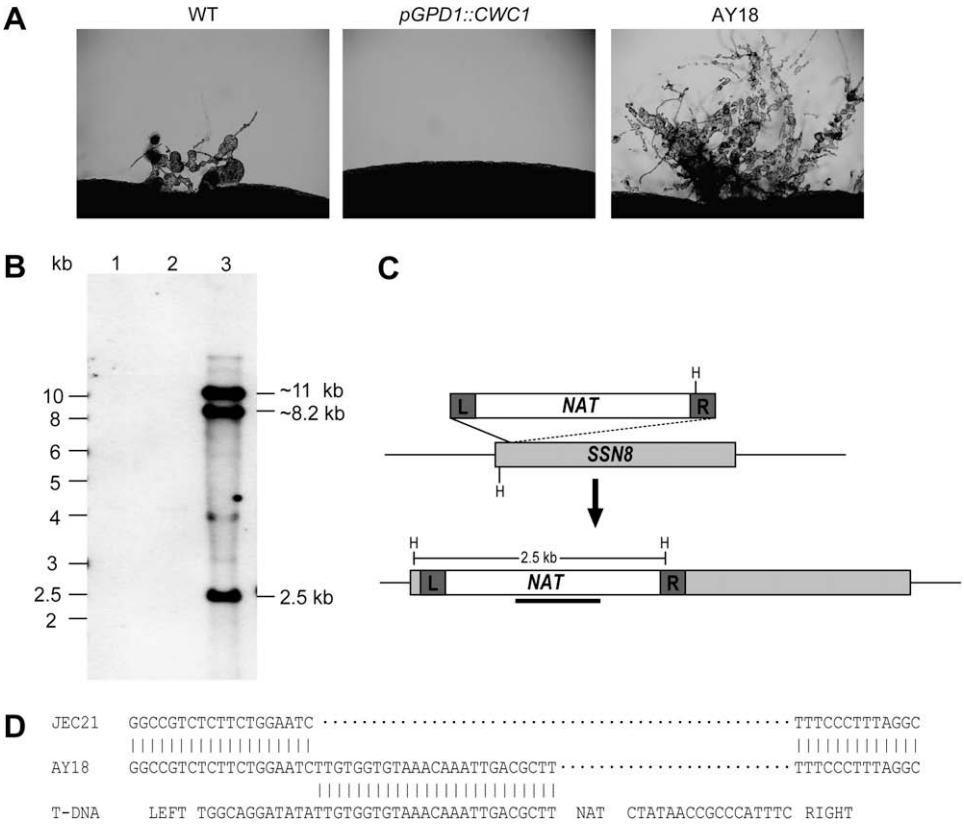

Fig. 6. Identification of the T-DNA integration site in the insertional mutant AY18. (A) AY18 strain showed the dramatic derepression of monokaryotic filamentation in the *CWC1* overexpression strain background. Monokaryotic fruiting assay was conducted on FA plate at 26 °C under light condition, and photos were taken after 5 days incubation at 100× magnification. (B) Genomic DNA from the *MAT*α wild-type strain (JEC21), P_{GPD1}-*CWC1* overexpression strain (YKC38), and AY18 strain were digested by HindIII, and hybridized by a probe in the *NAT* coding region as indicated in (C). (C) Schematic diagram of the T-DNA integration at the *SSN8* locus. Dashed line indicates that the flanking insertion site has not yet been verified. (D) Sequences of the wild-type JEC21, AY18 strain, and T-DNA left border were aligned.

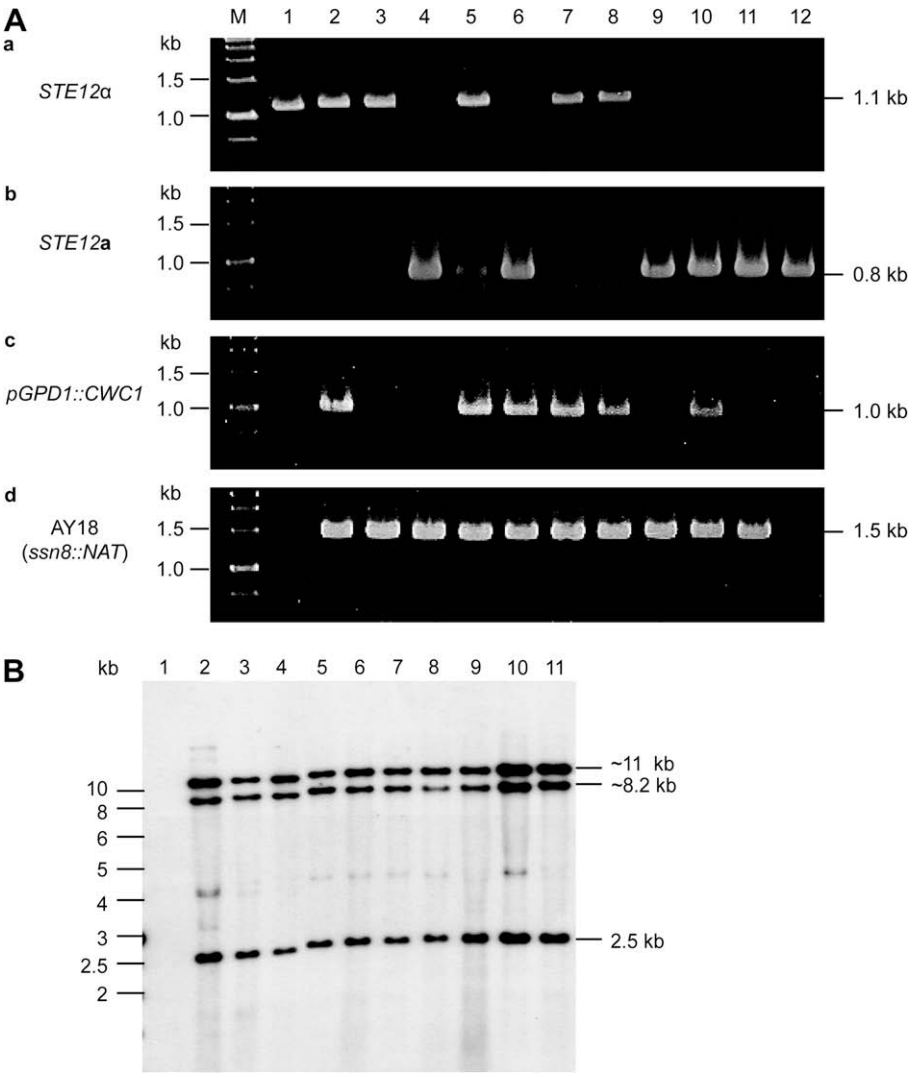

Fig. 7. Genotypic analyses of the progeny from the cross of the AY18 mutant and *MAT***a** wild-type strain. (A) PCR analyses of the progeny genotypes. Selective progeny obtained from the cross of the AY18 mutant and *MAT***a** wild-type strain was subjected to genomic DNA extraction and PCR amplification. Mating type was determined by the primers of the mating type specific *STE12*α (a) and *STE12***a** (b) genes. The presence of P_{GPD1}-*CWC1* overexpression cassette was confirmed in panel (c). T-DNA integration at the *SSN8* locus was confirmed in panel (d). Lane 1, JEC21; lanes 2–11, the progeny; lane 12, JEC20. (B) Southern blot analysis of the T-DNA insertion in the progeny. Genomic DNA was digested by HindIII, and hybridized by a probe in the *NAT* coding region. Lane 1, JEC21; lanes 2–11, the progeny.

NAT selectable marker and transformed into the *CWC1* overexpression strain YKC38 (Fig. 8A). The *ssn8* gene deletion mutant was verified by Southern hybridization with different restriction enzymes (Fig. 8B) and subjected to phenotypic comparisons with the AY18 mutant. We found that deletion of the *SSN8* gene in the *CWC1* overexpression strain reproduced mating filamentation and dramatic derepression of monokaryotic filamentation seen in the AY18 strain. Furthermore, complementation by the wild-type copy *SSN8* gene in the AY18 mutant and *SSN8*-deleted *CWC1* overexpression strain restored these filamentation phenotypes back to the *CWC1* overexpression strain (data not shown). Therefore, we conclude that T-DNA disruption at the *SSN8* gene was responsible for the phenotypes of the AY18 strain.

4. Discussion

Light displays profound effects on fungal biology. Evidences indicate that evolutionally conserved white collar proteins function as key blue light regulators in fungi across diverse taxonomical groups (Corrochano, 2007; Herrera-Estrella and Horwitz, 2007; Purschwitz et al., 2006). Structural comparisons of the WC-1 and WC-2 orthologues from two major fungal phyla, Ascomycete and Basidiomycete, suggest that these proteins might operate in a similar but not identical way in executing their regulatory mechanisms (Corrochano, 2007; Idnurm and Heitman, 2005; Lu et al., 2005).

To understand how Cwc1 and Cwc2 regulate light responses, we characterized their putative protein domains with conserved homology by deletion studies. *C. neoformans* Cwc1 was predicted to contain the chromophore-binding LOV domain and protein interacting PAS domains (Lu et al., 2005). Overexpression of the LOV domain-deleted $CWC1_{(568-680\triangle)}$ in the *cwc1* mutant background maintained the light insensitivity and displayed phenotypes similar to the original *cwc1* mutant (Fig. 1Bf and Bb). *C. neoformans cwc1* mutant containing the PAS domains-deleted $CWC1_{(767-929\triangle)}$ gene also exhibited phenotypes similar to the *cwc1* mutant (Fig. 1Bh and Bb). Since deletion of a segment of protein region may affect the general protein structure, the importance of specific region may not be truly revealed. However, our preliminary results found that deletion of a 148 amino acids region at the N-terminal part of Cwc1 showed no impact on the Cwc1 function (data not shown). Therefore, based on our results, we con-

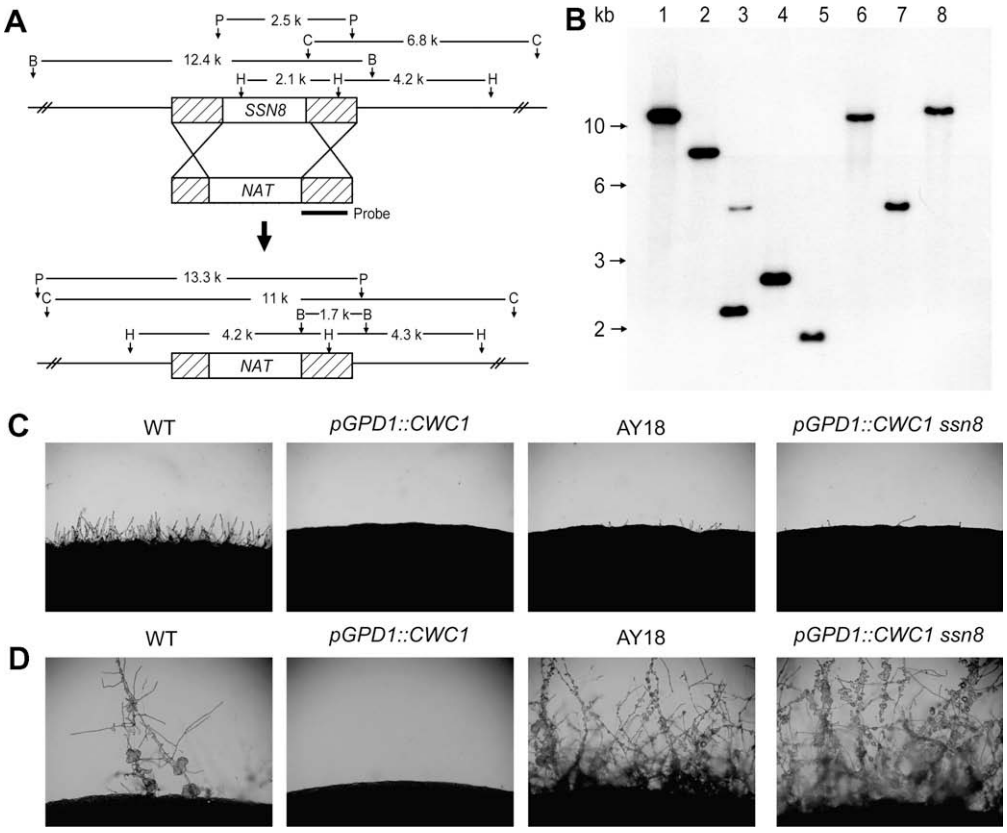

Fig. 8. Disruption of the *SSN8* gene in the P_{GPD1}-*CWC1* overexpression strain reproduces the mating and monokaryotic filamentation phenotypes of the AY18 mutant. (A) The *ssn8::NAT* disruption allele was created by replacing the *SSN8* coding region with the *NAT* dominant selectable marker. Hybridization probe for Southern blot analysis is indicated. (B) Genomic DNA from the *MAT*α wild-type (lanes 1–4) and *SSN8*-deleted YKC38 mutant strain (lanes 5–8) was digested with BamHI (lanes 1 and 5), ClaI (lanes 2 and 6), HindIII (lanes 3 and 7), and PstI (lanes 4 and 8) and subjected to Southern hybridization analysis using the DNA fragment downstream of the *SSN8* gene as indicated in (A). Size standards are indicated by kilobases (kb). (C) The *MAT*α wild-type (JEC21), *CWC1* overexpression strain (YKC38), AY18 mutant, and *SSN8* gene deleted YKC38 strain (YSC1) were crossed with the *MAT***a** wild-type strain (JEC20) on V8 agar at 26 °C under light condition, and photos were taken after 24 h incubation at 100× magnification. (D) The *MAT*α wild-type (JEC21), P_{GPD1}-*CWC1* overexpression strain (YKC38), AY18 mutant, and *SSN8* gene deleted YKC38 strain (YSC1) were spotted on FA medium and incubated at 26 °C under light condition. Photos were taken after 5 days incubation at 100× magnification.

clude that LOV and PAS domain play crucial role for the function of Cwc1.

Overexpression of the *CWC1*-truncated constructs in the wild-type strain showed interesting phenotypes. The wild-type strain containing the $CWC1_{(568–680\triangle)}$ overexpression construct showed phenotype similar to the *cwc1* mutant (Fig. 1Be and Bb). This result indicates that the non-functional LOV-deleted Cwc1 proteins had a complex poisoning effect and displayed a dominant negative effect over the wild-type Cwc1 proteins. The non-functional LOV-deleted Cwc1 proteins could compete with the wild-type Cwc1 proteins for binding to Cwc2, and proper association of the normal Cwc proteins was thus blocked. This complex poisoning phenomenon was not seen in the PAS domains-deleted $Cwc1_{(767–929\triangle)}$ wild-type strain and its filamentation level was slightly less than the wild-type strain (Fig. 1Bg). The phenotype suggests that the endogenous wild-type Cwc1 proteins can still interact normally with Cwc2 to inhibit filamentation, and the PAS domains-deleted Cwc1 proteins failed to compete for the Cwc2 proteins *in vivo*. The cause for slight reduction of the filamentation level in this strain is unknown. Whether alteration of the endogenous wild-type *CWC1* transcript level contributes to the phenotype requires further investigation.

Overexpression of the intact *CWC2* gene also inhibits sexual filamentation under light condition; however, the inhibition is not as dramatic as the *CWC1* overexpression strain (Lu et al., 2005). In our deletion studies, overexpression of the PAS domain-deleted $CWC2_{(72–140\triangle)}$ in the *cwc2* mutant showed the phenotype similar to the *cwc2* mutant (Fig. 2Bf and Bb), and the same construct in the wild-type strain displayed the phenotype similar to the wild-type strain (Fig. 2Be and Ba). These phenotypes suggest that the PAS domain-deleted Cwc2 proteins are not functional and also fail to interfere with the interaction between the wild-type Cwc1 and Cwc2 proteins. Therefore, the importance of PAS domain in the Cwc2 protein is confirmed.

Surprisingly, overexpression of the zinc finger motif-deleted $CWC2_{(349–373\triangle)}$ construct in the *cwc2* mutant background produced filaments similar but not to the same level as the *cwc2* mutant (Fig. 2Bh). This observation was repeatedly seen among different transformants and also verified in different experiments. In addition, overexpression of the same construct in the wild-type strain also showed a filamentation phenotype intermediate between the *cwc2* mutant and the wild-type strain (Fig. 2Bg). One possible explanation is that the truncated $Cwc2_{(349–373\triangle)}$ protein may still retain partial DNA-binding activity due to the flanking or other regions of the protein. Recent deletion experiment found that the last 19 amino acids of Cwc2, also located next to the zinc finger motif, were also crucial for the Cwc2 function (data not shown).

Previous studies by our group and others demonstrated that blue light inhibits sexual filamentation via the Cwc/Bwc complex in *C. neoformans* (Idnurm and Heitman, 2005; Lu et al., 2005). This negative effect was further supported by our overexpression study, in which overexpression of the *CWC1* or *CWC2* gene enhances the inhibition phenotype upon light activation (Lu et al., 2005). Based on these results, we have proposed two models to depict the possible regulatory network(s) in *C. neoformans*. In model 1, the light-

activated Cwc complex binds directly to the promoters and negatively regulates gene involved in the filamentation of mating process. In model 2, a repressor functions intermediately between the Cwc complex and downstream genes. In response to light, Cwc complex activates the transcription of the repressor and then the repressor subsequently inhibits genes involved in the mating filamentation. To further dissect this pathway, we have conducted *Agrobacterium* random mutagenesis screen in the *CWC1* overexpression strain. Our screen was designed to identify mutants suppressing the *CWC1* overexpression effect. The *CWC1* overexpression strain shows complete inhibition of sexual filamentation upon light irradiation and its dominant and tight phenotype allowed us to easily score the T-DNA mutants restoring the filamentous growth. However, only mutations with the dominant effect will be revealed in our mating screening. *Agrobacterium*-mediated transformation has been proven a powerful tool for functional genomics in fungi (de Groot et al., 1998; Michielse et al., 2005). In *C. neoformans*, *Agrobacterium*-mediated insertional mutagenesis displayed advantageous features including random integration, high transformation efficiency and improved stability over other transformation systems (Idnurm et al., 2004) and has also been successfully used to identify genes with novel functions in combination with available genomic sequence resources (Walton et al., 2005).

In our screen, we expected that genes mutated might be part of the Cwc complex or function as regulators in direct or indirect conjunction with the Cwc complex for light perception and transduction or light-regulated filamentation pathway. From our screen, we obtained a significant number of suppressor mutants. The number is not surprising, since that a large number of QTLs (quantitative trait loci) affecting filamentation has been demonstrated (Lin et al., 2006). Among the filamentous suppressors, most of them restored limited amount of filaments; however, over twenty suppressors produced mating filaments similar to, or even more than, the wild-type strain. Comparison of the filamentation phenotypes in the light and dark might reflect the roles or steps of these genes in controlling the processes. One type of the mutants we expected is the "blind" mutants, which should produce abundant and similar level of filaments in the light and dark. Although T-DNA has not been found to disrupt the *CWC1* overexpression, we did identify mutants affecting the *CWC2* gene. In addition to AZ5, we recently found three other mutants in level 5, in which T-DNAs were inserted at the promoter or coding region of the *CWC2* gene (results not shown). The light regulatory role of Cwc2/Bwc2 in *C. neoformans* was previously confirmed (Idnurm and Heitman, 2005). Interestingly, the *CWC2* gene was also originally discovered by an insertional mutagenesis approach with different settings. The finding of T-DNA insertion at the *CWC2* locus fell into the "blind" category and also validated our suppressor screening approach.

Identification of the suppressor strain AY18 is interesting and its different filamentous phenotypes in mating and monokaryotic fruiting are even more intriguing. Although three copies of T-DNA were found to be linked and integrate in the AY18 genome, we were able to identify the insertion site and demonstrate that the *C. neoformans SSN8* homologue is responsible for the suppression phenotypes. *S. cerevisiae* Ssn8p is a mediator component of the RNA polymerase II holoenzyme, which negatively regulates diverse physiological processes (Björklund and Gustafsson, 2005). The *SSN8* gene was originally identified as a suppressor of the *snf1* mutation involving in glucose catabolite repression (Carlson et al., 1984). To reveal the mechanism leading to the restoration of mating filamentation, the transcript level of *CWC1* in the AY18 or YSC1 strain was recently verified by quantitative real-time PCR analysis. We found that the *CWC1* gene remained at the overexpression level in these strain, suggesting the filamentation phenotypes were not due to down-regulation of the *CWC1* overexpression (results not shown). The expression study and phenotypic features of these strains suggest that *C. neoformans* Ssn8 possibly functions downstream of the Cwc complex via a more specific manner. Its dramatically derepressed monokaryotic filamentation phenotype also implies that Ssn8 might play a more critical role in the monokaryotic fruiting process.

In summary, the evolutionally conserved white collar proteins, Cwc1 and Cwc2, are two central blue regulators of *C. neoformans*. In this report, we characterized and confirmed the importance of putative domains identified in both proteins. By conducting a screen for suppressor mutants, we found genes involved in the blue light-inhibited filamentation pathways. In addition to the *CWC2* and *SSN8* genes, 15 other suppressor genes were identified by inverse PCR as listed in Table 3. According to the annotated information, they appear to be involved in chromatin modification, transcriptional regulation, bud site selection, meiosis and nutrient uptake and metabolism. Gene deletion of some of these genes, including the *SSN8* homologue, in the wild-type background exhibited no apparent, or slightly enhanced, mating phenotypes; on the other hand, overexpression of these genes consistently showed reduced mating phenotypes (results not shown). These preliminary results support our models in which negative regulators function in the pathway. Environmental signal such as light is one of the stimuli that controls filamentous growth of *C. neoformans*. Light was also demonstrated to influence mycelial growth and colony morphology of *T. borchii* and *N. crassa* (Ambra et al., 2004). In addition to unraveling the light signaling mechanism in *C. neoformans*, our studies might further provide insight into how hyphal growth is regulated in fungi.

Acknowledgments

We thank Drs. Alexander Idnurm and Joseph Heitman for kindly providing the *A. tumefaciens* strain LBA4404 bearing the plasmid pPZP-201BK. We also thank the genome sequence information provided at Stanford Genome Technology Center, the Institute for Genome Research, the Broad Institute, and Duke University. This work is supported by National Science Council Grants NSC94-2311-B-002-031, NSC95-2311-B-002-010, and NSC96-2311-B-002-022-MY3 to Wei-Chiang Shen.

References

Alspaugh, J.A., Perfect, J.R., Heitman, J., 1997. *Cryptococcus neoformans* mating and virulence are regulated by the G-protein alpha subunit GPA1 and cAMP. Genes Dev. 11, 3206–3217.

Ambra, R., Grimaldi, B., Zamboni, S., Filetici, P., Macino, G., Ballario, P., 2004. Photomorphogenesis in the hypogenous fungus *Tuber borchii*: isolation and characterization of *Tbwc-1*, the homologue of the blue-light photoreceptor of *Neurospora crassa*. Fungal Genet. Biol. 41, 688–697.

Ballario, P., Vittorioso, P., Magrelli, A., Talora, C., Cabibbo, A., Macino, G., 1996. White collar-1, a central regulator of blue light responses in *Neurospora*, is a zinc finger protein. EMBO J. 15, 1650–1657.

Björklund, S., Gustafsson, C.M., 2005. The yeast Mediator complex and its regulation. Trends Biochem. Sci. 30, 240–244.

Blumenstein, A., Vienken, K., Tasler, R., Purschwitz, J., Veith, D., Frankenberg-Dinkel, N., Fischer, R., 2005. The *Aspergillus nidulans* phytochrome FphA represses sexual development in red light. Curr. Biol. 15, 1833–1838.

Carlile, M.J., 1965. Photobiology of fungi. Annu. Rev. Plant Physiol. 16, 175–202.

Carlson, M., Osmond, B.C., Neigeborn, L., Botstein, D., 1984. A suppressor of *snf1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics 107, 19–32.

Casadevall, A., Perfect, J.R., 1998. *Cryptococcus neoformans*. ASM Press, Washington, DC.

Casas-Flores, S., Rios-Momberg, M., Bibbins, M., Ponce-Noyola, P., Herrera-Estrella, A., 2004. *BLR-1* and *BLR-2*, key regulatory elements of photoconidiation and mycelial growth in *Trichoderma atroviride*. Microbiology 150, 3561–3569.

Corrochano, L.M., 2007. Fungal photoreceptors: sensory molecules for fungal development and behaviour. Photochem. Photobiol. Sci. 6, 725–736.

de Groot, M.J.A., Bundock, P., Hooykaas, P.J.J., Beijersbergen, A.G.M., 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat. Biotechnol. 16, 839–842.

Dunlap, J.C., Loros, J.J., 2006. How fungi keep time: circadian system in *Neurospora* and other fungi. Curr. Opin. Microbiol. 9, 579–587.
Edman, J.C., Kwon-Chung, K.J., 1990. Isolation of the *URA5* gene from *Cryptococcus neoformans* var *neoformans* and its use as a selective marker for transformation. Mol. Cell. Biol. 10, 4538–4544.
Estrada, A.F., Avalos, J., 2008. The White Collar protein WcoA of *Fusarium fujikuroi* is not essential for photocarotenogenesis, but is involved in the regulation of secondary metabolism and conidiation. Fungal Genet. Biol. 45, 705–718.
Froehlich, A.C., Liu, Y., Loros, J.J., Dunlap, J.C., 2002. White Collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. Science 297, 815–819.
Froehlich, A.C., Noh, B., Vierstra, R.D., Loros, J., Dunlap, J.C., 2005. Genetic and molecular analysis of phytochromes from the filamentous fungus *Neurospora crassa*. Eukaryot. Cell 4, 2140–2152.
Griffith, G.W., Jenkins, G.I., Milner-White, E.J., Clutterbuck, A.J., 1994. Homology at the amino acid level between plant phytochromes and a regulator of asexual sporulation in *Emericella* (=*Aspergillus*) *nidulans*. Photochem. Photobiol. 59, 252–256.
Grimaldi, B., Coiro, P., Filetici, P., Berge, E., Dobosy, J.R., Freitag, M., Selker, E.U., Ballario, P., 2006. The *Neurospora crassa* White Collar-1 dependent blue light response requires acetylation of histone H3 lysine 14 by NGF-1. Mol. Biol. Cell 17, 4576–4583.
Guthrie, C., Fink, G.R., 1991. Guide to Yeast Genetics and Molecular Biology. Academic Press, San Diego.
He, Q., Cha, J., He, Q., Lee, H.C., Yang, Y., Liu, Y., 2006. CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the *Neurospora* circadian negative feedback loop. Genes Dev. 20, 2552–2565.
He, Q., Cheng, P., Yang, Y., Wang, L., Gardner, K.H., Liu, Y., 2002. White collar-1, a DNA binding transcription factor and a light sensor. Science 297, 840–843.
He, Q., Liu, Y., 2005. Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation. Genes Dev. 19, 2888–2899.
Herrera-Estrella, A., Horwitz, B.A., 2007. Looking through the eyes of fungi: molecular genetics of photoreception. Mol. Microbiol. 64, 5–15.
Huang, G., Chen, S., Li, S., Cha, J., Long, C., Li, L., He, Q., Liu, Y., 2007. Protein kinase A and casein kinases mediate sequential phosphorylation events in the circadian negative feedback loop. Genes Dev. 21, 3283–3295.
Hull, C.M., Heitman, J., 2002. Genetics of *Cryptococcus neoformans*. Annu. Rev. Genet. 36, 557–615.
Idnurm, A., Heitman, J., 2005. Light controls growth and development via a conserved pathway in the fungal kingdom. PLoS Biol. 3, e95.
Idnurm, A., Reedy, J.L., Nussbaum, J.C., Heitman, J., 2004. *Cryptococcus neoformans* virulence gene discovery through insertional mutagenesis. Eukaryot. Cell 3, 420–429.
Idnurm, A., Rodriguez-Romero, J., Corrochano, L.M., Sanz, C., Iturriaga, E.A., Eslava, A.P., Heitman, J., 2006. The *Phycomyces madA* gene encodes a blue-light photoreceptor for phototropism and other light responses. Proc. Natl. Acad. Sci. USA 103, 4546–4551.
Kihara, J., Moriwaki, A., Tanaka, N., Ueno, M., Arase, S., 2007. Characterization of the *BLR1* gene encoding a putative blue-light regulator in the phytopathogenic fungus *Bipolaris oryzae*. FEMS Microbiol. Lett. 266, 110–118.
Kwon-Chung, K.J., 1976. Morphogenesis of *Filobasidiella neoformans*, the sexual state of *Cryptococcus neoformans*. Mycologia 68, 821–833.
Kwon-Chung, K.J., Edman, J.C., Wickes, B.L., 1992. Genetic association of mating types and virulence in *Cryptococcus neoformans*. Infect. Immun. 60, 602–605.
Lee, K., Singh, P., Chung, W.C., Ash, J., Kim, T.S., Hang, L., Park, S., 2006. Light regulation of asexual development in the rice blast fungus, *Magnaporthe oryzae*. Fungal Genet. Biol. 43, 694–706.
Lin, X., Heitman, J., 2006. The biology of the *Cryptococcus neoformans* species complex. Annu. Rev. Microbiol. 60, 69–105.
Lin, X., Huang, J.C., Mitchell, T.G., Heitman, J., 2006. Virulence attributes and hyphal growth of *C. neoformans* are quantitative traits and the *MAT*α allele enhances filamentation. PLoS Genet. 2, e187.
Lin, X., Hull, C.M., Heitman, J., 2005. Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans*. Nature 434, 1017–1021.
Linden, H., Ballario, P., Macino, G., 1997. Blue light regulation in *Neurospora crassa*. Fungal Genet. Biol. 22, 141–150.
Linden, H., Macino, G., 1997. White collar 2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. EMBO J. 16, 98–109.
Liu, Y., He, Q., Cheng, P., 2003. Photoreception in *Neurospora*: a tale of two White Collar proteins. Cell. Mol. Life Sci. 60, 2131–2138.
Loftus, B.J., Fung, E., Roncaglia, P., Rowley, D., Amedeo, P., et al., 2005. The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. Science 307, 1321–1324.
Lu, Y.K., Sun, K.H., Shen, W.C., 2005. Blue light negatively regulates the sexual filamentation via the Cwc1 and Cwc2 proteins in *Cryptococcus neoformans*. Mol. Microbiol. 56, 480–491.
Michielse, C.B., Hooykaas, P.J., van den Hondel, C.A., Ram, A.F., 2005. *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Curr. Genet. 48, 1–17.
Mooney, J.L., Yager, L.N., 1990. Light is required for conidiation in *Aspergillus nidulans*. Genes Dev. 4, 1473–1482.
Moore, T.D., Edman, J.C., 1993. The α-mating type locus of *Cryptococcus neoformans* contains a peptide pheromone gene. Mol. Cell. Biol. 13, 1962–1970.
Perfect, J.R., Toffaletti, D.L., Rude, T.H., 1993. The gene encoding phosphoribosylaminoimidazole carboxylase (*ADE2*) is essential for growth of *Cryptococcus neoformans* in cerebrospinal fluid. Infect. Immun. 61, 4446–4451.
Purschwitz, J., Müller, S., Kastner, C., Fischer, R., 2006. Seeing the rainbow: light sensing in fungi. Curr. Opin. Microbiol. 9, 566–571.
Purschwitz, J., Müller, S., Kastner, C., SchÖser, M., Haas, H., Espeso, E.A., Atoui, A., Calvo, A.M., Fischer, R., 2008. Functional and physical interaction of blue- and red-light sensors in *Aspergillus nidulans*. Curr. Biol. 18, 255–259.
Sambrook, J., Russell, I., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Shen, W.-C., Davidson, R.C., Cox, G.M., Heitman, J., 2002. Pheromones stimulate mating and differentiation via paracrine and autocrine signaling in *Cryptococcus neoformans*. Eukaryot. Cell 1, 366–377.
Silva, F., Torres-Martinez, S., Garre, V., 2006. Distinct *white collar-1* genes control specific light responses in *Mucor circinelloides*. Mol. Microbiol. 61, 1023–1037.
Tan, K.K., 1978. Light-inducing fungal development. In: Smith, J.E., Berry, D.R. (Eds.), The Filamentous Fungi, vol. 3. E. Arnold, London, pp. 334–357.
Terashima, K., Yuki, K., Muraguchi, H., Akiyama, M., Kamada, T., 2005. The *dst1* gene involved in mushroom photomorphogenesis of *Coprinus cinereus* encodes a putative photoreceptor for blue light. Genetics 171, 101–108.
Toffaletti, D.L., Rude, T.H., Johnston, S.A., Durack, D.T., Perfect, J.R., 1993. Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. J. Bacteriol. 175, 1405–1411.
Walton, F.J., Idnurm, A., Heitman, J., 2005. Novel gene functions required for melanization of the human pathogen *Cryptococcus neoformans*. Mol. Microbiol. 57, 1381–1396.
Wang, P., Perfect, J.R., Heitman, J., 2000. The G-protein β subunit GPB1 is required for mating and haploid fruiting in *Cryptococcus neoformans*. Mol. Cell. Biol. 20, 352–362.
Wickes, B.L., Mayorga, M.E., Edman, U., Edman, J.C., 1996. Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the α-mating type. Proc. Natl. Acad. Sci. USA 93, 7327–7331.