

# Blue light negatively regulates the sexual filamentation via the Cwc1 and Cwc2 proteins in *Cryptococcus neoformans*

Ying-Ku Lu, Kai-Hui Sun and Wei-Chiang Shen\*

Department of Plant Pathology and Microbiology, National Taiwan University, 106 Taipei, Taiwan.

## Summary

*Cryptococcus neoformans* is a heterothallic basidiomycetous yeast that primarily infects immunocompromised individuals. Dikaryotic hyphae resulting from the fusion of the *MAT $\alpha$*  and *MAT $\alpha$*  mating type strains represent the filamentous stage in the sexual life cycle of *C. neoformans*. In this study we demonstrate that the production of dikaryotic filaments is inhibited by blue light. To study blue light photoreponse in *C. neoformans*, we have identified and characterized two genes, *CWC1* and *CWC2*, which are homologous to *Neurospora crassa wc-1* and *wc-2* genes. Conserved domain analyses indicate that the functions of Cwc1 and Cwc2 proteins may be evolutionally conserved. To dissect their roles in the light response, the *CWC1* gene deletion mutants are created in both mating type strains. Mating filamentation in the bilateral cross of *cwc1 MAT $\alpha$*  and *MAT $\alpha$*  strains is not sensitive to light. The results indicate that Cwc1 may be an essential regulator of light responses in *C. neoformans*. Furthermore, overexpression of the *CWC1* or *CWC2* gene requires light activation to inhibit sexual filamentation, suggesting both genes may function together in the early step of blue light signalling. Taken together, our findings illustrate blue light negatively regulates the sexual filamentation via the Cwc1 and Cwc2 proteins in *C. neoformans*.

## Introduction

All organisms are capable of sensing environmental fluctuations and make appropriate physiological adjustments via complex mechanisms. The capabilities to perceive light and adapt to its changes have been developed in organisms across different kingdoms. The processes of

blue light perception, signal transduction and related responses have been extensively studied in bacteria, algae, plants and fungi. The classical genetic system *Neurospora crassa* has been the leading model for studying the blue light photobiology in filamentous fungi (Linden *et al.*, 1997; Liu *et al.*, 2003).

*Neurospora crassa* can sense light in the ultraviolet and blue regions of the radiant energy spectrum, and in turn triggers diverse developmental, morphological and physiological responses (Degli-Innocenti and Russo, 1984). Blue light stimulates the formation of asexual sporulation structures and influences the number of conidial production (Lauter *et al.*, 1997). Blue light also regulates the development of protoperithecia and the number of perithecia formed, and the perithecial beaks display positive phototropism towards blue light (Harding and Melles, 1983; Linden *et al.*, 1997). Additionally, blue light induces the mycelial production of carotenoid pigment and acts as a signal to entrain the circadian rhythm (Sargent and Briggs, 1967; Harding and Turner, 1981).

Two *N. crassa* light-insensitive mutants, *white collar-1* (*wc-1*) and *white collar-2* (*wc-2*), were identified through extensive mutagenesis screens (Sargent and Briggs, 1967). *wc-1* and *wc-2* mutants exhibit pleiotropic phenotypes, and all the blue light-induced phenomena are abolished in the *wc-1* or *wc-2* null mutants (Linden *et al.*, 1997). In the *white collar* mutant backgrounds, known genes under the control of blue light all lose their light-inducible transcriptional activation (Nelson *et al.*, 1989; Schmidhauser *et al.*, 1990; Lauter and Russo, 1991; Arpaia *et al.*, 1993; 1995; Dunlap, 1996), suggesting that the gene products of *wc-1* and *wc-2* play pivotal roles in the regulation of blue light responses in *N. crassa*.

*Neurospora crassa wc-1* and *wc-2* genes have been cloned and characterized (Carattoli *et al.*, 1995; Ballario *et al.*, 1996; Linden and Macino, 1997). Analyses of the deduced amino acid sequences of these proteins revealed that they are PAS (Per-Arnt-Sim) domain-containing transcriptional factors with GATA type zinc finger DNA-binding domains. Subsequent studies demonstrated that WC-1 and WC-2 proteins form heterodimeric complex via the PAS domains to activate the transcription of downstream light-regulated genes (Ballario *et al.*, 1998; Cheng *et al.*, 2002). A specialized class of PAS domains known

Accepted 22 December, 2004. \*For correspondence. E-mail wshen@ntu.edu.tw; Tel. (+886) 223 630 231 ext. 2733; Fax (+886) 223 636 490.

as LOV (for Light-Oxygen-Voltage) was identified in WC-1 protein. The LOV domain of WC-1 was found to be similar to the chromophore-binding LOV domains of phototropins, which are blue light photoreceptors in plants (Huala *et al.*, 1997; Crosson and Moffat, 2001). WC-1 protein was later demonstrated to be the blue light photoreceptor in *N. crassa* (Froehlich *et al.*, 2002; He *et al.*, 2002) and a flavin species, flavin adenine dinucleotide (FAD), was shown to be the chromophore associated with WC-1 through binding to the LOV domain. This is the first demonstration of fungal blue light photoreceptor.

*Cryptococcus neoformans* is an opportunistic pathogenic yeast that infects both immunocompromised and immunocompetent individuals (Casadevall and Perfect, 1998). This fungus has recently become a model system of fungal pathogenesis, physiology and differentiation because of increasing clinical importance and well-developed molecular and genetic tools (Lengeler *et al.*, 2000; Hull and Heitman, 2002).

*Cryptococcus neoformans* is a heterothallic basidiomycete and grows vegetatively as a yeast. The sexual and asexual life cycles have been well described (Kwon-Chung, 1975; 1976; Alspaugh *et al.*, 2000). Under nitrogen limitation conditions, strains of opposite mating types, *MATa* and *MAT $\alpha$* , produce conjugation tubes and fuse to form dikaryotic filaments with fused clamp connections. The tips of the filaments further differentiate to form the basidia, where nuclear fusion and meiosis occur, and four chains of sessile basidiospores are finally produced. An asexual sporulation process called haploid fruiting or haploid filamentation was originally described in *MAT $\alpha$*  strains and this phenomenon has recently been observed also in *MATa* cells (Wickes *et al.*, 1996; Tschärke *et al.*, 2003). This process primarily occurs under nitrogen starvation and severe desiccation conditions and has been suggested as a way to relocate the *C. neoformans* cells to find their mating partners (Wickes *et al.*, 1996).

Studies of the *C. neoformans* genes involved in the mating process revealed that the production of dikaryotic filaments during the mating process is influenced by light. No known photoreponse has been previously described in *C. neoformans*. To further characterize this phenomenon, we examined the effectiveness of different wavelength of light and the molecular mechanism of photoreponse in *C. neoformans*. Here we report the identification and characterization of the *N. crassa* *wc-1* and *wc-2* homologues in *C. neoformans*. We found that *Cwc1* and *Cwc2* proteins are essential during signalling of blue light-regulated mating process in *C. neoformans* and may function in a way similar to *N. crassa* WC-1 and WC-2 proteins. Our results suggest that cellular components involved in the perception and control of light responses may be evolutionally conserved in this distantly related human fungal pathogen.

## Results

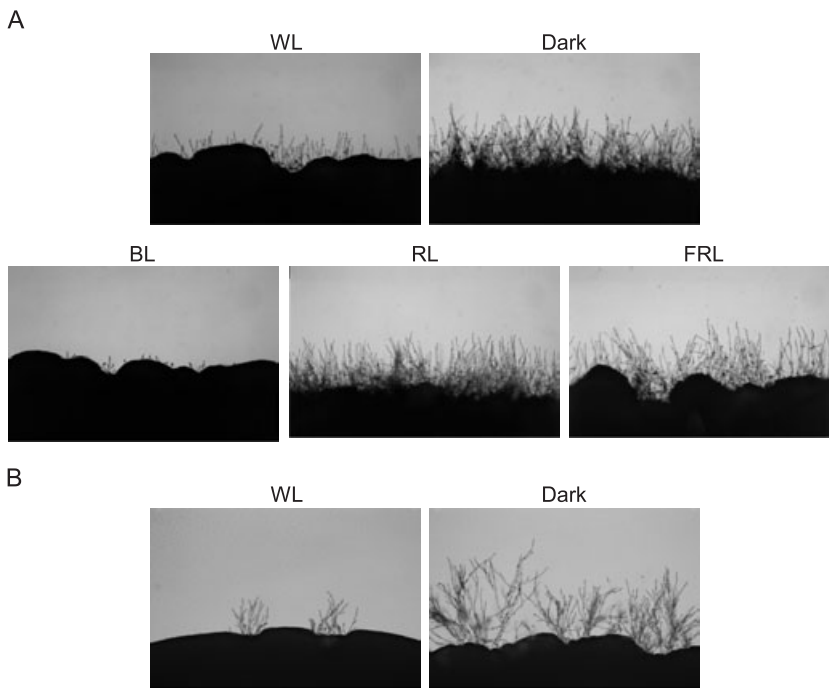
### *Blue light inhibits the production of dikaryotic mating filaments in C. neoformans*

*Cryptococcus neoformans* grows vegetatively as a yeast under normal growth conditions, and filamentous hyphae develop predominantly in the process of sexual differentiation. In an effort to characterize genes involved in the mating process, we found that the formation of dikaryotic filaments was inhibited by light (Shen *et al.*, 2002). To confirm this observation, we utilized the serotype D *MAT $\alpha$*  and *MATa* wild-type cells and compared the mating reactions under constant white light and dark conditions. We found that white light inhibited the development of the dikaryotic mating filaments, and the filaments developed under such condition were fewer and shorter (Fig. 1A). In contrast, abundant and longer mating filaments were developed in darkness after the same incubation period (Fig. 1A). Although the production of sexual filaments is apparently affected by light, the mating differentiation is not blocked by light. Filamentation can be observed after prolonged incubation, and the sexual progeny can still be obtained. In addition, light inhibition of dikaryotic filaments during the mating process is not restricted to V8 mating medium, and we have seen this phenomenon on other mating supporting media including synthetic low-ammonium dextrose (SLAD) and FA media (data not shown). Furthermore, the light sensitivity is not restricted to the serotype D *C. neoformans* strains. The mating filaments produced from the cross of the serotype A *MAT $\alpha$*  wild-type strain H99 and serotype D *MATa* wild-type strain JEC20 are also inhibited by light (Fig. 1B).

To determine the effectiveness of wavelength for triggering *C. neoformans* photoreponses, we set up illumination systems within three different wavelength ranges in the visible light spectrum including blue, red and far-red light (Peter *et al.*, 1998). The results indicate that blue light strongly inhibits the production and growth of dikaryotic filaments (Fig. 1A). On the other hand, the production of filaments is unaffected by red light and far-red light and filamentation under these two conditions is comparable to the sample observed in darkness (Fig. 1A). Taken together, our findings demonstrate that *C. neoformans* can sense light within the blue region of the visible light spectrum and blue light inhibits the production of dikaryotic mating filaments.

### *Identification and characterization of the C. neoformans CWC1 and CWC2 genes*

We demonstrate that *C. neoformans* is able to perceive blue light and regulates the filamentous morphogenesis associated with the sexual mating process. In fungi, molecular mechanisms of blue light perception and regu-



**Fig. 1.** Blue light inhibits the production of dikaryotic mating filaments in *C. neoformans*. A. Congenic serotype D wild-type *MATa* (JEC 20) and *MAT $\alpha$*  (JEC 21) strains were mixed and incubated on V8 mating agar for 2 days at 22°C with different illumination conditions. WL: the V8 mating plate was incubated under white light condition ( $84 \mu\text{mol m}^{-2} \text{s}^{-1}$ ); Dark: the V8 mating plate was incubated in the dark; BL, RL, FRL: the V8 mating plates were incubated under blue light ( $11 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), red light ( $27 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), far-red light ( $33 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) conditions respectively. The edges of mating spots were photographed at 100 $\times$  magnification.

B. The serotype A *MAT $\alpha$*  wild-type strain H99 and serotype D *MATa* wild-type strain JEC20 were mixed and incubated on V8 mating agar for 4 days at 22°C under white light (WL,  $7 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) or constant darkness conditions.

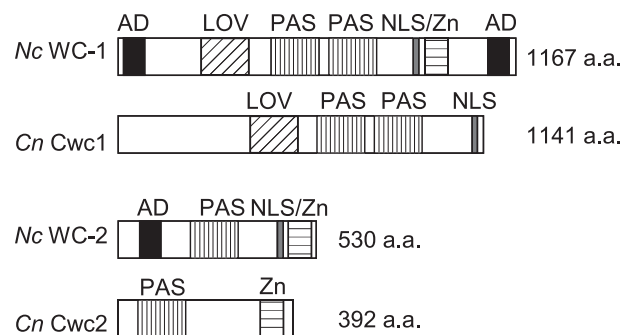
lation have been studied in great details in *N. crassa*, and WC-1 and WC-2 proteins have been shown to play the central roles in the photoresponses of *N. crassa*. To dissect the molecular mechanisms of light response in *C. neoformans*, we used *N. crassa* WC-1 and WC-2 amino acid sequences to conduct BLAST search analyses and found candidate homologues in the Stanford *C. neoformans* genome database. BLAST search with the retrieved sequences in GenBank further confirmed their homologues with *N. crassa* WC-1 and WC-2, and we therefore named the homologues *CWC1* (*Cryptococcus WC-1* homologue) and *CWC2* (*Cryptococcus WC-2* homologue). Oligonucleotide primers were designed to amplify the corresponding *CWC1* and *CWC2* fragments and the amplified products were used as probes to identify the respective genomic clones. Reverse transcription polymerase chain reaction (RT-PCR) was conducted to obtain the *CWC1* cDNA fragments. Sequencing analyses of *CWC1* genomic clone and RT-PCR products revealed a predicted coding region of 3591 bp with three introns, encoding for a protein of 1141 amino acid residues. Analyses of *CWC2* genomic sequence indicated a 1439 bp coding region encoding a 392-amino-acid protein with four introns.

Conserved domain and BLAST analyses revealed several important features in the Cwc1 and Cwc2 polypeptides. The predicted structural organizations of *C. neoformans* Cwc1 and Cwc2 proteins are shown and compared with the functional domains of *N. crassa* WC-1 and WC-2 (Fig. 2). A putative LOV domain, two putative PAS domains, and a putative nuclear localization

sequences are identified in the *C. neoformans* Cwc1 protein, whereas the Cwc2 protein includes a putative PAS domain and GATA type zinc finger DNA-binding domain.

Overall, the *C. neoformans* Cwc1 and *N. crassa* WC-1 proteins share 18% identity and 28% similarity. Sequence conservation is more significant among the predicted functional domains. The sequences of the LOV domains of *C. neoformans* Cwc1 and *N. crassa* WC-1 proteins are about 57% identity and 76% similarity, and the first and second PAS domains found in both proteins share 40% sequence identity and 55% similarity, and 57% identity and 76% similarity respectively.

On the other hand, the sequences of *C. neoformans* Cwc2 and *N. crassa* WC-2 proteins share 16% identity



**Fig. 2.** Schematic representation showing the domains organization of *C. neoformans* Cwc1 and *N. crassa* WC-1, and *C. neoformans* Cwc2 and *N. crassa* WC-2. AD, the putative activation domains; LOV, the Light-Oxygen-Voltage domains; PAS, the Per-Arnt-Sim domains; NLS, the putative nuclear localization signals; Zn, the zinc finger DNA-binding domains; a.a., amino acids.

and 27% similarity. Greater sequence conservation is found in the zinc finger DNA-binding domains, and the PAS domains among the two proteins only share 20% sequence identity and 41% similarity.

#### Disruption of the *C. neoformans* *CWC1* gene

The function of the *CWC1* gene in *C. neoformans* was further analysed by gene disruption. The *MAT $\alpha$*  wild-type copy of the *CWC1* gene was replaced with the *cwc1::URA5* disruption allele by homologous recombination (Fig. 3A). Mutant strains were screened by PCR and verified by Southern blot analysis. The hybridization results confirmed the gene replacement by the presence of 5.2 kb *EcoRI* hybridization signal due to the loss of *EcoRI* restriction site in the deleted region of *CWC1* gene and the absence of 2.9 kb wild-type fragment (Fig. 3B). The *MAT $\alpha$*  *cwc1* strain isolated from the cross of the *MAT $\alpha$*  *cwc1* strain and JEC34 (*MAT $\alpha$*  *ura5*) was also confirmed by Southern hybridization analysis (Fig. 3B). Furthermore, wild-type fragment of *CWC1* gene was reintroduced into the two gene disruption mutant strains. The *MAT $\alpha$*  *cwc1* and *MAT $\alpha$*  *cwc1* strains with the reintroduction of the wild-type fragment of *CWC1* gene were also obtained and verified (Fig. 3B).

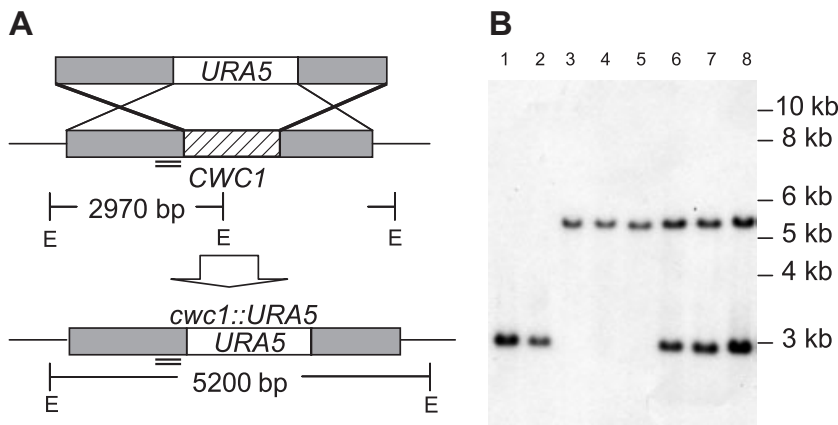
#### Deletion of the *CWC1* gene results in loss of light inhibition of filamentation during the mating process

The phenotypes of the *MAT $\alpha$*  *cwc1* and *MAT $\alpha$*  *cwc1* mutant strains were examined and the formation of dikaryotic filaments was compared under constant light and dark conditions. Mating between the wild-type *MAT $\alpha$*  (JEC20) and *MAT $\alpha$*  (JEC21) strains were used as control. Under constant darkness abundant sexual filaments were observed (Fig. 4A). In contrast, the inhibition of filamentation by light was observed on V8 mating medium at 22°C after 24 h incubation, and only a few filaments were observed at the edges of the mating strains in white light

condition (Fig. 4D). When the *MAT $\alpha$*  *cwc1* or *MAT $\alpha$*  *cwc1* strain was co-incubated with the opposite *MAT $\alpha$*  or *MAT $\alpha$*  mating type wild-type tester strains, more filaments were formed in light condition as compared with the cross between the *MAT $\alpha$*  and *MAT $\alpha$*  wild-type strains (Fig. 4E and F); however, the filaments were not as many as those formed in darkness (Fig. 4B and C). Interestingly, more dramatic production of mating filaments was observed in the bilateral cross of the *MAT $\alpha$*  *cwc1* and *MAT $\alpha$*  *cwc1* strains under constant light illumination (Fig. 4J), and the amount of the mating filaments under light was comparable to that formed in the dark (Fig. 4G). Finally, unilateral or bilateral cross of *MAT $\alpha$*  *cwc1* or *MAT $\alpha$*  *cwc1* reconstitution strains under light and dark conditions was conducted, and the extent of filamentation in these crosses was all comparable to the extent of filamentation in the wild-type strain background (Fig. 4H, I, K and L; data not shown). Taken together, our results demonstrate that deletion of the *CWC1* gene results in loss of light sensitivity, and the sexual filamentation is no longer inhibited by light.

#### The *CWC1* and *CWC2* genes are expressed at a very low level

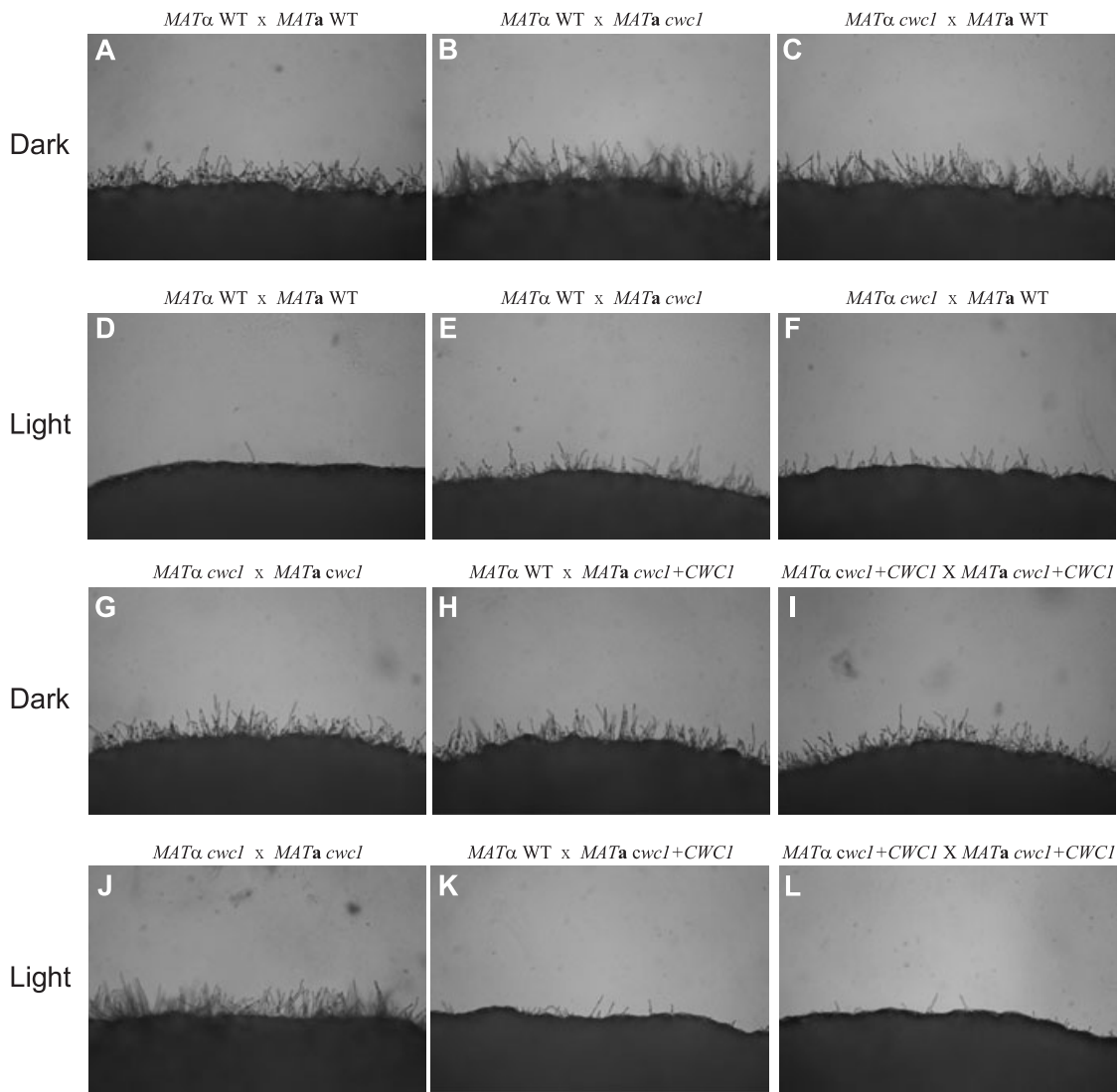
To understand the regulation of *CWC1* and *CWC2* in response to light, the steady-state level of mRNA expression under different illumination conditions was examined. Total RNA was extracted from the *MAT $\alpha$*  wild-type strain grown under conditions with constant light, constant darkness and constant darkness with 20 min or 2 h light induction before harvesting the cells during the 24 h incubation period in the yeast-peptone-dextrose (YPD) liquid medium. Twenty micrograms of total RNA from each sample were subjected to Northern hybridization analysis. However, under all conditions tested we failed to detect the *CWC1* and *CWC2* RNA transcripts (Fig. 5A). The results suggest that both of the *C. neoformans* *CWC1* and *CWC2* genes are expressed at a very low level.



**Fig. 3.** Disruption of the *CWC1* gene.

A. The *cwc1::URA5* deletion allele was created by replacing part of the *CWC1* coding region with the *URA5* selectable marker. The hybridization probe for Southern blot analysis is indicated by double line. E, *EcoRI*

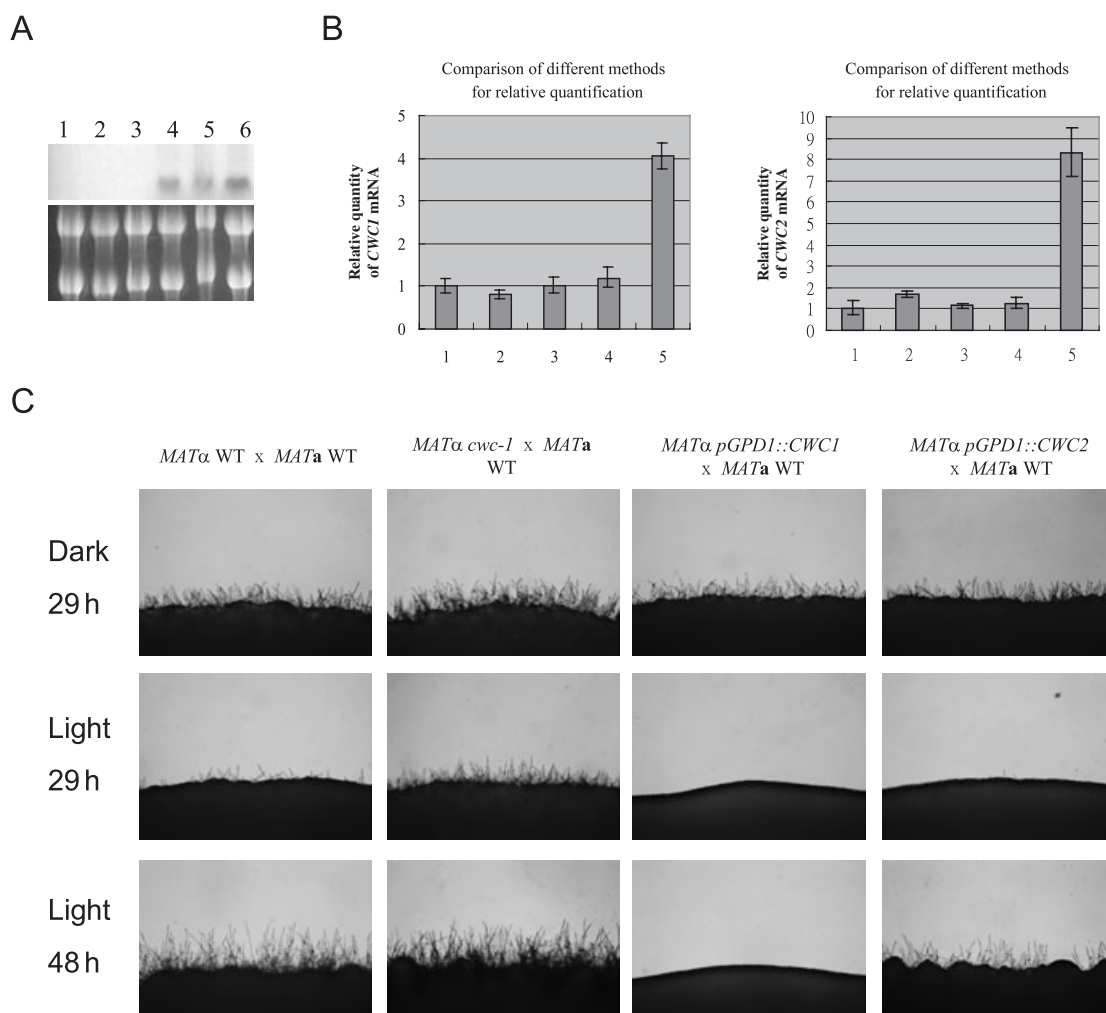
B. Genomic DNA from the *MAT $\alpha$*  wild-type (lane 1, JEC21), *MAT $\alpha$*  wild-type (lane 2, JEC20), *cwc1* *MAT $\alpha$*  mutant strains (lane 3 and 4, YKC7 and YKC12), *cwc1* *MAT $\alpha$*  mutant strain (lane 5, YKC29), *cwc1* + *CWC1* *MAT $\alpha$*  reconstituted strains (lanes 6 and 7, YKC32 and YKC33) and *cwc1* + *CWC1* *MAT $\alpha$*  reconstituted strain (lane 8, YKC34) was digested with *EcoRI* and subjected to Southern hybridization analysis using the *CWC1* probe indicated in (A). Size standards are indicated by kilobases (kb).



**Fig. 4.** Deletion of the *CWC1* gene results in loss of light inhibition of filamentation in the mating process. The *MATα* wild-type (JEC21), *cwc1* *MATα* mutant strain (YKC7), and *cwc1* + *CWC1* *MATα* reconstituted strain (YKC32) were individually crossed with the *MATa* wild-type (JEC20) or *cwc1* *MATa* mutant strain (YKC29) or *cwc1* + *CWC1* *MATa* reconstituted strain (YKC34) as indicated under constant darkness and white light ( $7 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) conditions. Mating was conducted on V8 agar at  $22^\circ\text{C}$ , and photos were taken after 24 h incubation at  $100\times$  magnification.

To further reveal the possible pattern of their regulation, we conducted the real-time PCR detection approach. The same amount of RNA from each sample used for Northern blot analysis was reverse transcribed with oligo-dT primer. Two sets of primer pairs optimized for real-time PCR analysis were designed in the *CWC1* and *CWC2* coding regions. To normalize the RNA sample, the constitutively expressed *C. neoformans* glyceraldehyde 3-phosphate dehydrogenase gene *GPD1* was used as control. The standard curves for all the three genes were experimentally determined and the dilution range for the analysis of each gene was also optimized. The resulting PCR amplification curves of *CWC1* and *CWC2* indicated that the steady states of *CWC1* and *CWC2* transcripts are very

low under the conditions examined. However, relative changes of the *CWC1* and *CWC2* mRNA levels under different illumination conditions can be detected. The results of real-time PCR analyses are shown in Fig. 5B. *C. neoformans* *CWC1* appears to be expressed at a constitutive level. On the other hand, slightly induction of the *CWC2* message was detected in the cells grown in darkness with 20 min light induction, and no significant difference is observed under other conditions. Taken together, our real-time PCR analyses indicate that both the *CWC1* and *CWC2* genes are expressed at very low levels. The *CWC1* gene is constitutively expressed, and the steady-state level of *CWC2* mRNA may possibly be regulated by light in *C. neoformans*.



**Fig. 5.** Inhibition of the sexual filamentation by *CWC1* and *CWC2* overexpression requires light activation.

**A.** Northern analysis of *P<sub>GPD1</sub>-CWC1* overexpression strains. Total RNA extracted from the *MAT $\alpha$*  wild-type (lane 1, JEC21), *cwc1* *MAT $\alpha$*  mutant strain (lane 2, YKC7), *cwc1* + *CWC1* *MAT $\alpha$*  reconstituted mutant strain (lane 3, YKC32) and *P<sub>GPD1</sub>-CWC1* overexpression strains (lane 4, YKC38; lane 5, YKC39; lane 6, YKC40) was subjected to Northern blot analysis. Hybridization probe used was as indicated in Southern analysis. RNA loading is shown by the ethidium-bromide-stained gel.

**B.** Relative real-time PCR quantification of *CWC1* (left) and *CWC2* (right) mRNA in the *MAT $\alpha$*  wild-type strain (JEC21), *CWC1* (YKC38) and *CWC2* (KHC6) overexpression strains. RNA samples of the *MAT $\alpha$*  wild-type strain were extracted from growth conditions in the constant darkness (1), and constant darkness with 20 min (2) or 2 h (3) light induction before harvesting the cells, and constant light (4) conditions during the 24 h incubation period in the YPD liquid medium. The results of RNA extracted from the *P<sub>GPD1</sub>-CWC1* (YKC38) and *P<sub>GPD1</sub>-CWC2* (KHC6) overexpression strains grown in the YPD liquid medium are shown in column (5). The results were normalized by the *GPD1* mRNA.  $n = 3$  for all groups.

**C.** The *CWC1* (YKC38) and *CWC2* (KHC6) overexpression strains were crossed with the *MAT $\alpha$*  wild-type (JEC20) under dark and light conditions. The *MAT $\alpha$*  wild-type (JEC21) and the *cwc1* *MAT $\alpha$*  mutant strain (YKC7) were co-inoculated as controls for the mating reaction. Mating was conducted on V8 agar at 22°C under constant dark and white light ( $7 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) conditions, and photos were taken after 29 and 48 h incubation as indicated at 100 $\times$  magnification.

#### *Inhibition of the filamentation by overexpression of CWC1 and CWC2 requires light activation*

To further reveal the molecular mechanism of *CWC1* function, the promoter of *C. neoformans* *GPD1* gene was used to examine the effect of *CWC1* overexpression. The fusion construct of candidate *C. neoformans* *CWC1* overexpression strains was verified by PCR. The expression of *CWC1* transcripts in the overexpression strains was con-

firmed by Northern hybridization analysis (Fig. 5A). Real-time PCR analyses revealed at least fourfold induction was observed among the *CWC1* overexpression strains (Fig. 5B and data not shown). The confirmed *CWC1* overexpressers were subjected to phenotypic characterization of dikaryotic filamentation. Interestingly, the presence of the *CWC1* transcripts and proteins was not enough for such inhibition to occur under constant darkness condition. Inhibition of the dikaryotic filaments fully depended

on light, and upon light induction formation of the dikaryotic mating filaments was completely repressed by *CWC1* overexpression even after 48 h prolonged incubation (Fig. 5C and data not shown).

Similar experiments were also conducted to examine the effect of *CWC2* overexpression. The *CWC2* overexpression strain was verified by PCR and subjected to real-time PCR analysis. An about eightfold elevation of the steady state of *CWC2* mRNA was detected in the *CWC2* overexpresser by real-time PCR analysis (Fig. 5B). Overexpression of the *CWC2* gene also failed to inhibit dikaryotic filament formation in darkness. Upon light illumination, the production of sexual filaments was repressed to some extent but not completely and some filaments were observed after prolonged light illumination (Fig. 5C). Taken together, we conclude that the inhibition of the sexual filamentation process by light in *C. neoformans* is mediated via the Cwc1 and Cwc2 proteins, and light activation is required for proper execution of molecular functions of the Cwc1 and Cwc2 proteins.

## Discussion

Light is an important environmental cue regulating diverse physiological and developmental processes. The effects of light on fungi have been well documented (Carlile, 1965; Tan, 1978). Most fungi respond to near UV and blue light, but for some fungi, including *Aspergillus nidulans*, biological responses to red and far-red light have been reported (Mooney and Yager, 1990; Griffith *et al.*, 1994). In this study, we report the first photoresponse of *C. neoformans* and demonstrate that a robust filamentation process associated with sexual differentiation in *C. neoformans* is negatively regulated by light. Using several filters, we determined that *C. neoformans* can sense blue light but is not influenced by red or far-red light. The perception of blue light results in inhibition of dikaryotic filaments formation, and hyphae produced under blue light are much sparser and shorter compared with other illumination conditions (Fig. 1A). This inhibition phenomenon appears to be universal, and is not restricted to certain *C. neoformans* serotypes or media (Fig. 1). In fact, we found that diploid filamentation associated with certain *C. neoformans* diploid strains is also inhibited by light (Sia *et al.*, 2000; data not shown).

Blue light photoresponses have been studied in great detail in *N. crassa* and interactions among the functional domains of two central regulatory proteins, WC-1 and WC-2, allow the light-induced processes to be delicately regulated. By reverse genetics approach, we identified the *N. crassa* *wc-1* and *wc-2* homologues, *CWC1* and *CWC2*, in *C. neoformans*. Deletion of the *CWC1* gene results in mutant strains insensitive to light. In the unilateral crosses between *MAT $\alpha$*  or *MAT $\alpha$*  *cwc1* mutant and the opposite

mating type wild-type strain, light inhibition still occurs to some extent due to the presence of one *CWC1* wild-type copy (Fig. 4E and F). In the bilateral cross of the *cwc1* *MAT $\alpha$*  and *MAT $\alpha$*  cells, light inhibition of mating filamentation is abolished, and more dramatic production of mating filaments is observed under constant light condition and the extent is comparable to that observed in cross between the wild-type *MAT $\alpha$*  and *MAT $\alpha$*  cells in darkness (Fig. 4J and A). These findings suggest that *CWC1* is an important regulator of light response in *C. neoformans* and light-dependent inhibition of sexual filamentation is mediated by the level of Cwc1 inside the cells.

The perception of blue light in *N. crassa* is mediated by the photoreceptor WC-1 (Froehlich *et al.*, 2002; He *et al.*, 2002). *N. crassa* strains with the deleted or point-mutated LOV domain version of WC-1 protein lose light responses (Ballario *et al.*, 1998; He *et al.*, 2002). Conservation of 11 amino acid residues important for chromophore binding was identified in the LOV domain of *N. crassa* WC-1 (Christie *et al.*, 1999). In *C. neoformans* Cwc1 protein, a putative LOV domain was identified (Fig. 2). Sequence alignment of Cwc1 LOV domain with LOV domains identified from other homologues found that these 11 amino acid residues are also conserved in Cwc1. The findings strongly suggest that the LOV domain of Cwc1 may share the conserved function and involve in chromophore binding.

In addition, alignment of LOV domains revealed a segment of 9–11 amino acid residues is inserted between  $\alpha$ 'A and  $\alpha$ C loop in certain LOV domains containing homologues including *N. crassa* WC-1 (Crosson *et al.*, 2003). This structure was suggested to possibly accommodate the larger, terminal adenine moiety of FAD, which has been demonstrated to be the flavin species associated with *N. crassa* WC-1 (Froehlich *et al.*, 2002; He *et al.*, 2002; Crosson *et al.*, 2003). This 11-amino-acid segment is also present in *C. neoformans* Cwc1. Whether FAD is the chromophore binding to Cwc1 remains to be determined by fluorescence spectroscopic analysis.

To reveal the function of *CWC2* gene in the photoresponses of *C. neoformans*, the *cwc2::URA5* disruption allele was also created and transformed. Preliminary phenotypic characterization indicated *cwc2* mutants displayed similar phenotypes as *cwc1* mutants. The result suggests that Cwc2 may also function in the blue light response pathway in this basidiomycetous pathogen.

The results of overexpression experiments are intriguing. Strains overexpressing *CWC1* gene display normal sexual filamentation in darkness as the wild-type *MAT $\alpha$*  cells. However, complete inhibition of mating filaments is observed upon light activation (Fig. 5C). This light-driven change phenotype further suggests that Cwc1 functions as a blue light photoreceptor in *C. neoformans* and the Cwc1 proteins must be converted to an active form by light

to perform its function. The observation that overexpression of *CWC2* dramatically but not completely inhibited the sexual filamentation upon light exposure is also interesting (Fig. 5C). Lack of conserved chromophore binding LOV domain in *Cwc2* suggests *Cwc2* itself is unlikely to function as a molecule for light perception. In *N. crassa*, the WC-1 and WC-2 proteins dimerize via the PAS domains (Ballario *et al.*, 1998; Cheng *et al.*, 2002), and WC-1 has also demonstrated to be the limiting factor in the complex (Cheng *et al.*, 2001). The presence of PAS domain in *Cwc2* suggests that it may interact with a less abundant light-activating component to properly execute its function.

From the presence of PAS domains in *Cwc1* and *Cwc2* and similar phenotypes observed in the *CWC1* and *CWC2* gene deletion or overexpression strains, we hypothesize that *C. neoformans* *Cwc1* and *Cwc2* possibly form the complex via the conserved protein dimerization PAS domains to activate downstream related genes. However, domain organization in *C. neoformans* *Cwc* proteins somewhat differs from that in *N. crassa* WC proteins. First, the zinc finger DNA-binding domain is not found in *Cwc1*, but present in *Cwc2* in *C. neoformans*. In *N. crassa*, the zinc finger DNA-binding domains are present in both WC-1 and WC-2 proteins. However, light induction requires only one intact zinc finger DNA-binding domain from either WC-1 or WC-2 (Cheng *et al.*, 2003). Second, the nuclear localization sequence has only identified in *Cwc1*. In *N. crassa*, the nuclear localization sequences are also found in both WC-1 and WC-2 proteins. Localization study has demonstrated that WC-1 is localized exclusively in the nucleus, and WC-2 is found in both nuclear and cytoplasmic fractions (Schwerdtfeger and Linden, 2000). Further experiments will be required to address the subcellular distribution of *Cwc* proteins. Finally, no corresponding activation domain has been identified in *C. neoformans* *Cwc1* and *Cwc2* proteins. Further structural analysis of the *Cwc* proteins should help to define regions important for their regulation. Overall, sequence analyses revealed that even though the conserved photoreponse proteins are present in these two evolutionarily distinct organisms, and some underlying molecular execution of *Cwc1* and *Cwc2* proteins may be different from WC-1 and WC-2 proteins of *N. crassa*. How *Cwc1* and *Cwc2* proteins function together in *C. neoformans* remains to be answered.

The expression of *CWC1* and *CWC2* genes was also investigated. The steady-state mRNA level of both genes was very low and failed to detect by Northern analysis (Fig. 5A). Using real-time PCR technique, we revealed that the *CWC1* gene is constitutively expressed, and the expression of *CWC2* gene is slightly upregulated in response to light and then goes down to the basal level after 2 h (Fig. 5B). Whether this upregulation is significant needs further examination. The expression levels of *wc-1*

and *wc-2* genes in *N. crassa* appears to be higher compared with those of *CWC1* and *CWC2* in *C. neoformans*, and both *wc-1* and *wc-2* is transcriptionally induced upon 20 min light irradiation by Northern analysis (Ballario *et al.*, 1996; Linden and Macino, 1997). Additionally, the expression of *wc-1* is autoregulated and PAS domain-mediated WC-1/WC-2 interaction is essential for maintaining the steady-state level of *N. crassa* *wc-1* transcript (Ballario *et al.*, 1996; Cheng *et al.*, 2002). To further understand the regulation of *CWC* genes, we are currently examining the *CWC2* expression in the *cwc1* mutant background to determine whether the light induction of *CWC2* is dependent on the function of *Cwc1*.

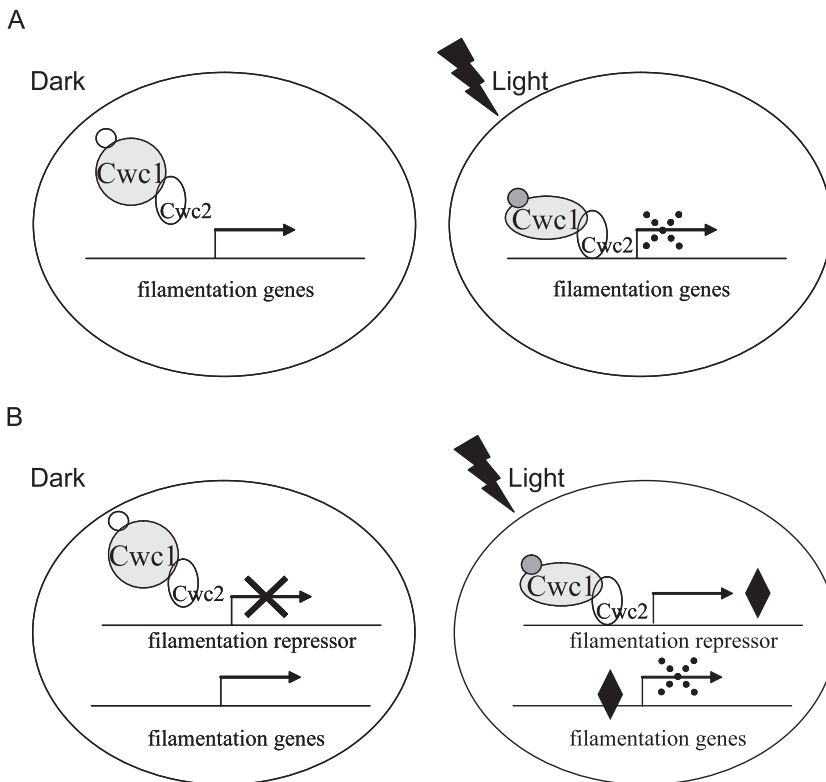
To summarize, in this study negative regulation of sexual filamentation by blue light is demonstrated in *C. neoformans*. Two conserved proteins, *Cwc1* and *Cwc2*, are identified and shown to be involved in the regulation of light response in this pathogenic fungus. However, the specific roles of these two proteins need further investigation. On the other hand, based on our current results we propose two models to describe the molecular control of photoreponses by the *Cwc* proteins in *C. neoformans* (Fig. 6). In both models, *Cwc1* functions as a blue light photoreceptor to mediate light responses through complexing with *Cwc2*. The unidentified chromophore molecule, possibly FAD, is non-covalently associated with the *Cwc1/Cwc2* complex via the LOV domain of *Cwc1*. Upon light induction, covalent modification occurs between the chromophore and LOV domain and results in the conformational change of the *Cwc1/Cwc2* complex. The activated complex in turn binds to the promoters of blue light-regulated genes to repress the expression of filamentation genes. However, it is unclear at the moment whether the activated complex directly represses the filamentation or indirectly represses the process via the action of a downstream repressor. In model 1, the activated *Cwc1/Cwc2* complex directly binds to the promoters of filamentation genes and negatively regulates the filamentation process. In model 2, an intermediate repressor is transcriptionally activated by the light-induced *Cwc1/Cwc2* complex and then inhibits the production of dikaryotic filaments associated with the mating process. To reveal the real situation, a subtractive screen approach is currently underway to hopefully identify genes under direct control of *Cwc1/Cwc2* and also involved in light-regulated filamentation or additional biological processes in *C. neoformans*.

## Experimental procedures

### *Strains, media and growth conditions*

All *C. neoformans* strains used in this study are listed in Table 1, including two congenic serotype D *MATa* and *MAT $\alpha$*  strains JEC20 and JEC21, their auxotrophic derivatives and serotype A strain H99 (Edman and Kwon-Chung, 1990;





**Fig. 6.** Blue light negatively regulates the sexual filamentation via the Cwc1 and Cwc2 proteins in *C. neoformans*. Two models describing the blue light photoregulation in *C. neoformans* are proposed.

A. In model 1, the light-activated Cwc1/Cwc2 complex directly binds to the promoters and negatively regulates genes involved in the filamentation process.

B. In model 2, an intermediate repressor is transcriptionally activated by the light-activated Cwc1/Cwc2 complex and subsequently inhibits the production of dikaryotic filaments associated with the mating process.

Moore and Edman, 1993; Wang *et al.*, 2000). All strains were handled according to standard techniques and media (Guthrie and Fink, 1991; Alspaugh *et al.*, 1997). YPD medium, synthetic (SD) medium, V8 agar, SLAD medium and filament agar were prepared as described (Wickes *et al.*, 1996; Alspaugh *et al.*, 1997). *C. neoformans* strains were routinely maintained on YPD at 30°C and mating was conducted on V8 agar at 22°C. Irradiation systems with different wavelengths were set up as previously described (Peters *et al.*, 1998). Red light ( $27 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was generated from FL20SRF fluorescent tubes (National, Osaka, Japan) with a red, plastic filter (Shinkolite A No. 102, Mitsubishi Rayon, Tokyo, Japan). Far-red light ( $33 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was generated from FL20S-FR74 fluorescent tubes (Toshiba) wrapped with one layer of Polycolor No. 22 film and one layer of Polycolor No. 72 film (Tokyo Butai Shomei, Tokyo, Japan). Blue light ( $11 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was generated using FL20S.B fluorescent tubes (Toshiba). White light ( $84$  or  $7 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was generated from FL20BR fluorescent tubes (China Electric, Taipei, Taiwan).

#### Identification of the *C. neoformans* CWC1 and CWC2 homologues and molecular manipulation

The *C. neoformans* serotype D CWC1 and CWC2 gene was identified in the *C. neoformans* genome database with *N. crassa* WC-1 and WC-2. Two sets of PCR primers, WC1 (5'-GGGCACGAATGGAGTGGTTTA-3') and WC4 (5'-GCGATCAAGCCCTATTAAGGA-3') and WC272 (5'-GACCTTCCTATACACTGT-3') and WC284 (5'-GCAGACACGTCTAGCTCTGTT-3'), were designed and

used to amplify partial fragments of the *C. neoformans* CWC1 and CWC2 genes respectively. For CWC1, a 1583 bp PCR product of the expected size was subsequently used as a hybridization probe to identify the genomic clones from a JEC21 BAC library (Research Genetics). A 12 kb *Bam*HI genomic fragment was subcloned into pBluescript SK (+) (Stratagene) to generate a plasmid, pYKL1. The sequence of a 3790 bp region containing the *C. neoformans* CWC1

**Table 1.** Strains used in this study.

Strain	Description	Reference
JEC20	MATa	Kwon-Chung <i>et al.</i> (1992)
JEC21	MAT $\alpha$	Kwon-Chung <i>et al.</i> (1992)
JEC34	MATa <i>ura5</i>	Moore and Edman (1993)
JEC43	MAT $\alpha$ <i>ura5</i>	Moore and Edman (1993)
H99	MAT $\alpha$	Wang <i>et al.</i> (2000)
YKC7	MAT $\alpha$ <i>cwc1::URA5 ura5</i>	This Study
YKC12	MAT $\alpha$ <i>cwc1::URA5 ura5</i>	This Study
YKC29	MATa <i>cwc1::URA5 ura5</i>	This Study
YKC25	MAT $\alpha$ <i>ura5 cwc1</i> (5-FOA')	This Study
YKC30	MATa <i>ura5 cwc1</i> (5-FOA')	This Study
YKC32	MAT $\alpha$ <i>ura5 cwc1 + CWC1-URA5</i>	This Study
YKC33	MAT $\alpha$ <i>ura5 cwc1 + CWC1-URA5</i>	This Study
YKC34	MATa <i>ura5 cwc1 + CWC1-URA5</i>	This Study
YKC38	MAT $\alpha$ <i>pGPD1::CWC1-URA5 ura5</i>	This Study
YKC39	MAT $\alpha$ <i>pGPD1::CWC1-URA5 ura5</i>	This Study
YKC40	MAT $\alpha$ <i>pGPD1::CWC1-URA5 ura5</i>	This Study
KHC6	MAT $\alpha$ <i>pGPD1::CWC2-URA5 ura5</i>	This Study

gene was verified with a series of designed sequencing primers. RT-PCR was performed and the amplified products were sequenced to verify the intron–exon junctions and determine a putative open reading frame. All nucleic acid manipulations were performed using standard procedures (Sambrook and Russell, 2001).

#### *Disruption, reintroduction and overexpression of the C. neoformans CWC1 gene*

To disrupt the *C. neoformans CWC1* gene, a *cwc1::URA5* disruption allele was constructed by replacing part of the *CWC1* open reading frame with the *URA5* marker. In brief, a *SalI*-mutated version of pBluescript SK (+) was first created and used to clone the 12 kb *Bam*HI *CWC1* genomic fragment from pYKL1. The resulting plasmid pYKL2 was subsequently restriction digested with *Xba*I and self-ligated to remove the 5' part of 6 kb region to create pYKL3. pYKL3 was restriction digested with *Xba*I, blunt-ended by fill-in reaction, ligated with the *Sma*I *URA5* fragment released from plasmid pRCD69, and finally created pYKL4. The 4.5 kb fragment of the *cwc1::URA5* disruption allele was released from pYKL4 by *Xba*I and *Eco*RI double digestion and introduced into the *MAT $\alpha$  ura5* strain JEC43 by biolistic transformation as described (Toffaletti *et al.*, 1993). One microgram of purified DNA was used for each bombardment in the transformation experiment. The resulting uracil prototrophic transformants were picked and screened by PCR and Southern blot analyses. To isolate *MAT $\alpha$  cwc1* strains, the *MAT $\alpha$  cwc1* strain was crossed with *C. neoformans* JEC34 strain (*MAT $\alpha$  ura5*). The progeny were isolated on SD medium lacking uracil and the *MAT $\alpha$*  strains containing the disruption construct were identified by PCR and Southern blot analyses.

The *C. neoformans CWC1* reconstitution clone was made first by digesting pYKL2 with restriction enzyme *Sma*I. The 11 kb vector containing fragment was gel-purified and self-ligated to generate pYKL5. pYKL5 was linearized by *Bam*HI and then ligated with the *Bam*HI *URA5* fragment released from plasmid pRCD69, and finally created reconstitution clone pYKL6. The *ura5* version of the *cwc1 MAT $\alpha$*  and *MAT $\alpha$*  mutant strains used for reconstitution were generated by selecting the original mutants on 5-fluoroorotic acid (5-FOA) medium, which is toxic to *URA5* cells. The *CWC1* reconstitution plasmid pYKL6 was also biolistically transformed into the *ura5 cwc1 MAT $\alpha$*  and *MAT $\alpha$*  mutant strains. Uracil prototrophic transformants were picked, screened by PCR and verified by Southern blot analysis.

To overexpress the *CWC1* gene, the promoter region of *C. neoformans GPD1* gene was amplified using PCR primers YK1 (5'-TATCCTATCTACAAAATGCCGC TCTAGAAAC CAGTCA-3') and YK2 (5'-AAAAAGGATCCATGTATTATG CAAGT ATACTCCTAGAAGGA-3'). The resulting 1.9 kb PCR product was purified, digested with *Xba*I and *Bam*HI and cloned into pBluescript SK (+) (Stratagene) to generate pYKL7. The *URA5* marker of pRCD69 was released by *Xho*I, filled-in, ligated with the *Sac*I digested and blunt-ended pBluescript SK (+) and generate pYKL8.

The 4.7 kb *CWC1* fragment containing the coding region and the 0.5 kb downstream region was amplified by primers WC241 (5'-CGGGATCCATGTCAACA AACCTCACTTCT-3') and YK3 (5'-GTCATCAATAAGCTTGATGTGAGT-3'). The

PCR product was purified and digested with *Bam*HI and ligated with pYKL8. The clone with correct orientation was verified and biolistically transformed into the *MAT $\alpha$  ura5* strain JEC43. Uracil prototrophic transformants were picked and screened by PCR. The candidate clones were further verified by Northern blot and real-time PCR analyses.

#### *Overexpression of the C. neoformans CWC2 gene*

To overexpress the *CWC2* gene, pYKL8 with the *C. neoformans GPD1* promoter and *URA5* selectable marker were also used. The 2 kb *CWC2* fragment was amplified by primers WC325 (5'-CGGGATCCAATGTCC CTCCTCGCCGAGTCT-3') and WC326 (5'-TCCCCCGG GAAGGCCGAAATGAAATAAGCGT-3'). The PCR product was purified and digested with *Bam*HI and *Sma*I and ligated with pYKL8 to generate pKHS1. The clone was verified and biolistically transformed into the *MAT $\alpha$  ura5* strain JEC43. Uracil prototrophic transformants were picked and screened by PCR. The candidate clones were further verified by real-time PCR analysis.

#### *Isolation of genomic DNA and Southern blot analysis*

Genomic DNA used for Southern analysis was prepared by a large-scale isolation protocol described previously (Perfect *et al.*, 1993). DNA was digested with *Eco*RI and electrophoresed in a 0.8% 1 $\times$  TAE agarose gel. DNA was transferred to nylon membrane (Immobilon<sup>TM</sup>-Ny+, MILLIPORE) by capillary action. Southern hybridization was performed with a 0.8 kb *WC1* probe generated by PCR amplification with the primers YK4 (5'-CCAGCTTGGACCATGGCTCCA GATCAG-3') and YK5 (5'-GGCGGGATATCGTAGTGGGT GCTGTAGAAGGG-3'). Probe was labelled by Prime-It<sup>®</sup> II Random Primer Labeling Kit (Stratagene) with [ $\alpha$ -<sup>32</sup>P]-dCTP (NEN Life Science Products) according to the manufacturer's instruction.

#### *Gene expression analysis*

Strains subjected to gene expression analysis were first grown on YPD at 30°C for 2 days and then inoculated into YPD liquid medium. Cultures were shaken at 150 r.p.m. at 30°C with different illumination conditions for 22 h. Illumination conditions included constant white light, constant darkness, or constant darkness with white light irradiation for 20 min or 2 h right before harvesting the cells. The *CWC1* and *CWC2* overexpression strains were also followed the same procedure and kept in darkness.

Cells were quickly harvested at the end of incubation, frozen in liquid nitrogen and freeze-dried. Total RNA was isolated from the freeze-dried cells using the TRIzol total RNA isolation reagent according to manufacturer's instructions (Invitrogen). For Northern blot analysis, 20  $\mu$ g 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<sup>TM</sup>-Ny+, MILLIPORE) and hybridized in hybridization buffer [0.12 M Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2), 0.25 M NaCl, 1 mM EDTA (pH 8), 7% SDS and 50% formamide]. The same 0.8 kb *CWC1* probe

described in Southern analysis was also used in Northern hybridization analysis.

Real-time PCR analysis was performed using ABI 7000 Sequence Detection System (PE Applied-Biosystems) in the presence of SYBR-green. The optimization of the real-time PCR reaction was performed according to the manufacturer's instructions but scaled down to 25  $\mu$ l per reaction. The PCR was conducted with the SYBR<sup>®</sup> Green PCR Master Mix (PE Applied-Biosystems, 4309155). After optimization, 100 nM PCR primers were used for the detection and quantification. PCR primers WC329 (5'-TATCCTTCGTTCCGCC-3') and WC330 (5'-ATCGGTGTTAGCACAG-3'), WC318 (5'-CGACAGATACTCCACCG-3') and WC319 (5'-AGGACCTTTC TCCATT-3'), and WC316 (5'-TTCTGAGAGCCCTGAGT-3') and WC317 (5'-GGCATCAACAC CAGCA-3') were used for the *C. neoformans* *CWC1*, *CWC2* and *GPD1* genes respectively.

### Mating assay

Strains for mating and haploid fruiting assays were first grown on YPD at 30°C for 2 days. Mating reactions were conducted by co-incubating cells with desired partners on V8, SLAD or filament agar medium at 22°C in the light or dark for 1–5 days. The mating testers JEC20 (*MATa*) and JEC21 (*MAT $\alpha$* ) were used for serotype D strains and serotype A strain H99 was crossed with JEC20. Dikaryotic filaments at the periphery of the mating spot were evaluated under microscope. Pictures were taken 1–4 days post incubation.

### Nucleotide sequence accession number

The sequence of the *C. neoformans* *CWC1* gene has been submitted to GenBank and its accession number is AY660969.

### Acknowledgements

We thank Dr Hsu-Liang Hsieh for kindly providing the facility with different irradiation systems. We thank Cristl Arndt and Dr Deborah S. Fox for technical assistance. We also thank Drs Shin-Hung Yeh, Janine Lin and Ya-Fen Chen for comments on the manuscript. We finally appreciate the sequence information provided by the *C. neoformans* Genome Project at Stanford Genome Technology Center. This work is supported by National Science Council Grants NSC93-2311-B-002-032 to Dr Wei-Chiang Shen.

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