行政院國家科學委員會專題研究計畫 成果報告

解析白色念珠菌 CaCDC7 在型態形成上的新穎功能(第 3 年)
研究成果報告(完整版)

計 畫 類 別 ：個別型
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執 行 期 間 ：99 年 08 月 01 日至 100 年 07 月 31 日
執 行 單 位 ：中山醫學大學生物醫學科學學系（所）

計 畫 主 持 人：謝家慶

計畫參與人員：碩士級-專任助理人員：曾子玲
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中 華 民 國 100 年 10 月 31 日

中文摘要：

Candida albicans CDC7 (CaCDC7) 編碼一種與演化同源基因產物結構相似之絲氨酸/酥胺酸蛋白質激酶，其功能依賴 DBF4 產物，Cdc7-Dbf4 具高度保留控制細胞週期中 DNA 複製起始之功能。與出芽酵母菌 CDC7 為其必要基因且調控 DNA 複製不同，白色念珠菌 CDC7(CaCDC7)似為菌絲之生長抑制者。
我們提出 CaCDC7 是白色念珠菌型態形成的主要開關的假說，經提出的三年計劃驗證此假說。我們已證實 CaCDC7 調節次單元基因 CaDBF4 為必要基因且同樣為白色念珠菌酵母菌至真菌絲轉換抑制者，顯示 CaCDC7 與 CaDBF4 的功能相關性。第二年，我們發現雙倍體的白色念珠菌 CaDBF4 等位基因產生多一份重複是由尿嘧啶合成酶篩選記號基因-清除片匣誘導。利用酵母菌雙雜合系統我們確認 CaCDC7 與 CaDBF4 直接的功能相關性。另外，利用外源持續表現 CaCDC7 在 CaDBF4 匱乏或持續表現 CaDBF4 在 CaCDC7 匱乏下形成菌絲無法被抑制證實 CaDBF4 與 CaCDC7 功能上相互依賴。第三年，我們證實 CaCdc7 激酶之催化活性及其磷酸化為其功能所需。我們也進行了以 CaCDC7 為餌以酵母菌雙雜合試驗篩選數個後選基因，目前正進行其與 CaCDC7 功能關係的確認。

英文摘要：

The Saccharomyces cerevisiae CDC7 gene product shares structural similarity with that of Candida albicans CDC7 (CaCDC7), which is a serine/threonine protein kinase. Cdc7 together with Dbf4 acts to control DNA replication of the cell division cycle in S. cerevisiae. Unlike S. cerevisiae CDC7 being essential and required for initiation of DNA replication in S. cerevisiae, C. albicans CDC7 (CaCDC7) is a filament-suppressor in C. albicans.
We have postulated that CaCDC7 is a key morphological switch in C. albicans and have proposed the three-year project to validate this hypothesis. We have already verified that the CaCDC7 regulatory subunit encoded gene CaDBF4 is a suppressor of yeast-to-hyphae transition in C. albicans, suggesting that CaCDC7 and CaDBF4 are functionally associated. In the second year, we found that the CaDBF4 allele inducibly duplicated by Ura-blaster. By using yeast two-hybrid assay, we confirmed that CaCdc7 physically interact with CaDbf4, indicating that they are indeed directly associated functionally. By introducing plasmid that carries CaDBF4 capable of constitutively expressing CaDBF4 in cells whose endogenous CaDBF4 is repressed, the hyphal development of the cells, indicating that CaDBF4 and CaCDC7 are functionally interdependent. In the third year, we verified that the catalytic activity of CaCdc7 and phosphorylation of CaCdc7 are required for the function of CaCdc7. We also performed the yeast two-hybrid assay with CaCDC7 as bait and identified several potential candidate genes whose functions are currently being characterized.

附件一

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

解析白色念珠菌CaCDC7在型態形成上的新穎功能(3/3)

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號：NSC 97-2320-B-040-014-MY3
執行期間：　2010　年　8 月　1 日至　2011 年　7 月　31 日

計畫主持人：謝家慶
計畫參與人員：簡莛、沈逸婷、賴威仲
共同主持人：

成果報告類型(依經費核定清單規定繳交)：□精簡報告　■完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年■二年後可公開查詢

執行單位：私立中山醫學大學

中　華　民　國　100　年　10　月　31　日

行政院國家科學委員會專題研究計畫成果報告

解析白色念珠菌*CaCDC7*在型態形成上的新穎功能
Elucidation of a novel role of *Candida albicans* *CaCDC7* for morphogenesis

計畫編號：NSC 97-2320-B-040-014-MY3
執行期限：99 年 8 月 1日至100 年7 月31 日
主持人：謝家慶 私立中山醫學大學生物醫學科學學系

摘要:

關鍵字：白色念珠菌；*CDC7*；絲氨酸/酥胺酸蛋白質激酶；*DBF4*；細胞周期；型態形成; 酵母菌至真菌絲轉換; 尿嘧啶合成酶篩選記號基因-清除片匣

Candida albicans CDC7 (*CaCDC7*) 編碼一種與演化同源基因產物結構相似之絲氨酸/酥胺酸蛋白質激酶，其功能依賴 *DBF4* 產物，Cdc7-Dbf4 具高度保留控制細胞週期中 DNA 複製起始之功能。與出芽酵母菌 *CDC7* 為其必要基因且調控 DNA 複製不同，白色念珠菌 *CDC7*(*CaCDC7*)似為菌絲之生長抑制者。

我們提出 *CaCDC7* 是白色念珠菌型態形成的主要開關的假說，經提出的三年計劃驗證此假說。我們已證實 *CaCDC7* 調節次單元基因 *CaDBF4* 為必要基因且同樣為白色念珠菌酵母菌至真菌絲轉換抑制者，顯示 *CaCDC7* 與 *CaDBF4* 的功能相關性。第二年，我們發現雙倍體的白色念珠菌 *CaDBF4* 等位基因產生多一份重複是由尿嘧啶合成酶篩選記號基因-清除片匣誘導。利用酵母菌雙雜合系統我們確認 *CaCDC7* 與 *CaDBF4* 直接的功能相關性。另外，利用外源持續表現 *CaCDC7* 在 *CaDBF4* 匱乏或持續表現 *CaDBF4* 在 *CaCDC7* 匱乏下形成菌絲無法被抑制證實 *CaDBF4* 與 *CaCDC7* 功能上相互依賴。第三年，我們證實 *Ca*Cdc7 激酶之催化活性及其磷酸化為其功能所需。我們也進行了以 *CaCDC7* 為餌以酵母菌雙雜合試驗篩選數個後選基因，目前正進行其與 *CaCDC7* 功能關係的確認。

Abstract

Key words: *Candida albicans*; *CDC7*; protein serine/threonine kinase; cell cycle; morphogenesis; yeast-to-hyphae transition; Ura-blaster

The *Saccharomyces cerevisiae CDC7* gene product shares structural similarity with that of *Candida albicans CDC7* (*CaCDC7*), which is a serine/threonine protein kinase. Cdc7 together with Dbf4 acts to control DNA replication of the cell division cycle in *S. cerevisiae*. Unlike *S. cerevisiae CDC7* being essential and required for initiation of DNA replication in *S. cerevisiae*, *C. albicans CDC7* (*CaCDC7*) is a filament-suppressor in *C. albicans*.

We have postulated that *CaCDC7* is a key morphological switch in *C. albicans* and have proposed the three-year project to validate this hypothesis. We have already verified that the *CaCDC7* regulatory subunit encoded gene *CaDBF4* is a suppressor of yeast-to-hyphae transition in *C. albicans*, suggesting that *CaCDC7* and *CaDBF4* are functionally associated. In the second year, we found that the *CaDBF4* allele inducibly duplicated by Ura-blaster. By using yeast two-hybrid assay, we confirmed that *Ca*Cdc7 physically interact with *Ca*Dbf4, indicating that they are indeed directly associated functionally. By introducing plasmid that carries *CaDBF4* capable of constitutively expressing *CaDBF4* in cells whose endogenous *CaDBF4* is repressed, the hyphal development of the cells, indicating that *CaDBF4* and *CaCDC7* are functionally interdependent. In the third year, we verified that the catalytic activity of *Ca*Cdc7 and phosphorylation of *Ca*Cdc7 are required for the function of *Ca*Cdc7. We also performed the yeast two-hybrid assay with *CaCDC7* as bait and identified several potential candidate genes whose functions are currently being characterized.

目錄

I. Introduction 前言

Candida albicans is an opportunistic human fungal pathogen and a natural diploid without conventional sexual cycle. It causes disease in healthy and immunocompromised hosts. *Saccaromyces cerevisiae* has been used as a comparative model for *C. albicans* due to compatible between the two in gene function, morphogenesis, and many other aspects. Considerable efforts have made towards elucidating the molecular mechanism in the control morphological plasticity among the ellipsoid blastospore to various filamentous forms including germ tubes, pseudohyphae, and true hyphae (*1*) in *C. albicans* as it has been known to be associated with virulence and pathogenesis, even though they could sometimes be decoupled (*2*). Completion of the *C. albicans* genome sequencing in 2004 has facilitated study of *C. albicans* by comparative genomics with the model yeast *Saccaromyces cerevisiae* whose genome had been completely sequenced in 1996.

Research progress has revealed an unanticipated complexity in that at least three positive and five negative pathways control morphological transition in *C. albicans* (*3-5*). To add even more impediment, Cdks and many cyclins with their regulators have also been shown to play roles in the control of such morphological transition in *C. albicans* (*6*). As such, a central and underlying issue as to how these genes and environmental factors are intertwined to modulate morphogenesis remains incomplete understood. Intriguingly, different cell cycle regulators appear to coordinate various morphological states. Significantly, an intriguing question has recently been revealed by us and others that some key cell cycle genes conserved throughout evolution play no essential role on cell cycle but morphogenesis in *C. albicans* (*7-12*).

Consequently, the long term overall goal of our project is to use budding yeast *S. cerevisiae* as a comparative model in exploring the molecular mechanism that connect cell cycle progression and morphogenesis in *C. albicans*. How the different morphological states are coordinated with cell cycle progression and the extent of divergence of such regulation in *C. albicans* from that in *S. cerevisiae* are to be addressed.

II. Objective and specific scientific aims 研究目的

We were interested in study *C. albicans* morphogenesis by exploring other cell cycle genes, particularly those involved in G1/S transition. We have focused on another *S. cerevisieae* homologue, *CDC7*, the *DBF4*-dependent kinase (DDK) (*13*) for its conserved role in the initiation of DNA replication (*14, 15*). We want to determine the extent of conservation of *CaCDC7* function on initiation of DNA replication. Particularly, our previous studies have shown that *CaCDC7* is unable to rescue the temperature sensitivity of *S. cerevisiae* strain carrying a temperature-sensitive mutant of *cdc7*. We wonder whether *CaCDC7* still conserves a central role in initiation of DNA replication or plays a role in morphogenesis in addition to initiation of DNA replication that is required to characterize directly in *C. albicans*.

Our initial results confirmed that *C. albicans CDC7* (*CaCDC7*) kinase negatively

regulates yeast-to-hyphae transition, contrasting to its homologues across evolutionary spectrum being essential for initiator of DNA replication. Consequently, in the current proposal, we postulate that *CaCDC7* is a key morphological switch in *C. albicans* and propose a three-year project to validate this hypothesis. Three specific aims are proposed. The first one is to verify *CaCDC7* together with its regulatory subunit encoded gene *C. albicans DBF4* (*CaDBF4*) regulating filamentous growth negatively and whether they are essential genes. The second one is to assess the requirement of kinase/regulatory activity and the dependency of *Ca*Cdc7 on the *Ca*Dbf4 for the function of *Ca*Cdc7. The final one is to determine *CaCDC7* associated regulation during morphogenesis. We have completed all the aims that we have set out to do.

III. Literature Reviews 文獻探討

The yeast-to-hyphae transition by the above environmental cues in *C. albicans* is mediated by several signaling pathways, the most defined of which are MAP kinase and cAMP-based PKA ones (*16*) that target Cph1 and Egf1 transcription factors, respectively (*17, 18*). However, whereas a number of factors control hyphal formation independent of the known pathways in *C. albicans* (*19*), hyphal growth involves in regulation afar the level of transcription (*20*), indicating presence of other regulatory networks and underline the complexity of the response systems. Several cell cycle regulators that play role in morphogenesis have recently been characterized in *C. albicans.* These include *CDC2*-related kinase *CRK1*, which promotes hyphal formation (*21*) and the polo-like kinases (PLKs) *CaCDC5*, a mitotic regulator that induces hyphal formation (*22*), and the *CDC2*-related kinase *CRK1*, which advances hyphal formation (*21*). Moreover, cyclins have been found to be involved in morphogenesis. *C. albican*s Cln1, a homologue of *S. cerevisiae* Cln1 appears to be a positive inducer of hyphal type (*23*), and *C. albicans* Hgc1, a homolog of *S. cerevisiae* Cln1/Cln2, is required though insufficient to induce hyphal growth (*24*). By contrast, the essential gene of *C. albicans CLN3* in its absence appears to enhance hyphal and pseudohyphal growth determined by specific conditions (*24-26*). Furthermore, two *C. albicans* G1 cyclin proteins, Clb2 and Clb4 have also been shown to act as negative regulator of polarized growth (*8*).

To date, the yeast-to-hyphae transition by the above environmental cues in *C. albicans* is mediated by several signaling pathways, the most defined of which are MAP kinase and cAMP-based PKA ones (*16*) that target Cph1 and Egf1 transcription factors, respectively (*17, 18*). In addition, Cdks and many cyclins with their regulators have also been shown to play roles in the control of morphological transition in *C. albicans* (*6*). As such, a central and underlying issue as to how these genes and environmental factors are intertwined to modulate morphogenesis remains incomplete understood. Intriguingly, different cell cycle regulators appear to coordinate various morphological states, and the most important of which is the cyclin Hgc1-dependent Cdc28 kinase that determines polarized growth (*27, 28*), inhibition of cell separation (*29*), or septin ring dynamic (*30*). In addition, *HGC1* expression is activated

either directly by Efg1 (*24*) or indirectly through Efg1-Eed1-Ume6 for long-term maintenance (*31, 32*), providing a link between morphogenesis and signaling pathway. Moreover, phosphorylation of the septin Cdc11 by Gin4, followed by cyclin Ccn1 dependent Cdc28 is required for suppression of depolarization (*33*). Significantly, we and others have recently revealed that some key cell cycle genes conserved throughout evolution play no essential role on the cell cycle control but morphogenesis in *C. albicans* (*7-12*).

We have examined the extent of conservation between *C. albicans* and *S. cerevisiae* in the control of the mitotic cell cycle at G1 phase by isolating *C. albicans* genes involved in this specific point. We have previously identified *S. cerevisiae* homologues of *CDC28*, two G1 cyclins in *C. albicans* (*34*). However, our and others' studies have shown that *C. albicans CDC4* (*CaCDC4*), contrasting to its *S. cerevisiae* counterpart whose function is required for progression from G1 to S phase of the mitotic cell cycle, suppresses filamentation (*7, 12*). We have hence identified several novel Cdc4-associated proteins (*35*). Consequently, we were interested to investigate further on other genes, which are also needed for cells to advance from G1 to S phase. Catalytic subunit encoded by *CDC7*, together with its regulatory subunit encoded by *DBF4*, known as Dbf4-dependent kinase (DDK), was a candidate that we were particularly interested as it plays an essential role on the initiation of DNA replication. *C. albicans CDC7* (*CaCDC7*) encodes a protein with sequence homology to *C. cerevisiae* Cdc7, being required for progression through S phase of mitotic cell cycle in *S. cerevisiae* (*36*). The protein product of *S. cerevisiae CDC7* has been shown to be a protein kinase that is periodically regulated via phosphorylation by other protein kinases (*37*) and by interaction with the *DBF4* gene product (*38, 39*). The mechanism by which Cdc7 protein regulates the initiation of DNA replication has now largely been elucidated. The pre-loaded MCM2-7 complex at origins is an inactive form during G1 phase and is then activated during S phase that is associated recruitment of many other factors to the origin in the S phase cyclin-dependent kinase (S-CDK) and Dbf4-dependnet Cdc7 kinase (DDK) dependent manner (*40*). Significantly, DDK, has been conserved throughout evolution, which confirms the essential role in the initiation of DNA replication among eukaryotes (*41*). To verify the role of DDK being required for the initiation of DNA replication in *C. albicans*, we have characterized *CaCDC7* and *C. albicans DBF4* (*CaDBF4*).

IV. Experimental designed and Methods 研究方法

1. Constriction of strain capable of conditionally depletion of *CaDBF4* and *CaCDC7*. To determine whether *CaDBF4* is not an essential gene and is a negative regulator of hyphal growth as *CaCDC7*, we have generated strains capable of expressing *CaDBF4* conditionally. We first constructed strains with one *CaDBF4* allele deleted. The plasmid pDDB57 with the dpl200 flanked by *CaURA3*, together with primers having sequences homologous to *URA3*-dpl200 and the up- and down-stream sequences of *CaDBF4* was PCR amplified. Such an Ura-blaster cassette was then introduced into a *C. albicans* auxotrophic strain BWP17 (*ura3*

arg4 his1) for Ura+ cells to obtain *Ca*DBF4 +/*U*3–. The strain was verified by Southern blotting analysis.

To make strains capable of conditionally expressing *CaDBF4* under *MET3* promoter control, the partial *CaDBF4* was amplified by PCR and cloned into the pFA-HIS1-Met3p plasmid. The plasmid was then linearized by a unique restriction site within the partial *CaDBF4* on the plasmid. By transforming linearized plasmid into *C. albicans* strain *Ca*DBF4 +/*U3*– for His+ prototroph, the *CaDBF4 M3*/U3– strain was obtained. However, the *CaDBF4* allele appeared to be induced duplication by the Ura-blaster. As a result, one more additional deletion of *CaDBF4* was performed to obtain *CaDBF4 M3*/*U3*–/– and the final strain whose Ura-blaster cassette was removed by addition of 5-FOA in the medium to obtain *CaDBF4* M3/–/–. The expression of *CaDBF4* under the *MET3* promoter control was tested in medium with or without methionine/cysteine. Verification of two alleles of *CaDBF4* and the Ura-blaster induced the third copy of *CaDBF4* was sought by Sothern blotting analysis. Similar approach was conducted to obtain *CaCDC7 M3*/– with one *CaCDC7* allele deleted and the other under the control of *MET3* promoter control. Southern blotting analysis was used to confirm the strain *CaCDC7 M3*/–.

2. Comparison of morphological alteration in the absence of *CaCDC7* and *CaDBF4*. Morphological consequences of the strains *Ca*DBF4 +/*U3*– in the presence or absence of *CaDBF4* were examined and compared with those of strain *CaCDC7 M3*/*U3*– capable of depleting *CaCDC7*. Similarity in phenotypes between cells depleted with *Ca*Cdc7 and *Ca*Dbf4 would suggest that they act on the same pathway. The construction of *C. albicans* strain with both *CaDBF4* alleles deleted followed the procedure as the *CaCDC7* double deletion. Such *Cadbf4* homozygous null mutant would be the definite verification of *CaDBF4* being the essential gene.

3. Construction plasmids and strains for verification physical interaction between *Ca*Cdc7 and *Ca*Dbf4 and a search of *Ca*Cdc7-interactors by yeast two-hybrid assay. One way to confirm functional association between two genes is to see if they are physically interacted. *CDC7* and *DBF4* are known to be highly conserved and encode protein kinase of catalytic subunit and regulatory subunit, respectively, to form *DBF4* dependent kinase (DDK). We have sought to test if *Ca*Cdc7 and *Ca*Dbf4 are interacted physically in which their functional relationship would be further verified. The yeast two-hybrid assay was used. Plasmid constructs capable of either expressing Gal4 activation domain fused N-terminally with *Ca*Dbf4 (pACT2-*CaDBF4*) or Gal4 DNA binding domain fused N-terminally with *Ca*Cdc7 (pGBKT7-*CaCDC7*) were made and introduced into each of haploid *S. cerevisiae* strain with opposite mating type and with reporter systems to assay the activation of the system, hence the interaction between *Ca*Cdc7 and *Ca*Dbf4 once being fused to become diploid. As the CUG codon in *C. albicans* specifies serine rather than leucine, which are specified universally by almost all other organisms including the budding yeast used in the two-hybrid assay, alternative assay using fluorescence fusion protein were sought. This includes construction of

plasmids capable of either expressing YFP fused C-terminally with *Ca*Cdc7 or CFP fused C-terminally with *Ca*Dbf4 in the presence of doxycycline. The plasmids were linearized and introduced into *C. albicnas* at *ADH1* or *OP4* locus. The interaction between *Ca*Cdc7-YFP and *Ca*Dbf4-CFP was assessed by co-localization of fluorescence of YFP and CFP.

4. Determine the functional interdependency by constitutively expressing *CaCDC7* in the absence of *CaDBF4* and vice versa. To determine the functional dependency between *CaCDC7* and *CaDBF4*, strains capable of conditionally repressing one gene while constitutively expressing another were sought to construct. If the *Ca*Cdc7 and *Ca*Dbf4 indeed form DDK, the phenotypic consequence resulted from depletion of one gene would not be rescued by the other gene. The ORFs of either *CaCDC7* or *CaDBF4* was cloned into the vector p6HF-Act1 to obtain p6HF-Act1-*CaCDC7* and p6HF-Act1-*CaDBF4*, respectively, each of which is capable of constitutively expressing *CaCDC7* or *CaDBF4* in *C. albicans*. The plasmid p6HF-Act1-*CaCDC7* were linearized and introduced into *C. albicans* strain *CaDBF4 M3*/–/– that targeted and integrated at the *RP10* locus. The cells were grown in medium with methionine and cysteine and were assessed microscopically. Similarly, the plasmid p6HF-Act1-*CaDBF4* was introduced into *CaCDC7 M3*/– and examined morphological alteration under the *MET3* repressed condition.

5. Construction strains capable of constitutively expressing the *Ca*Cdc7 catalytic inactivated or phophoacceptor deficient mutant.

The site-directed mutagenesis with QuickChange® II-E Site-Directed Mutagenesis Kit (Stratagene) was used to introduce mutations in the *CaCDC7* gene in the p6HF-based plasmid. Each of the plasmids p6HF-Act1-*CaCDC7*, p6HF-Act1-*CaCDC7* (K232R), and p6HF-Act1-*CaCDC7* (T437A) was linearized and introduced into *C. albicans* strain *CaDBF4 M3*/–/– that targeted and integrated at the *RP10* locus. The cells were grown in the SD medium with methionine and cysteine and were assessed microscopically.

V. Results and Discussions 結果與討論

1. The third copy of *C. albicans CaDBF4* is induced by Ura-blaster cassette

The *CaDBF4* +/U3– strain was obtained by introducing the *URA3*-dpl200 flanked the up- and down-stream sequences of *CaDBF4* for Ura+ prototrophs. The *CaDBF4* M3/U3– strain was obtained by introducing linearized pFA-HIS1-Met3p plasmid containing partial *CaDBF4* at a unique restriction site within the partial *CaDBF4*. Surprisingly, the *CaDBF4* M3/U3– strain was unable to form hyphae when *CaDBF4* was repressed. To verify the correctness of the strains made, Southern blotting analysis was performed. We were surprised to find that *C. albicans* possessed three copies of *CaDBF4* allele (**Figure 1**). In addition, one *CaDBF4* allele appears to have an extra 400-bp downstream region than those of the other two, when

examined from the *C. albicans* database (**Figure 1**). To verify that the presence of the third copy of *CaDBF4* is indeed genuine and that such phenomenon is not strain dependent, we performed introduction *CaDBF4* by Ura-blaster more than once into either BWP17 or its parental CAI4 strain and analyzed at least two independent Ura+ isolates (**Figure 2**). As shown in Fig. 2, presence of an extra copy of *CaDBF4* can be seen. To exclude the possibility of Ura-blaster being able to induce duplication of *CaDBF4*, markers other than *URA3* carried by the Ura-blaster were used to delete *CaDBF4* and introduced into *C. albicans*. It appeared that all the markers other than *URA3* were unable to induce an extra copy of *CaDBF4* (**Figure 3**), indicating that the genome of *C. albicans* in fact possess only two alleles and that the third copy of *CaDBF4* was induced by the introduction of Ura-blaster.

2. *CaDBF4* suppresses yeast-to-hyphae transition in *C. albicans*

To determine the role of *CaDBF4* in *C. albicans*, the *CaDBF4 M3/U3*− strain was used to introduce a cassette where *CaDBF4* was deleted by an *ARG4* maker and selected for Arg+. The correctness of the strains was verified by Southern blotting analysis (**Figure 4**). The *CaDBF4* M3/U3− strain was renamed as *CaDBF4* +/M3/U3− and the strain being further deleted with *ARG4* maker as *CaDBF4 M3/U3*−/−. In addition, the *CaDBF4 +/U3*− strain was remained as *CaDBF4 +/+/U3*−. To test the repressibility of *CaDBF4* of the strain *CaDBF4* M3/U3−/−, cells of *CaDBF4 M3/U3*−/− were grown with or without 2.5 mM methionine/cycsteine and were subjected to extraction of RNA and RT-PCR analysis. Under repressed condition, cells of *CaDBF4 M3/U3*−/−, the expression of *CaDBF4* was indeed greatly reduced as compared with the derepressed condition (**Figure 5A**), suggesting that only one of three *CaDBF4* alleles under the control of *MET3* and the other two were deleted. To determine the phenotypic consequence of *CaDBF4 M3/U3*−/− under repressed condition, cells of *CaDBF4 M3/U3*−/− were grown with or without 2.5 mM methionine/cycsteine, and the phenotypic consequence was examined microscopically. It appeared that cells form germ tubes after 4 hrs of repression and continue to grow as hyphae from 8 hrs onward up to 24 hrs under repressed condition (**Figure 5B**). To definitely determine that *CaDBF4* is the gene for suppression of yeast-to-hyphae transition, we performed a rescued assay where a constitutive *ACT1*-driven *CaDBF4* was introduced into *CaDBF4 M3/U3*−/− strain and the cells of the strain were grown as yeast form even in the presence of methinone and cysteine, confirming that *CaDBF4* is responsible foe the inhibition of hyphal growth in *C. albicans*. Importantly, up to this point, we were unable to obtain *Cadbf4* homozygous null mutant, suggesting that *CaDBF4* is essential in *C. albicans*. By introducing a cassette carrying a *ACT1*-driven *CaDBF4* and a BSA inducible recombinase gene *FLP* together with *SAT1* gene that provide resistant to nouseothrecin flanked with the *FRT* into *C. albicans* cells lacking *CaDBF4*, no colonies were formed after addition of BSA for inducibly popping out the cassette. This result confirms that *CaDBF4* is indeed an essential gene.

3. *CaCDC7* suppresses yeast-to-hyphae transition in *C. albicans*

To determine the role of *CaCDC7* in *C. albicans*, the *CaCDC7 M3/U3–* strain was constructed by using the Ura-blaster approach. *CaCDC7 M3/–* strain was also obtained after cells of *CaCDC7 M3/–* strain treated 5-FOA for Ura–. The correctness of the strains was verified by Southern blotting analysis (**Figure 6**). To test the repressibility of *CaCDC7* of the strain *CaCDC7 M3*/*U3–*, cells of the strain were grown with or without 2.5 mM methionine/cycsteine and were subjected to extraction of RNA and RT-PCR analysis. Under repressed condition, cells of either *CaCDC7M3/U3–* (data not shown) or *CaCDC7M3/–*, the expression of *CaCDC7* was indeed greatly reduced as compared with the derepressed condition as compared with those of strains BWP17 (*CaCDC7* +/+) and *CaCDC7* +/– that has one *CaCDC7* allele being deleted by using the Ura-blaster approach for Ura+, followed by treatment with 5-FOA for Ura– (**Figure 7A**), suggesting that the expression of *CaCDC7* is solely controlled by *MET3* promoter and the other allele was deleted. To determine the phenotypic consequence of *CaCDC7 M3/–* under repressed condition, cells of *CaCDC7 M3/–* were grown with or without 2.5 mM methionine/cycsteine, and the phenotypic consequence was examined microscopically. It appeared that cells form germ tubes after 4 hrs of repression and continue to grow as hypha from 8 hrs onward up to 24 hrs under repressed condition (**Figure 7B**). These observations were comparable to those of cells repressing *CaCDC7* expression (**Figure 7B**), although *C. albicans* cells repressing *CaCDC7* expression exhibited some branching which were lacking in cells repressing *CaDBF4* expression (**Figure 5B**), suggesting that *CaCDC7* and *CaDBF4* function as DDK for yeast-to-hyphae transition and that *CaCDC7* may have additional roles in morphogenesis. To definitely determine that *CaCDC7* is the gene for suppression of yeast-to-hyphae transition, we performed a rescued assay where a constitutive *ACT1*-driven *CaCDC7* was introduced into *CaCDC7 M3/–* strain and the cells of the strain were grown as yeast form even in the presence of methinone and cysteine, confirming that *CaCDC7* is responsible for the inhibition of hyphal growth in *C. albicans*. Also, we were unable to obtain *Cacdc7* homozygous null mutant, suggesting that *CaCDC7* is, like *CaDBF4*, is an essential gene.

4. *Ca*Dbf4 and CaCdc7 are physically interacted

Protein products encoded by *CDC7* and *DBF4* have been known function together as a Dbf4-dependent kinase (DDK) for the initiation of DNA replication. Although our study suggests that DDK in *C. albicans* plays an important role in morphogenesis, we wonder whether presence of *C. albicans* DDK for the function of hyphae-supression in *C. albicans.* The definitive determination of *Ca*Cdc7 and *Ca*Dbf4 form DDK for their function is to assay for their direct interaction. The yeast two-hybrid assay was adopted. As shown in **Figure 8**, diploid S. cerevisiae cells were able to grow on plate without histidine in the presence of 20 mM 3- amino-1,2,4-triazole (3-AT), the inhibitor of imidazoleglycerol-phosphate dehydratase encoded by the reporter gene *HIS3*, suggesting that the *Ca*Cdc7 and *Ca*Dbf4 are physically interacted. In parallel, due to the CUG codon being specified to serine rather than the universal leucine in *C. albicans*, we also adopted an alternative approach where different fluorescence proteins fused with either *Ca*Cdc7 or *Ca*Dbf4 simultaneous were expressed in *C. albicans* in

the presence of doxycycline, the co-localized fluorescence was used as an indication of interaction between the two proteins. As can be seen in **Figure 9**, the *Ca*Cdc7-YFP and *Ca*Dbf4-CFP were able to co-localize in the nucleus of *C. albicans*, suggesting that they form DDK to function and that DDK act on the nucleus. The result also suggest that *Ca*Cdc7 and *Ca*Dbf4 remain in the nucleus when cells grow as yeast form, possibly by activating the expression of yeast-specific genes, or repressing the expressing of hyphae-specific genes, either directly or indirectly.

5. Epistasis analysis reveals that *CaCDC7* and *CaDBF4* are functionally interdependent

To assess the functional dependency between *CaCDC7* and *CaDBF4*, plasmids p6HF-Act1-*CaCDC7* and p6HF-Act1-*CaDBF4* that are capable of constitutively expressing either *CaCDC7* or *CaDBF4* were made and introduced into *CaDBF4 M3*/–/–, which was made by treating the cells of strain *CaDBF4 M3*/–/– with 5-FOA for Ura–, and *CaCDC7 M3*/–, respectively. The ability of introduced one of DDK gene that can be constitutively expressed to suppress loss of the other DDK gene due to be repressed in the methionine and cysteine was assessed. As shown in **Figure 10**, constitutively expressing *CaCDC7* was unable to suppress loss of *CaDBF4*, suggesting that the function of *CaDBF4* requires the presence of *CaCDC7*. Similar results in which constitutively expressing *CaDBF4* was unable to suppress loss of *CaCDC7* were observed. The epistasis analyses confirm that *CaCDC7* and *CaDBF4* encode proteins to form functional DDK, hence are functionally interdependent. We have introduced a tet-on expression system that made by us (*42*) (see the final section of conclusion remarks) into BWP17 where *CaCDC7* or *CaDBF4* were massively overproduced in the presence of serum that induces hyphal growth. Cells under such condition grew as hypha, suggesting that either DDK controlling yeast-to-hyphae transition is not on the serum-induced signalling pathway, probably through other pathways or the blockage of the serum-induced filamentous growth could be bypassed by other factors. It is equally possible that DDK controls yeast-to-hypae transition via novel pathways.

6. The kinase activity and phosphorylation of *Ca*Cdc7 is required for function of DDK on suppression of yeast-to-hyphae transition

To investigate whether the ability of cells to suppress yeast-to-hyphae transition requires kinase activity and phosphorylation of DDK, plasmids p6HF-Act1-*CaCDC7* (K232R) and p6HF-Act1-*CaCDC7* (T437A), capable of constitutively expressing either the catalytically inactive *Ca*Cdc7 (K232R) or the phophoacceptor deficient *Ca*Cdc7 (T437A), were introduced into cells of the strain *CaCDC7 M3*/–. Cells of these strains were grown in medium in the presence or absence of methionine and cysteine. It is apparent that the cells expressing *ACT1*-driven mutant of either *Ca*Cdc7 (K232R) or *Ca*Cdc7 (K232R) when the expression of endogenous *CaCDC7* under the control of *MET3* promoter was repressed were grown as hyphal forms, suggesting that the catalytic activity of *Ca*Cdc7 and phosphorylation of *Ca*Cdc7

are required for the function of *Ca*Cdc7 on the suppression of yeast-to-hyphae transition.

7. Conclusion remarks

We confirmed that both *CaCDC7* and *CaDBF4* suppress yeast-to-hyphae transition and *CaDBF4* gene with allelic heterology prone to be duplicated by the Ura-blaster in *C. albicans*. In addition, we verified that the interaction between the *Ca*Cdc7 and the *Ca*Dbf4 by the yeast two-hybrid assay, together with that fact that neither cells of the strain repressed with *CaCDC7* were rescued by the constitutive expressed *C. albicans DBF4* nor vice versa, confirming that *CaCDC7-CaDBF4* is functionally interdependent. Moreover, cells of the *C. albicans* strain expressing either the catalytically inactive *Ca*Cdc7 (K232R) or the phophoacceptor deficient *Ca*Cdc7 (T437A) showed hyphal growth, confirming that the catalytic activity of *Ca*Cdc7 and phosphorylation of *Ca*Cdc7 are required for the function of *Ca*Cdc7 on the suppression of yeast-to-hyphae transition. These results will be drafted to two manuscripts and are intended to submit back-to-back to the "Nature" as our preferred Journal for publication. Furthermore, we conducted a yeast two-hybrid assay with *CaCDC7* as bait and identified two *CaCDC7*-interactors encoded by *CST20* and *MIG1*, upstream of the signaling pathway targeting to Cph1 and repressor of the glucose metabolism, respectively. Since the former is known to be activated the filament induced agents, the latter regulates glucose metabolism, if proved to be the true functional interactors with *Ca*Cdc7, would provide evidence of how *Ca*Cdc7 regulate morphogenesis and the possibility of glucose metabolism is involved in the regulation of morphogenesis mediated by *Ca*Cdc7. Further study is required to the mechanism underlying morphogenesis mediated by *CaCDC7*.

During the three-year period, we have also constructed a system to allow for inducible expression of a desired *C. albicans* gene with the advantage of convenience of cloning. It also allows the presence of a recyclable *URA3* marker and the detectable expression of fusion or epitope-tagged protein. The system has been published (*42*) and is attached as an appendix. The newly developed tet-on system has a potential wide application as the system has been requested by groups from China, USA, UK, and Switzerland since its publication on last November, suggesting the applicability of the system. Based on the system, we have devised a system where *C. albicans* cells bi-directionally express specific cDNAs in a doxycycline (Dox)-induced manner. The specific genes affecting morphology can be revealed by being either activated due to overexpressing relevant cDNA or inhibited as a result of anti-sense effect from related cDNA in the presence of Dox.

VI. References 參考文獻

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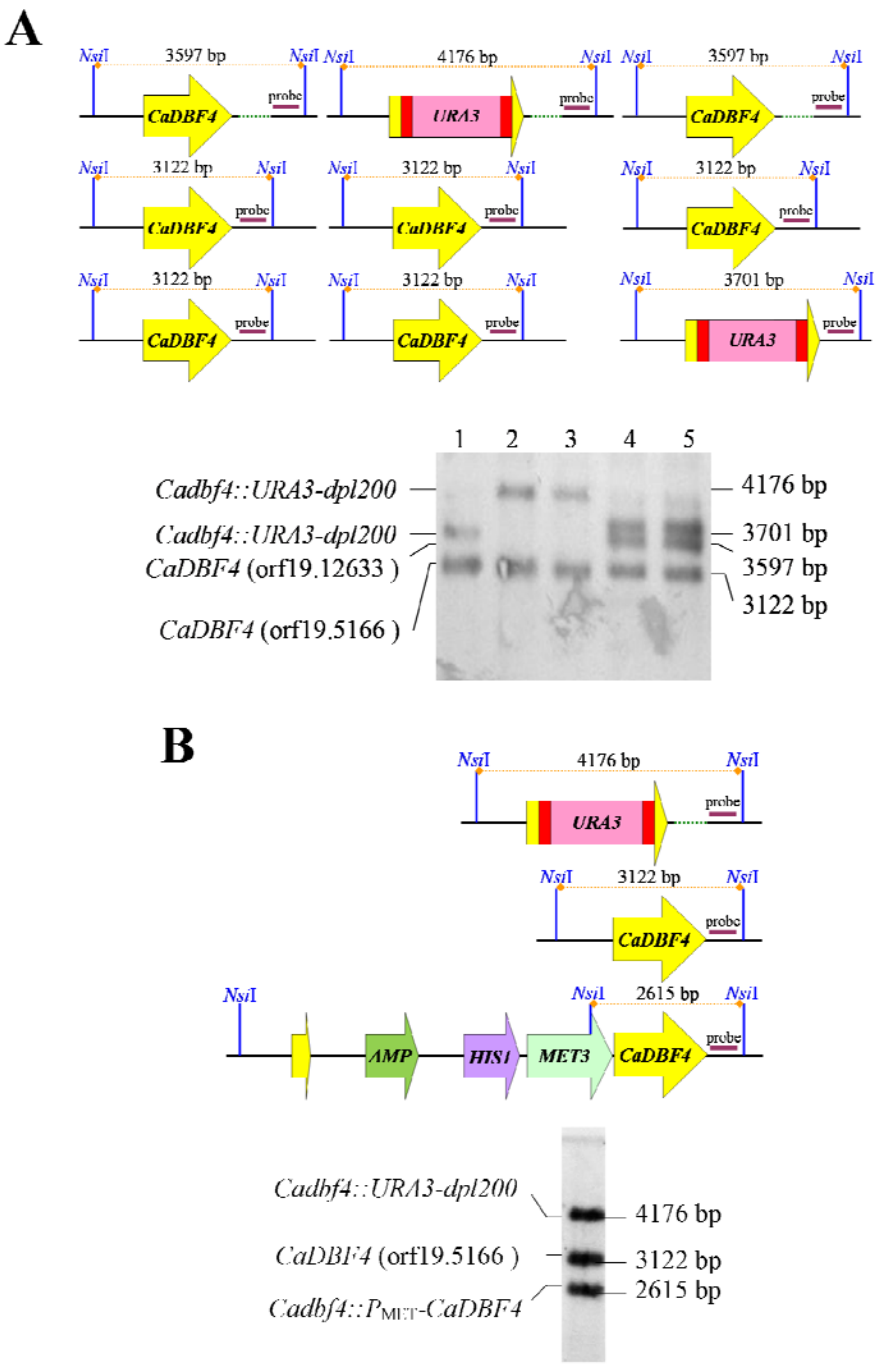

Figure 1. *C. albicans* have three copies of *CaDBF4*. **A.** Cells of all the strains were grown in YEPD to exponential. The genomic DNA was extracted and subjected to *Nsi* I digestion before electrophoresis and Southern blotting analysis. Note that the large *CaDBF4* allele has an extra downstream region of about 400 bp. The sample on lane 1 is from strain BWP17. The samples on lane 2 and lane 3 are from two independent isolates with Ura-blaster targeting the large *CaDBF4* allele. The samples on 4 and 5 are from two independent isolates with Ura-blaster targeting the small *CaDBF4* allele. **B.** When introducing the cassette where the expression of *CaDBF4* is under the control of the *MET3* promoter control that is methionine/cysteine repression, presence of the third copy of *CaDBF4* is revealed and confirmed by the size change from 3122 bp to 2615 bp. Regions used as probes are shown in red bars.

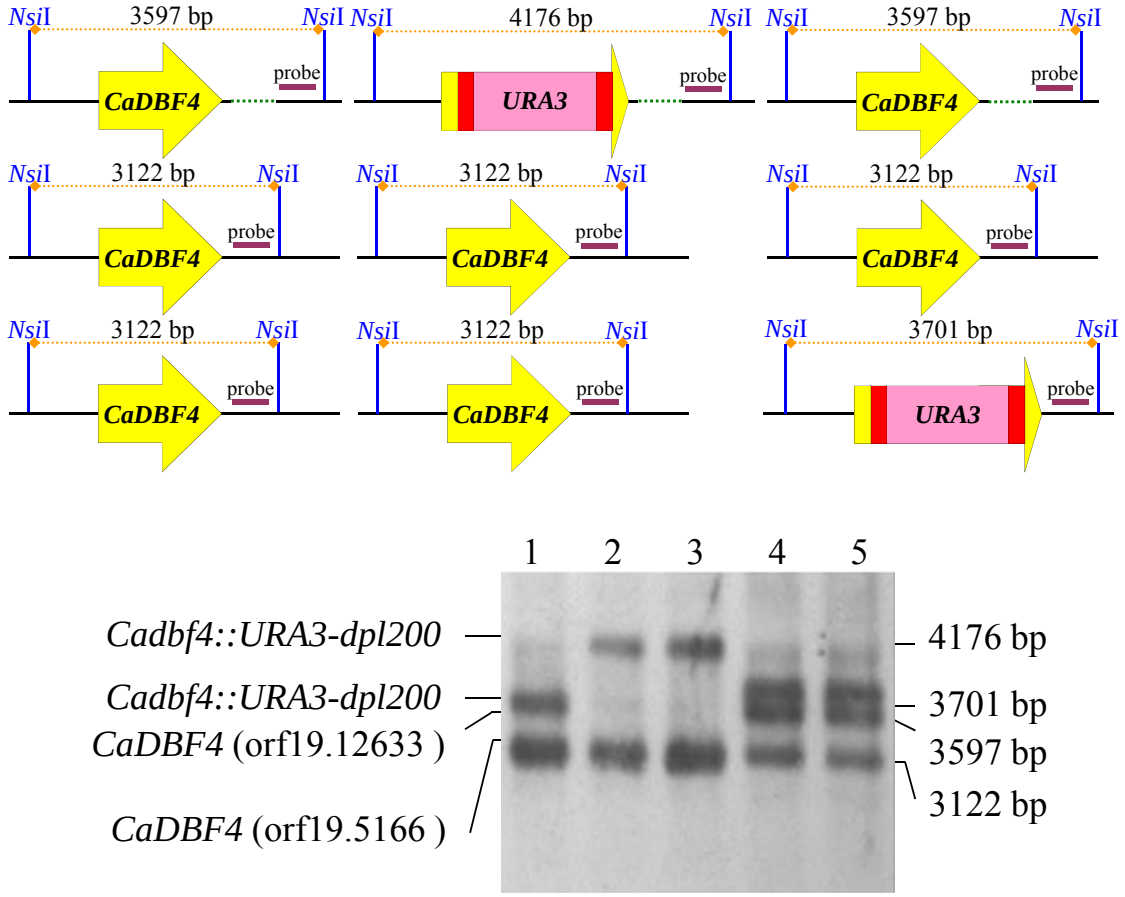

Figure 2. *C. albicans* having three copies of *CaDBF4* is not strain-specific. Cells of strain CAI4 (*ura3*) were deleted with *CaDBF4* by Ura-blaster and were subjected to Southern blotting analysis by the size change of *Nsi*I. Note that CAI4 strain also contains *CaDBF4* gene with heterologous alleles and the large *CaDBF4* allele has an extra downstream region of about 400 bp. The sample on lane 1 is from CAI4. The samples on lane 2 and lane 3 are from two independent isolates with Ura-blaster targeting the large *CaDBF4* allele. The samples on 4 and 5 are from two independent isolates with Ura-blaster targeting the small *CaDBF4* allele. Regions used as probes are shown in red bars.

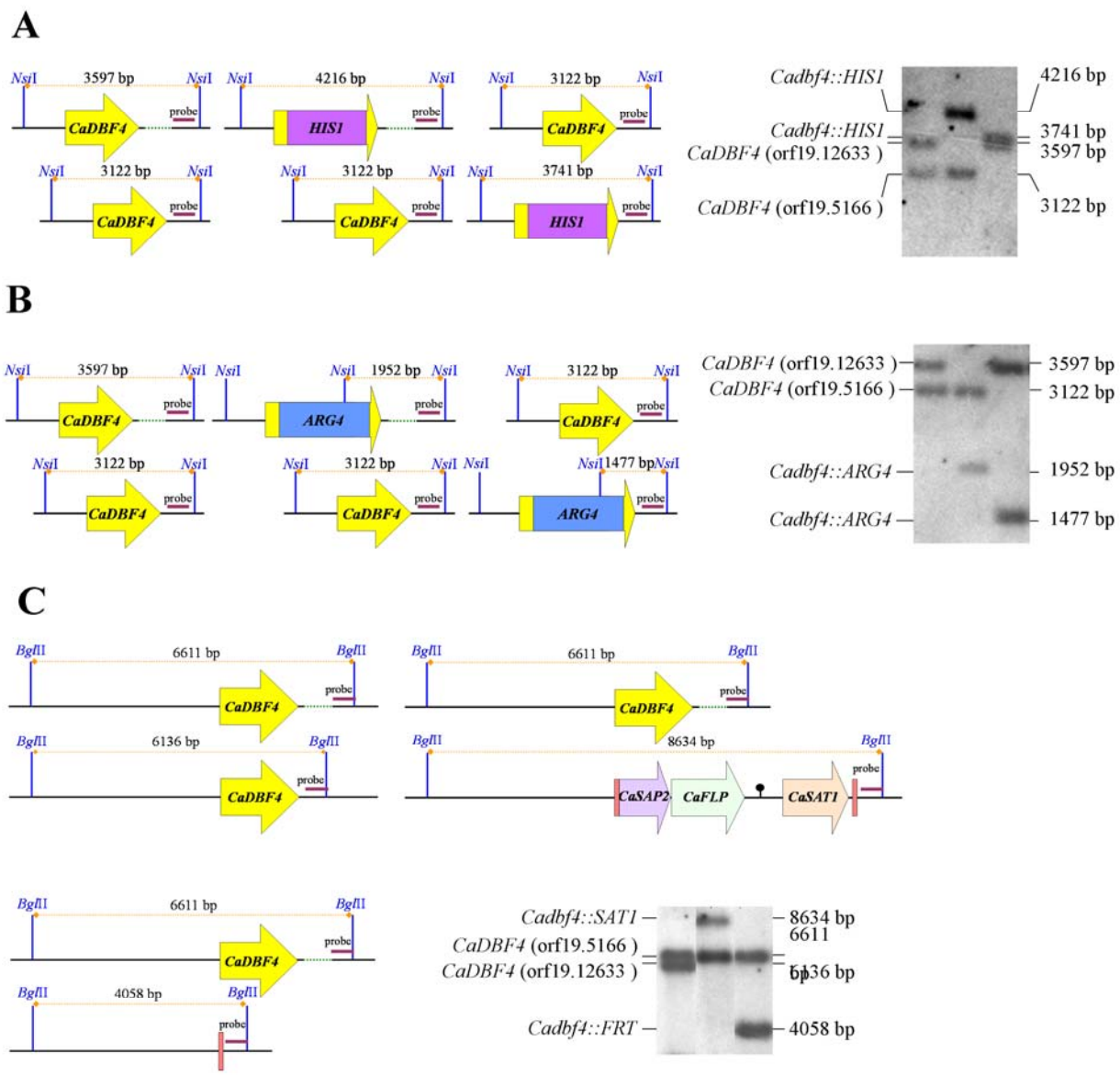

Figure 3. The extra copy of *CaDBF4* is inducibly duplicated by the introduction of Ura-blaster cassette. Cells of *C. albicans* BWP17 strain were introduced with each of *HIS1*(**A**), *ARG4* (**B**), and *FLP* flipper (**C**) cassette flanked with the up- and down-stream region of *CaDBF4*. As the assumption based on previous results that *CaDBF4* gene has three alleles were incorrect in these analysis, only two heterologous alleles are shown in **A**, **B**, and **C** of the structure of *CaDBF4* locus. The *HIS1* or the *ARG4* cassette appeared to targeting both the large or small allele of *CaDBF4*. In the case of using *FLP* flipper cassette, the one specifically targeting the small *CaDBF4* allele is shown. The *SAT1* selection marker gene can be popped out and left only the *FRT* by growing cells on plate with BSA. Regions used as probes are shown in red bars.

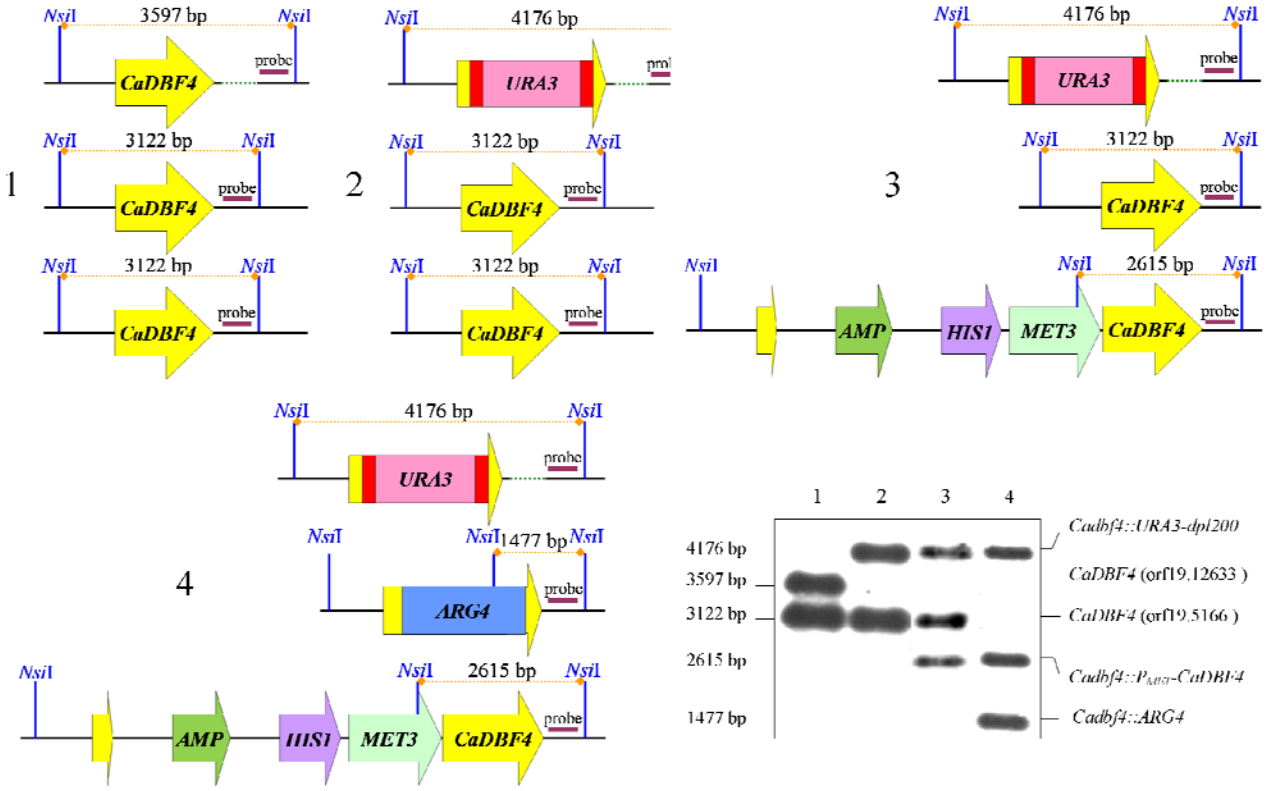

Figure 4. Construction of *C. albicans* strain capable of repressing the expression of *CaDBF4*. Cells of BWP17 (*CaDBF4* +/+/+) (**1**) was consecutively introducing cassette of Ura-blaster (**2**) to obtain *CaDBF4* +/*U3*–/+, *MET3* driven *CaDBF4* (**3**) to obtain *CaDBF4 M3/U3*–/+, *ARG4* (**4**) to obtain *CaDBF4 M3/U3*–/–. The genomic DNA from cells of each strain was extracted and subjected to *Nsi* I digestion before electrophoresis and Southern blotting analysis (the bottom panel). Regions used as probes are shown in red bars.

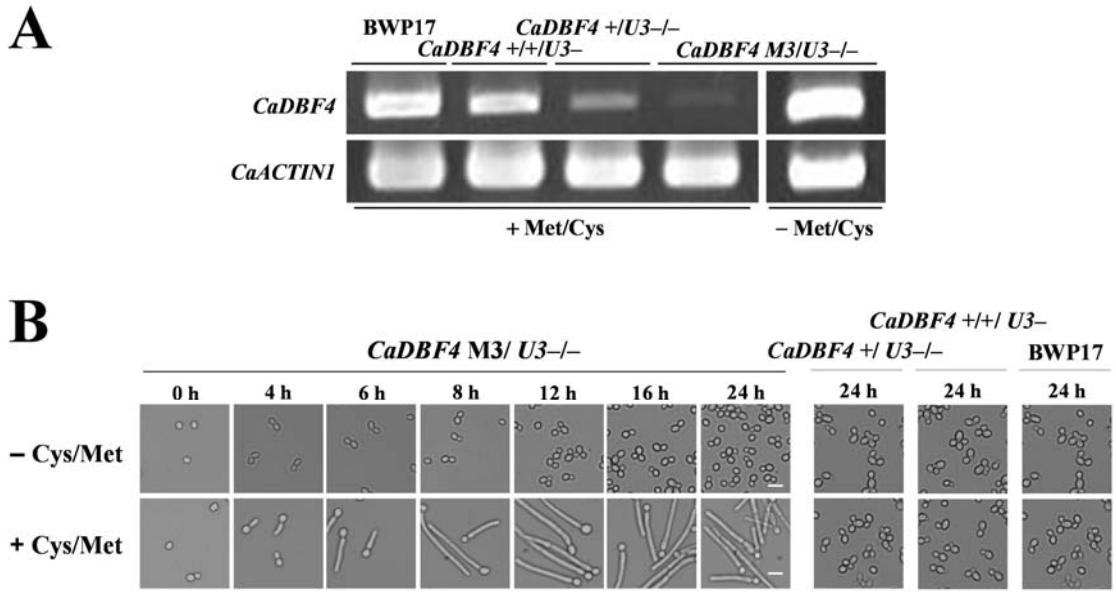

Figure 5. *CaDBF4* suppresses yeast-to-hyphae transition. Several strains are used. *CaDBF4* +/+/+ (BWP17) is an auxotrophic parental strain used in all strains construction. *CaDBF4* +/+/– is a strain with one *CaDBF4* deleted by Ura-blaster cassette for Ura+ (*CaDBF4* +/+/*U3*–), after which the cells were treated with 5-FOA to popped out the *URA3*. *CaDBF4* +/–/– is a strain with one *CaDBF4* deleted by Ura-blaster and the other by *ARG4*. *CaDBF4 M3/U*–/– is a strain with two of three *CaDBF4* alleles deleted with respective Ura-blaster (and treatment with Ura-blaster) and *ARG4* makers and the third allele being under the control of *MET3* promoter that is repressible by 2.5 mM methionine and cysteine. Cells of theses strains were grown in the SD medium with required supplements in the absence (–Met/Cys) or presence (+Met/Cys) of each of 2.5 mM methionine and cysteine for indicated times prior to assessment of morphological alteration under microscope (**B**). Bars represent 10 μm. Cells at 24 h of culture were also collected and subjected to RT-PCR to verify the repression of *CaDBF4* (**A**).

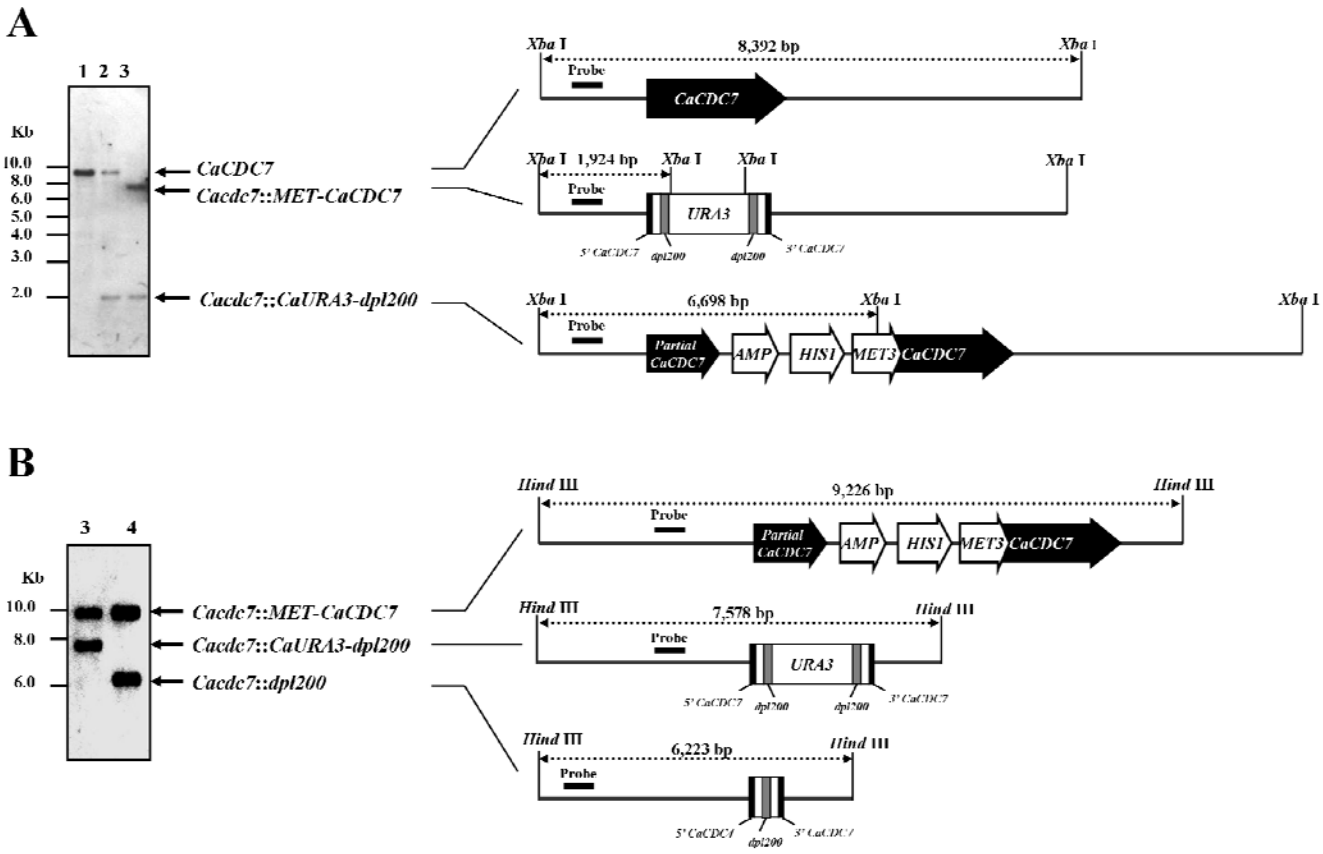

Figure 6. Construction of *C. albicans* strain capable of repressing the expression of *CaCDC7*. Cells of BWP17 (*CaDBF4* +/+) (lane 1) was consecutively introducing cassette of Ura-blaster to obtain *CaCDC7* +/*U3*– (lane 2) and *MET3* driven *CaCDC7* to obtain *CaCDC7 M3/U3*– (lane 3), after which the *CaCDC7 M3/U3*– was treated with 5-FOA to obtain *CaCDC7 M3/*– (lane 4) to obtain *CaDBF4 M3/U3*–/–. The genomic DNA from cells of each strain was extracted and subjected to either *Xba*I (**A**) or *Hind* III (**B**) digestion before electrophoresis and Southern blotting analysis (the bottom panel). Regions used as probes are shown in red bars.

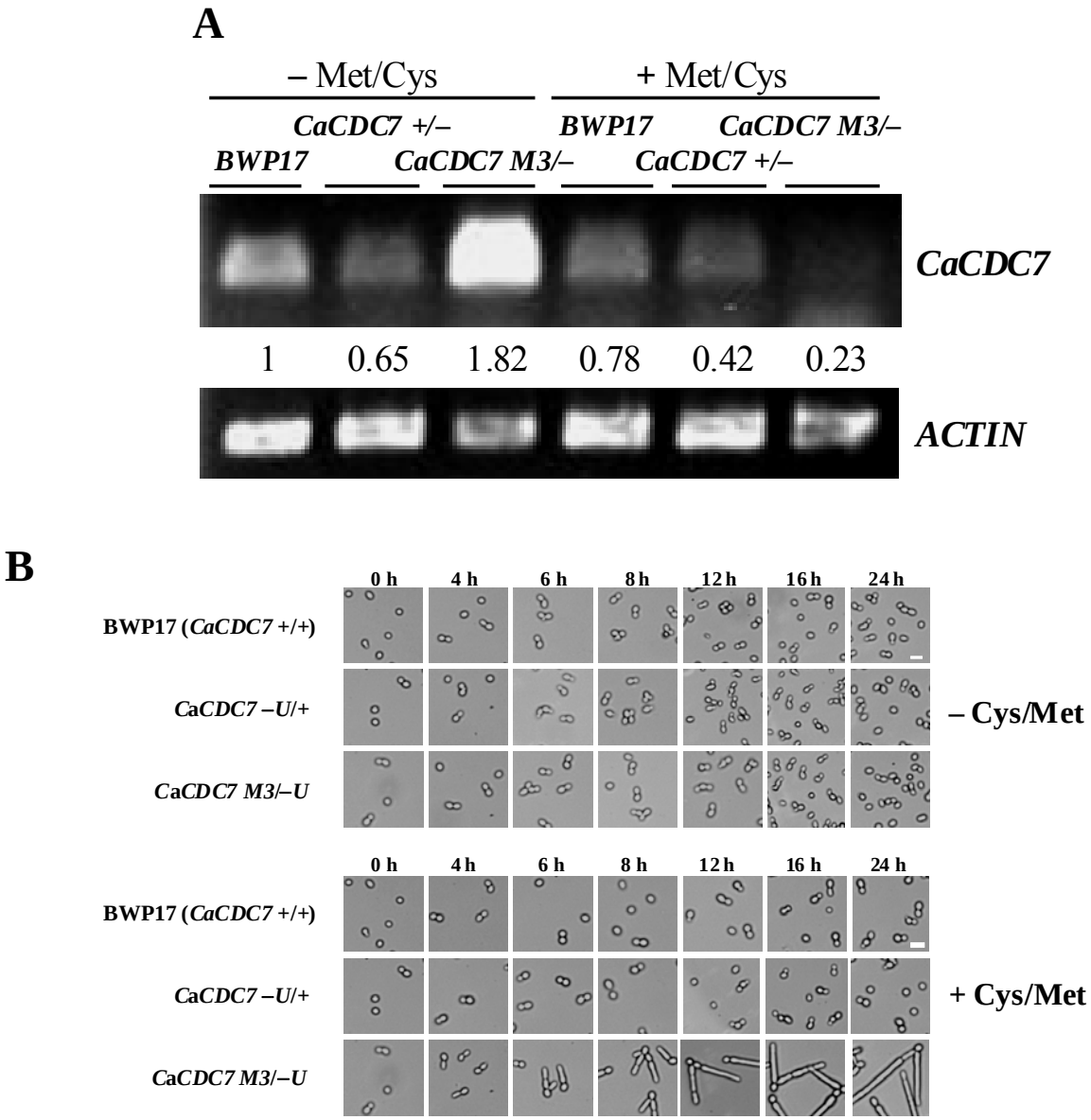

Figure 7. *CaCDC7* suppresses yeast-to-hyphae transition. *CaCDC7*+/+ (BWP17) is an auxotrophic parental strain used in all strain construction. *CaCDC7* +/*U3*– is a strain with one *CaCDC7* deleted by Ura-blaster cassette, designated *U3*–. *CaCDC7* +/– is a strain from strain *CaCDC7* +/*U3*– treated with 5-FOA to remove *URA3*. *CaCDC7 M3/*– is a strain from *CaCDC7* +/– that was introduced with a cassette such that the expression *CaCDC7* is under the control of *MET3* promoter that is repressible by 2.5 mM methionine and cysteine. Cells of theses strains were grown in the SD medium with required supplements in the absence (–Met/Cys) or presence (+Met/Cys) of each of 2.5 mM methionine and cysteine for indicated times prior to assessment of morphological alteration under microscope (**B**). Bars represent 10 μm. Cells at 24 h of culture were also collected and subjected to RT-PCR to verify the repression of *CaCDC7* (**A**). The numbers shown are relative folds of change in expression of those strains to BWP17 under derepressed condition (–Met/Cys), normalized by *ACTIN* expression

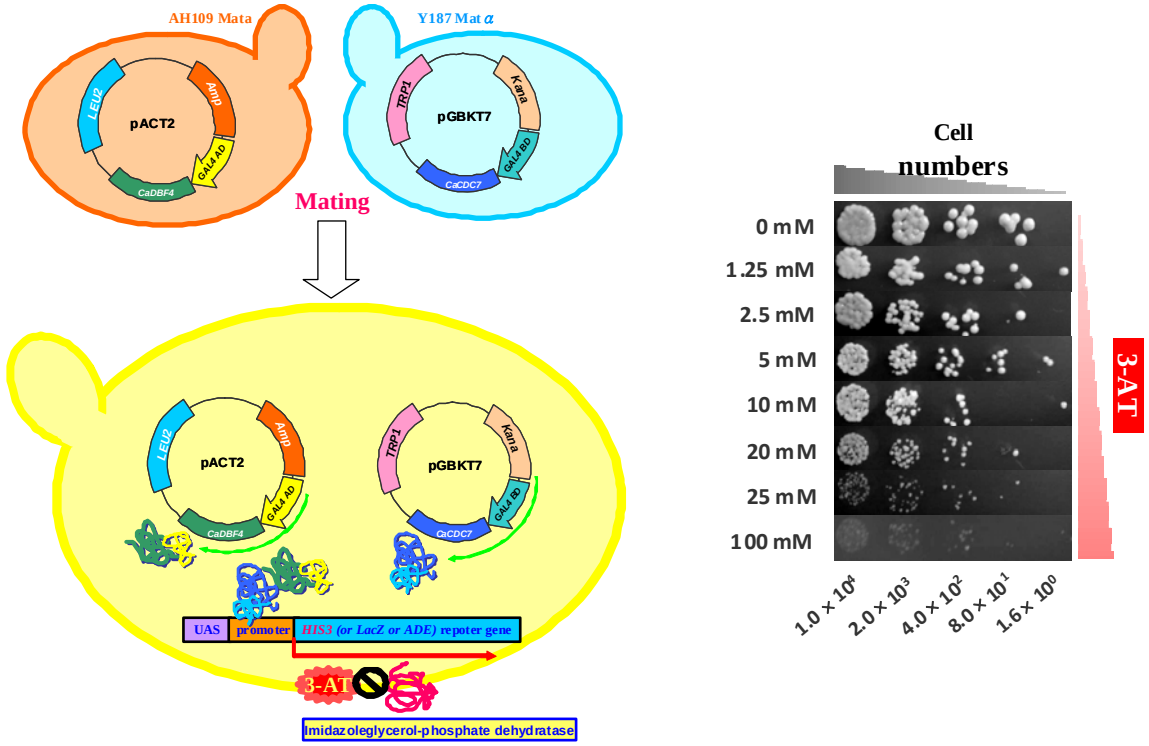

Figure 8. Strong interaction occurs between *Ca*Cdc7 and *Ca*Dbf4. Serially diluted *S. cerevisiae* diploid cells, as shown, carrying pGBKT7-*CaCDC7* and pACT2-*CaDBF4* plasmids, which were transformed into Y187 and AH109, respectively, were grown semi-solid agar plates with selective media lacking histidine but with indicated concentration of 3-aminio-triazole (3-AT), the antagonist of *HIS3* gene product. The cells were able to proliferate normally at the concentration of 20 mM due to relatively strong interaction between *Ca*Cdc7 and *Ca*Dbf4.

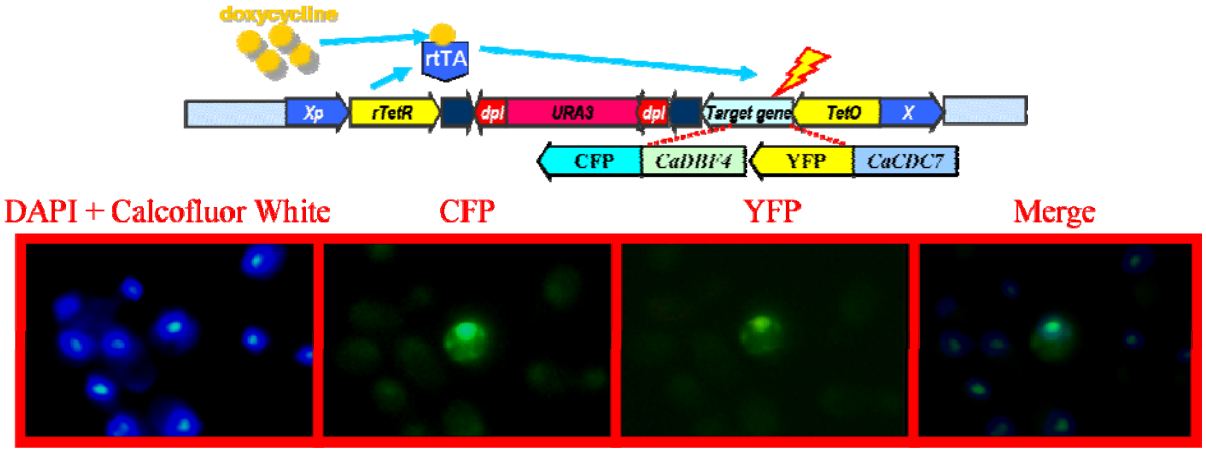

Figure 9. *Ca*Dbf4 and *Ca*Cdc7 are co-localized in the nucleus of *C. albicans* cells. Each of the *Ca*Dbf4 and *Ca*CDC7 was expressed as C-terminal fusion protein to either the cyan fluorescence protein (*Ca*Dbf4-CFP) or the yellow fluorescence protein (*Ca*Cdc7-YFP) by the Tet-on system in *C. albicans* cells. The Tet-on system is induced in the presence of 40 μg/ml doxycycline in which constitutively expressed rtTA bound with doxycycline binds to the Tet operator to allow downstream gene expression. Upon simultaneously induced expression of *Ca*Cdc7-YFP and *Ca*Dbf4-CFP, fluorescence of each of CFP and YFP was seen in the nucleus of *C. albicans* cells with yeast type, suggesting that *Ca*Cdc7 interacts with *Ca*Dbf4 and that they remain in the nucleus when cells grow as yeast form.

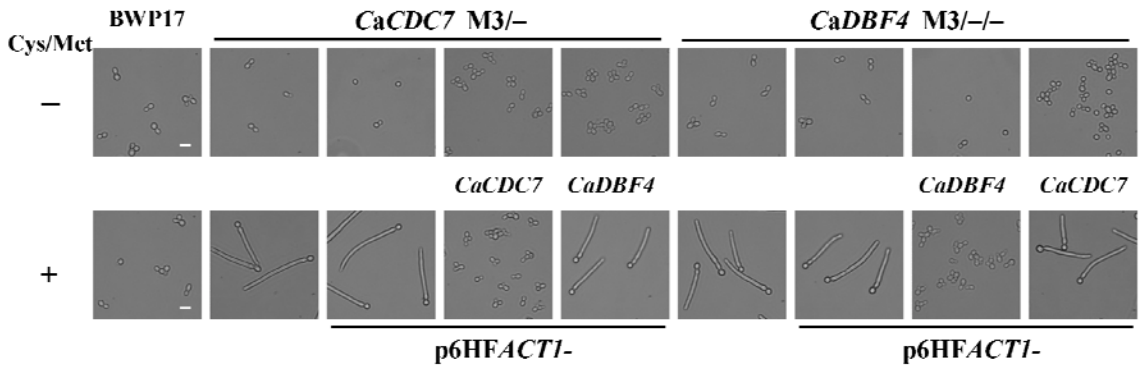

Figure 10. Loss of one member of DDK cannot be suppressed by the constitutive expression of the other member of DDK. While hyphal growth due to repressed expression of *CaDBF4* could not be suppressed by the *ACT1*-driven expression of *CaCDC7*, hyphal growth due to repressed expression of *CaCDC7* also could not be suppressed by the *ACT1*-driven expression of *CaDBF4*. Cells of strain *CaDBF4* M3/–/– was transformed with plasmid p6HF-Act1-*CaDBF4*, or p6HF-Act1-*CaCDC7* capable of constitutively expressing *CaDBF4* and *CaCDC7*, respectively. Cells of strain *CaCDC7* M3/– was transformed with plasmid p6HF-Act1-*CaCDC7*, or p6HF-Act1-*CaDBF4* capable of constitutively expressing *CaCDC7* and *CaDBF4*, respectively. Cells of strains *CaDBF4* M3/–/– and *CaCDC7* M3/– with plasmids were grown in the SD medium with required supplements in the presence of each of 2.5 mM methionine and cysteine for indicated times to assessment of morphological alteration under the microscope. Bars represent 10 μm.

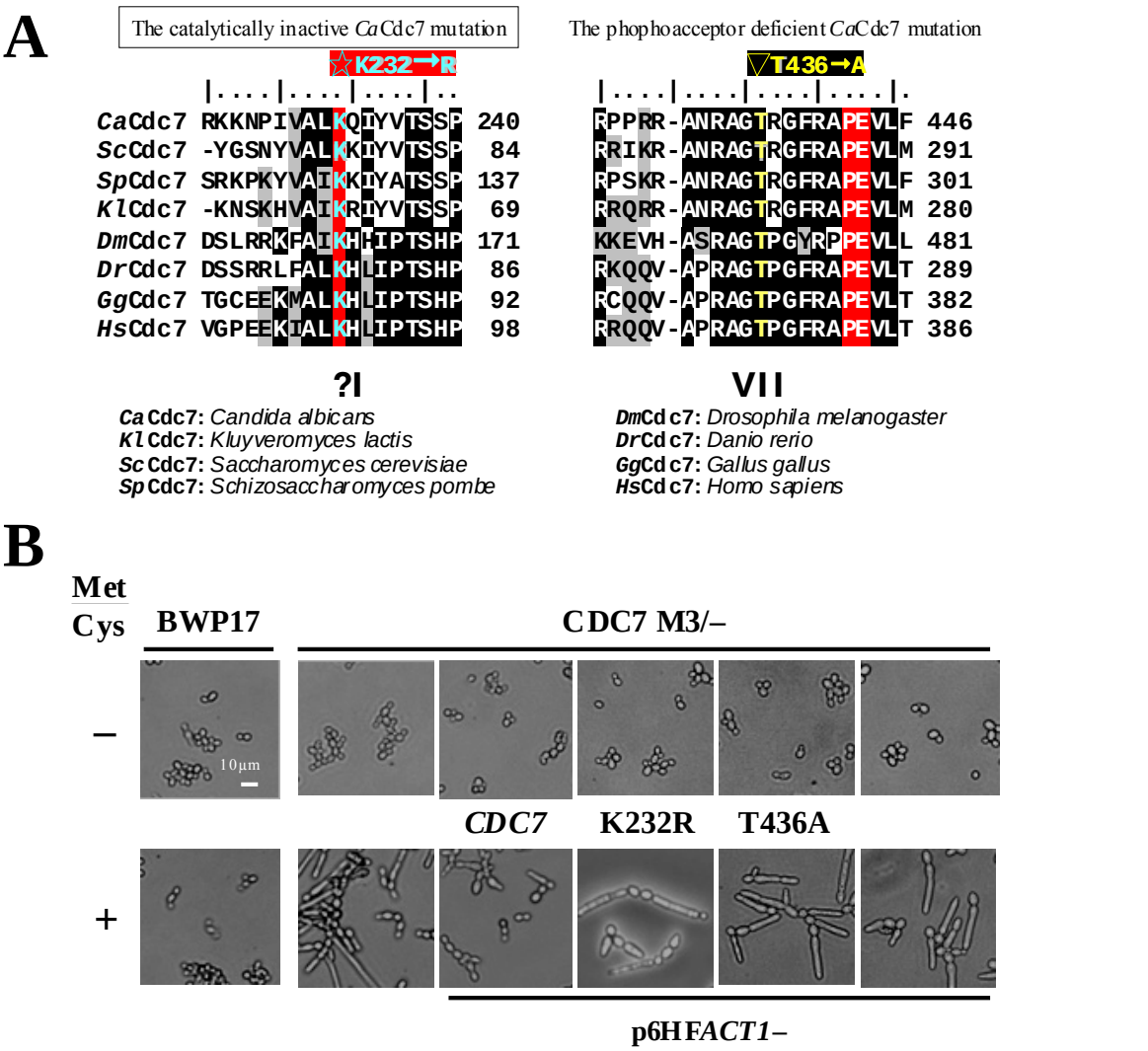

Figure 11. The catalytical activity and phsophorylation of *Ca*Cdc7 are essential for the suppression of yeast-to-hyphae transition in *C. albicans*. Cells of strain *CaCDC7* M3/– was transformed with either the empty p6HF-Act1 or the p6HF-Act1-*CaCDC7*, capable of constitutively expressing wild-type *Ca*Cdc7 (*CaCDC7*), catalytically inactive *Ca*Cdc7 (K232R) or the phophoacceptor deficient *Ca*Cdc7 (T437A). Cells of each strain were grown in the SD medium with required supplements in the presence (+ Met/Cys) of each of 2.5 mM methionine and cysteine for 12 h prior to assessment of morphological alteration under microscope. Bars represent 10 µm.

Appendix 附錄

As attached 如附檔

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Genome Screen Report

Construction of *Candida albicans* Tet-on tagging vectors with a Ura-blaster cassette

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Abstract

It has been difficult to develop molecular tools for studying the fungal pathogen *Candida albicans* because this species uses a non-standard genetic code and is diploid without a complete sexual cycle. Vector systems with regulatable promoters to produce conditional mutants, epitope tags for protein detection and recyclable selection markers are useful for functional study of genes. However, most currently available vectors contain only a subset of desired properties, which limits their application. To combine several useful properties in one vector, the vector pTET25 was initially modified into pTET25M, so that the *URA3* gene flanked by *dpl*200 could be used repetitively. To enable more choices for cloning, a multiple cloning site was introduced at both ends of *GFP* in pTET25M. GFP expression was induced by doxycycline in a dose- and time-dependent manner when the plasmid was introduced into *C. albicans* with or without *URA3*. The applicability of the vectors was verified by constructing strains capable of expressing either the N-terminal GFP fusion of Cdc10 or the C-terminal GFP fusion of Cdc11. Additionally, by replacing the *GFP* gene of pTET25M with DNA sequence encoding Cdc10 with an epitope tag of six histidine residues at the C-terminus, doxycycline-induced expression of *CDC10* was achieved when the expression vector was introduced into *C. albicans*. This new system allows for inducible expression of a desired *C. albicans* gene with the advantage of convenience of cloning. It also allows the presence of a recyclable *URA3* marker and the detectable expression of fusion or epitope-tagged protein.

Keywords: ***Candida albicans*; Tet-on tagging vector; Ura-blaster cassette; GFP fusion protein**

Introduction

The yeast *Candida albicans* is a member of the normal microflora on the mucosal surfaces of the gastrointestinal and genitourinary tracts in healthy humans. However, it is also the primary fungal pathogen in humans (Magee, 1998). In particular, *C. albicans* triggers systemic candidiasis in immunocompromised patients. Molecular analysis of *C. albicans* has been hindered because it is diploid in nature, does not have a complete sexual cycle and uses non-standard codons. However, many approaches have been undertaken to facilitate the functional study of *C. albicans*. To date, several gene disruption strategies have been developed for *C. albicans* (Berman and Sudbery, 2002), the most common of which is the use of a Ura-blaster cassette (Wilson *et al*., 2000) that allows the reuse of the auxotrophic *C. albicans URA3* selection marker. The Ura-blaster cassette

has also been incorporated into vector systems to sequentially introduce or delete genes to assess their functional interactions.

In addition, a number of gene expression vectors have been established, including repressible systems using the *MET3* promoter with methionine and cysteine (Care *et al.*, 1999), the *MAL2* promoter with glucose (Backen *et al.*, 2000) or the tetracycline operator (*TetO*) with tetracycline (Nakayama *et al.*, 2000) and inducible systems such as the tetracycline-inducible gene expression (Tet-on) system (Park and Morschhauser, 2005). Regulatable expression systems are especially useful for assessing the consequences of the presence or absence of gene expression. Gene function can be determined by either inducing the overexpression of a specific gene that disturbs the regulatory mechanism of its normal function, or repressing the expression of a gene of interest that reveals the functional consequence of depletion of the gene product.

Epitope tagging has become a common tool for detecting, purifying and studying the function of proteins (Fritze and Anderson, 2000). Several single epitope tags, such as glutathione *S*-transferase (Smith and Johnson, 1988), green fluorescent protein (GFP) (Cubitt *et al.*, 1995; Heim *et al.*, 1995), haemagglutinin (HA) (Field *et al.*, 1988), six histidine residues (6×His) (Hagan and Stirling, 1998), human c-Myc (Evan *et al.*, 1985) and FLAG tags (Brizzard *et al.*, 1994; Overholt *et al.*, 1997; Prickett *et al.*, 1989), as well as double epitope tags, such as 6×His–FLAG (Huang *et al.*, 2001; Robeva *et al.*, 1996), are widely used in a variety of organisms. In *C. albicans*, tagging vectors have recently been developed for the HA epitope of Tet-off (Nakayama *et al.*, 2000), GFP (Cormack *et al.*, 1997), FLAG (Umeyama *et al.*, 2002), and 6×His–FLAG systems (Kaneko *et al.*, 2004).

To facilitate the detection, purification and functional studies of *C. albicans* gene products, we constructed expression tag vectors that can be used in *C. albicans*. The vectors allow proteins to be ectopically expressed in a doxycycline-dependent manner with either a C-terminal or N-terminal GFP tag or a C-terminal 6×His epitope. The vectors also possess a Ura-blaster cassette to allow a *URA3* marker to be reintroduced. The function and applicability of the vectors were assessed using Cdc10 and Cdc11 proteins, members of the septin family that are components of the septum complex at the bud neck between mother and daughter cells (Sudbery, 2001).

Materials and methods

Strains, growth conditions and DNA manipulation

The auxotrophic *C. albicans* strain BWP17 (Wilson *et al.*, 1999), shown in Table 1, was used in strain construction. The *C. albicans* strains used and generated in this study are also shown in Table 1. All strains were grown in either a complete rich medium of yeast extract–peptone–glucose (YEPD) or in a synthetic minimal medium with or without 50 μg/ml uridine, as previously described (Tseng *et al.*, 2010). The strains were stored as frozen stocks in 20% glycerol at −80 °C. Transformants were selected on synthetic minimal medium without uridine and screened for integrants by yeast colony PCR (Wang *et al.*, 1996), using the oligonucleotide primers pNIM-inte detect F and pNIM-inte detect R, listed in Table 2.

The *Escherichia coli* strain DH5α was used as a host for plasmid DNA construction and routine plasmid maintenance and amplification. Bacterial cultures were grown in L-broth or L-broth supplemented with 50 μg/ml ampicillin, as required. Plasmid DNA was purified using the Gene-Spin™-V^2 Miniprep Purification Kit (Protech, Taipei, Taiwan). The oligonucleotide primers used for vector construction are listed in Table 2.

Generation of the Ura-blaster cassette

To introduce a Ura-blaster cassette, the DNA fragment of the *C. albicans URA3* marker from the doxycycline-inducible pTET25 vector (Park and Morschhauser, 2005) was removed by digestion with *Pst*I and *Sal*I and cloned into the pUC19 vector to generate pUC19-URA3. The DNA fragment of *dpl*200 in *URA3* was amplified by PCR from pUC19-URA3 using the primers CaURA3-dpl200-*Xho*I and CaURA3-dpl200-*Bam*HI (Table 2), digested with *Xho*I and *Bam*HI, and cloned into pUC19-URA3 to generate pUC19-URA3-dpl200. To introduce the *Bgl*II cloning site downstream of *C. albicans GFP* to pTET25, a DNA fragment containing partial *TetO, GFP* with *Sal*I and *Bgl*II sites, and the *ACT1* transcription termination sequence (*ACT1t*) from pNIM1 was amplified with

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Table 1. *C. albicans* strains used in this study

Name of strain	Parental strain	Genotype	Plasmid	Protein expressed	Reference
BWP17		*ura3::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	None	None	(Wilson *et al*., 1999)
JSCA0050	BWP17	*ADH1/adh1::Ptet-GFP : URA3*	pTET25	GFP	This study
JSCA0051	BWP17	*ADH1/adh1::Ptet-GFP : URA3-dpl200*	pTET25M	GFP	This study
JSCA0051F	JSCA0051	*ADH1/adh1::Ptet-GFP : dpl200*	pTET25M	GFP	This study
JSCA0052	BWP17	*ADH1/adh1::Ptet-GFP : URA3-dpl200*	pTET25M-N	GFP	This study
JSCA0053	BWP17	*ADH1/adh1::Ptet-GFP : URA3-dpl200*	pTET25M-NC	GFP	This study
JSCA0063	BWP17	*ADH1/adh1::Ptet-GFP-CDC10:URA3-dpl200*	pTET25M-GFP-CDC10	N-terminal GFP fusion Cdc10	This study
JSCA0063F	JSCA0063	*ADH1/adh1::Ptet-GFP-CDC10:dpl200*	pTET25M-GFP-CDC10	N-terminal GFP fusion Cdc10	This study
JSCA0064	BWP17	*ADH1/adh1::Ptet-CDC11-GFP : URA3-dpl200*	pTET25M-CDC11-GFP	C-terminal GFP fusion Cdc11	This study
JSCA0064F	JSCA0064	*ADH1/adh1::Ptet-CDC11-GFP : dpl200*	pTET25M-CDC11-GFP	C-terminal GFP fusion Cdc11	This study
JSCA0073	BWP17	*ADH1/adh1::Ptet-CDC10:URA3-dpl200*	pTET25M-CDC10-CH	C-terminal 6×His tag Cdc10	This study

Table 2. Synthetic oligonucleotide primers used in this study

Name	Primer sequence
CaURA3-dpl200 *Bam*HI	AAT***GGATCC***CCAGATATTGAAGGTAAAAGG
CaURA3-dpl200 *Xho*I	ATT***CTCGAG***CTAGAAGGACCACCTTTGAT
TET25M *Kpn*I	CAA***GGTACC***GAACCATCGTGAGTGTAA
TET25M *Bam*HI	GAA***GGATCC***CGACATTTTATGATGGAA
CaGFP-N-MCS	AATT*GTCGACTCGAGATATCCAGCTAGCGGCCGC*GTGACCATGAGTAAGGGAGAAGAAC
CaGFP-C-MCS	CCG*TGATCA*TTATGC*AGGCCTAGATCTTAAGCTGACGTCCGGA*CCTTTGTATAGTTCATCCATGCC
CaGFP-MCS-R	CTCTAGTTTTGACGCTCG
pNIM1-inte detect F	CATGTCAAAGGATTCAAC
pNIM1-inte detect R	GTATGGTGCCTATCTAAC
CaCDC10 *Aat*II	ATCA***GACGTC***ATGTCCATCGAAGAACCTAGT
CaCDC10 *Bgl*II	ACGC***AGATCT***TTATCTAGCAGCAGCAGTACC
CaCDC11 *Nhe*I	CTACTA***GCTAGC***GATTACTTTTACTTCATTTTCTG
CaCDC11 *Xho*I	CCGCCG***CTCGAG***ATGAATTATTCTACTGAAAATGT
CaCDC10-Tag-*Sal*I	AAA***GTCGAC***GTCAGCGGCCGCATGTCCATCGAAGAACCTAGTAC
CaCDC10-6xHis-*Bcl*I	CGA***TGATCA***TTAATGGTGATGATGGTGATGCCCGGGAGATCTTCTAGCAGCAGCAGTACCTGTAG

Sequences in bold italics donate sites of restriction enzymes.

the primers TET25M-*Kpn*I and TET25M-*Bam*HI (Table 2) by PCR. It was then digested with *Kpn*I and *Bam*HI and cloned into pUC19-URA3-dpl200 to generate pUC19-TET25M. A DNA fragment containing *GFP, ACT1t* and partial *URA3* was cut out of pUC19-TET25 by digestion with *Sal*I and *Eco*RI and cloned into pTET25 to generate pTET25M (Figure 1), the sequence of which was deposited into GenBank (Accession No. HM776584). Importantly, the coding sequence of *GFP* in pTET25M has been modified to allow for CUG-codon adaptation (Morschhauser *et al*., 1998).

Introduction of the multiple cloning sites

To enable the expression of a desired protein as a GFP fusion protein, restriction cloning sites

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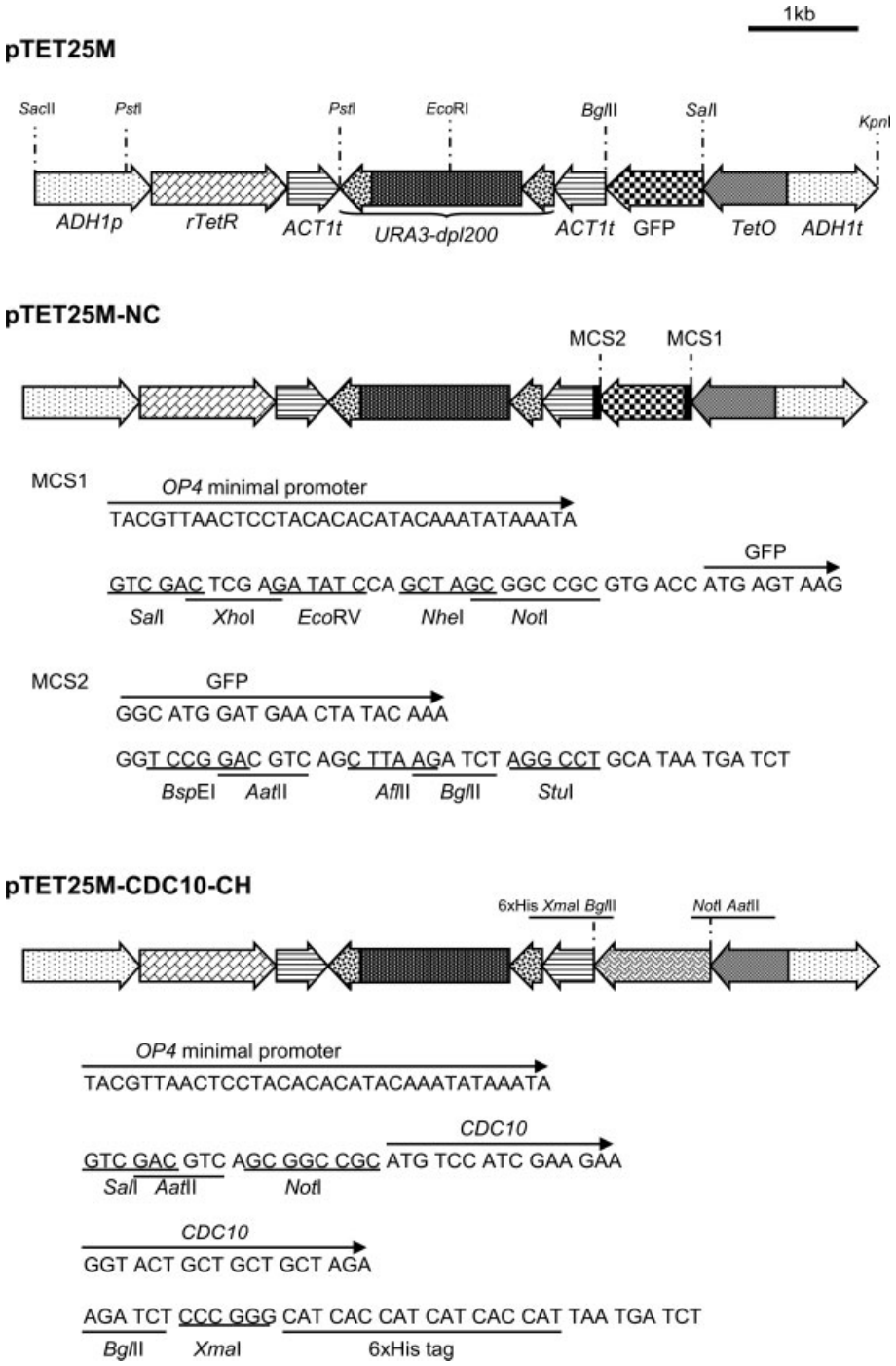

Figure 1. Maps of the pTET25M vector and its derivatives. Only the *Kpn*I/*Sac*II DNA cassettes, which are based on the pBluescript, used to integrate into the *ADH1* locus of *C. albicans*, are shown. The restriction sites shown on pTET25M are those that are relevant to its construction. The sequences shown under pTET25M-NC and pTET25M-CDC10-CH are relevant multiple cloning sites, featuring the unique restriction sites, the reading frames and the flanking sequences. The gene name followed by a small letter 'p' represents a promoter, whereas that followed by a small letter 't' denotes a transcription termination sequence. *URA3-dpl200* contains a functional *C. albicans URA3* gene with an extra downstream sequence upstream of the *URA3* gene. *GFP* and *rTetR* genes use the *C. albicans*-adapted codons. *TetO* contains a fusion of the minimal promoter of the *OP4* gene and an operator sequence capable of binding the *rTetR*-encoded protein in the presence of doxycycline to induce gene expression

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were introduced at both ends of the *GFP* coding sequence in the pTET25M vector. A DNA fragment was amplified from pTET25M by PCR with a forward primer, CaGFP-N-MCS (Table 2), containing the restriction sites for *Sal*I, *Xho*I, *Eco*RV, *Nhe*I and *Not*I and a reverse primer, CaGFP-C-MCS (Table 2), containing the restriction sites for *BspE*I, *Aat*II, *Afl*II, *Bgl*II, *Stu*I and *Bcl*I. A stop codon was also included. The PCR amplicon containing *GFP* was sequentially digested with *Sal*I and *Bcl*I and cloned into the pTET25M vector that had been digested with *Sal*I and *Bgl*II to cut out its copy of *GFP* to generate pTET25M-NC (Figure 1), which contains multiple cloning sites at both ends of the *GFP* coding sequence. Another DNA fragment was amplified by PCR with the primers CaGFP-N-MCS and CaGFP-MCS-R (Table 2), digested with *Sal*I and *Bgl*II, and cloned into pTET25M to generate pTET25M-N, with multiple cloning sites at one end of the *GFP* coding sequence that allows the generation of a C-terminal GFP fusion protein.

Generation of the 6×His epitope and GFP fusion proteins

The coding sequence of either *CaCDC10* or *CaCDC11* was amplified by PCR with the primer pairs of either CaCDC10-*Aat*II and CaCDC10-*Bgl* II or CaCDC11-*Nhe*I and CaCDC11-*Xho*I (Table 2). The two PCR amplicons were digested with either *Aat*II and *Bgl*II or *Nhe*I and *Xho*I. The digested products were subsequently cloned into pTET25M-NC to generate pTET25M-GFP-CDC10, which is capable of encoding an N-terminal GFP fusion of Cdc10, and pTET25M–CDC11–GFP, which is capable of encoding a C-terminal GFP fusion of Cdc11. To incorporate a 6×His epitope at the C-terminus of a protein, a new reverse primer, CaCDC10-6×His-*Bcl*I (Table 2), which is capable of encoding 6×His, was introduced. In addition, the reverse primer was incorporated into the *Xma*I and *Bgl*II restriction sites. A new forward primer, CaCDC10-Tag-*Sal*I (Table 2), was incorporated into the *Aat*II and *Not*I sites. A DNA fragment of *CaCDC10*-CH was amplified by PCR from pTET25M-GFP-CDC10 with the new pair of primers. Subsequently, the *CaCDC10*-CH PCR amplicon was digested with *Sal*I and *Bcl*I and cloned into pTET25M to generate pTET25M-CDC10-CH (Figure 1). The *C. albicans CDC10* in the pTET25M-CDC10-CH vector can be replaced by coding sequences of other genes with several restriction sites of choice (Figure 1).

Candida albicans transformation and selection

Transformation of *C. albicans* cells and selection of transformants were performed as previously described (Shieh *et al*., 2005). Briefly, the DNA fragment of the Tet-on part containing the gene of interest was removed from the plasmid by digestion with *Sac*II and *Kpn*I, purified using the G-M™ Gel Extraction System (Viogene, Taipei, Taiwan) and then introduced into *C. albicans* cells by the LiAc-PEG-ssDNA method (Gietz and Woods, 2006) with some modification (Walther and Wendland, 2003). The DNA was specifically integrated into the *ADH1* locus of the *C. albicans* genome as a stable integrant; the Ura^+ prototrophs on minimal medium plates lacking uridine (Shieh *et al*., 2005) and the integrants were determined by colony PCR, using the diagnostic primers pNIM1-inte detect F and pNIM1-inte detect R (Table 2). To recycle the *C. albicans URA3* cassette, the Ura^+ prototrophs were grown on a plate with 5-FOA (1 mg/ml) and uridine (50 μg/ml), and the Ura^- auxotrophs were selected. Correct insertion of the *URA3* gene and removal of the *URA3* gene after 5-FOA selection were verified by PCR with the specific primers CaURA3-dpl200 *Bam*HI and CaURA3-dpl200 *Xho*I (Table 2).

Western blot analysis

To detect expression of the tagged *Ca*Cdc10 and the *Ca*Cdc10 and *Ca*Cdc11 fusion proteins, *C. albicans* strains were grown at 30 °C in YEPD overnight, diluted (1 : 10) into fresh YEPD, grown to log phase before addition of 40 μg/ml doxycycline (Sigma) and then grown for 3 h, unless otherwise stated. Total protein was extracted from the cultured cells as previously described (Shieh *et al*., 2007). The proteins were resolved by SDS–PAGE and electrophoretically transferred onto PVDF membranes (Perkin-Elmer, Boston, MA, USA). The membranes were probed with an anti-6×His epitope monoclonal antibody (Novagen) or an anti-GFP polyclonal antibody (Abcam). Proteins were detected using either a peroxidase-conjugated goat anti-mouse IgG (Millipore) or a peroxidase-conjugated donkey anti-goat IgG (Santa Cruz).

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The signal was visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Microscopy

C. albicans cells were grown at 30 °C in YEPD overnight, diluted (1 : 10) into fresh YEPD, grown to log phase prior to addition of 40 μg/ml doxycycline (Sigma) and then grown for 3 h before being analysed by microscopy. The cells were collected and washed with sterile PBS prior to analysis. Both DIC and the green fluorescence images were assessed using a ZEISS Axioskop 2 microscope (Zeiss, Jena, Germany). Digital images were acquired using a MicroFirr digital camera (Olympus, Melville, NY, USA) and processed using the imaging software Optronics PictureFrame 2.1 (Optronics, Goleta, CA, USA). The micrographs were digitized and processed using Adobe Photoshop software.

Results and discussion

Modification of a tetracycline-inducible gene expression system

The tetracycline-inducible gene expression system introduced in *C. albicans* in the current study originated from the system developed by Park and Morschhauser (2005), in which a reverse tetracycline-controlled transactivator (rtTA) (Park and Morschhauser, 2005) and GFP (Morschhauser *et al.*, 1998) were adapted to the codon usage of *C. albicans*. The pTET25 plasmid with a *URA3* selection marker and the pNIM1 plasmid with a dominant *SAT1* selection marker were obtained from Park and Morschhauser. However, the inability to reuse the markers in the same system limits the functional analysis of genes. To make the *URA3* selection marker in pTET25 recyclable, a Ura-blaster cassette (Wilson *et al.*, 2000) was introduced into pTET25, which allows 5-FOA-induced intrachromosomal recombination. To facilitate further cloning procedures, subsequent cloning was made to generate pTET25M (Figure 1). This is the initial product of the modified version of pTET25. The pTET25M vector possesses *Sal*I and *Bgl*II restriction sites flanking the *GFP* coding sequence, which allow for the introduction of other restriction sites.

To verify the preservation of doxycycline-inducible GFP expression in pTET25M, both pTET25M and pTET25 were linearized by *Sac*II and *Kpn*I and introduced into the *C. albicans* strain BWP17 for Ura^+ prototrophs. Stable integrants with either pTET25 or pTET25M targeting the *ADH1* locus were verified by colony PCR with specific primers. Importantly, the level of doxycycline-induced GFP expression was comparable between the integrant of pTET25 (JSCA0050) and that of pTET25M (JSCA0051), as shown by the western blot results (Figure 2A). To determine whether doxycycline-inducible GFP expression in cells with *URA3* removed from the Ura-blaster cassette of pTET25M was preserved, the strain JSCA0051F without *URA3* was obtained from the strain JSCA0051 by 5-FOA treatment. It was then verified by yeast colony PCR. The ability of doxycycline-inducible GFP expression in JSCA0051F was verified (Figure 2A). Importantly, the induced GFP expression levels in JSCA0051 and JSCA0051F were comparable (Figure 2A). In addition, GFP expression levels were similar in both cells of the integrants, as observed by fluorescence microscopy (Figure 4). These results suggest that no significant alteration in the regulation of GFP expression occurs in cells with the pTET25M vector in the absence of *URA3*.

To further analyse GFP expression in cells with pTET25M, two aspects of doxycycline inducibility, namely dose and time, were examined. Cells capable of expressing GFP, either with *URA3* (JSCA0051) or without *URA3* (JSCA0051F), were grown in YEPD with 40 μg/ml doxycycline, and GFP expression was assessed at various time points up to 36 h. GFP expression in both strains gradually increased until 24 h, after which time levels remained by and large the same until 36 h (Figure 2B), suggesting that the doxycycline-induced GFP expression in cells with pTET25M with or without *URA3* is controlled in a time-dependent manner. In addition, cells capable of expressing GFP (JSCA0051) were grown in YEPD with various amounts of doxycycline, and GFP expression was assessed at 3 h. The GFP expression in JSCA0051 cells exhibited a dose-dependent response, with a peak at 40 μg/ml and a dip at 2.5 μg/ml doxycycline (Figure 2C). Therefore, we

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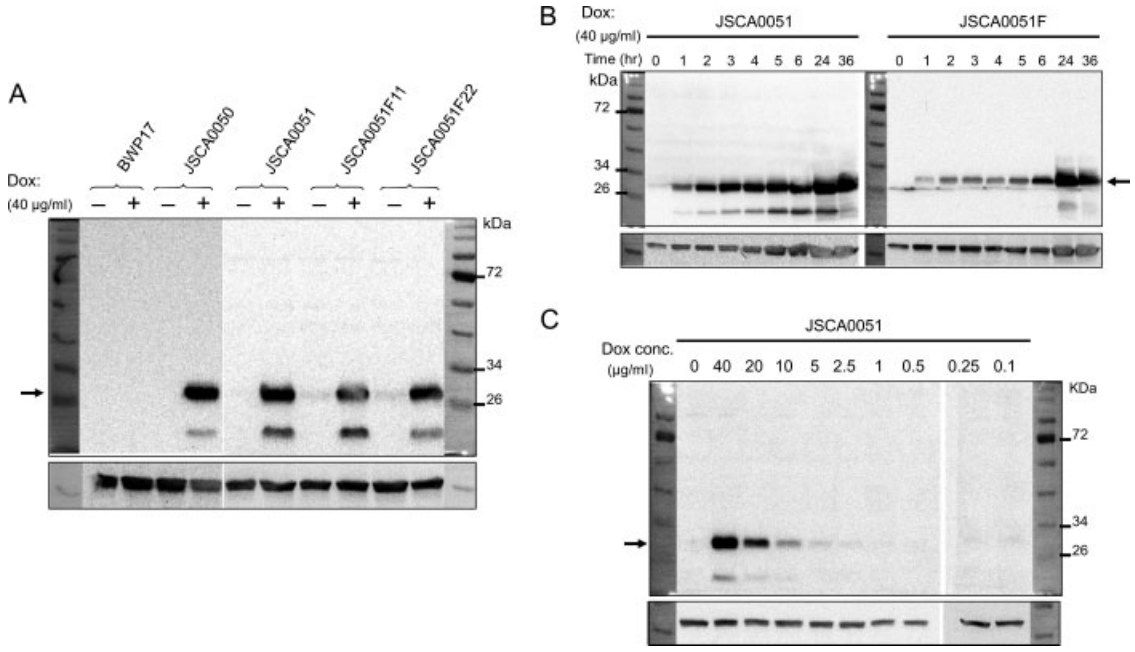

Figure 2. Western blotting analysis to assess protein expression of *C. albicans* strains based on the cassette of the pTET25M vector. (A) The doxycycline-induced expression of GFP in the strain with (JSCA0051) or without (JSCA0051F) *URA3*. The cells were grown in YEPD to log phase, and the protein was induced by adding 40 μg/ml doxycycline for 3 h before western blot analysis. The strain JSCA0050 carrying the original cassette of the pTET25 vector was used as a positive control. +, doxycycline-induced state; −, non-induced state. Two independent isolates (JSCA0051F11 and JSCA0051F22) of the strains without *URA3* were examined. (B) Demonstration of the time-dependent manner of doxycycline-induced protein expression. Both the JSCA0051 and JSCA0051F (without *URA3*) strains were examined in parallel. (C) Demonstration of the dose-dependent manner of doxycycline-induced protein expression. JSCA0051 cells were grown in YEPD to log phase before adding the indicated concentration of doxycycline to induce protein expression for 3 h. Arrows indicate the predicted positions to which the GFP proteins migrated

concluded that the pTET25M vector carrying the Ura-blaster cassette was successfully established.

Verification of the efficacy of the modified tetracycline-inducible gene expression system

To enhance the application of a vector that lacks an adequate number of restriction sites, multiple restriction sites were introduced flanking the *GFP* coding sequence in the pTET25M vector to generate pTET25M-NC (Figure 1). In addition, by introducing multiple cloning sites at only one end, an accompanying plasmid, pTET25M-N, was also generated. To examine the preservation of doxycycline-inducible GFP expression in pTET25M-N and pTET25M-NC, both plasmids were linearized by *Sac*II and *Kpn*I and introduced into the *C. albicans* strain BWP17 for Ura$^+$ prototrophs. The stable integrants with either pTET25M-N (JSCA0052) or pTET25M-NC (JSCA0053) targeting the *ADH1* locus were verified by colony PCR with specific primers. The ability of doxycycline-inducible GFP expression was verified (Figure 3A) and was found to be comparable to that of the JSCA0051 strain carrying pTET25M (Figure 2A).

The *C. albicans* genes *CDC10* and *CDC11* were chosen to validate the preservation of functional GFP fusion proteins expressed from pTET25M-NC. The *CDC10* gene was cloned into pTET25M-NC at the *Aat*II and *Bgl*II sites to obtain pTET25M-GFP-CDC10, capable of encoding an N-terminal GFP fusion Cdc10 protein. The *CDC11* gene was cloned into pTET25M-NC at the *Nhe*I and *Xho*I sites to obtain pTET25M-CDC11-GFP, capable of encoding a C-terminal GFP fusion Cdc11 protein. The two plasmids were then linearized by *Sac*II and *Kpn*I and introduced into the *C. albicans* strain BWP17 for Ura$^+$ prototrophs. The stable integrants with either pTET25M-GFP-CDC10 (JSCA0063) or pTET25M-CDC11-GFP (JSCA0064) targeting the

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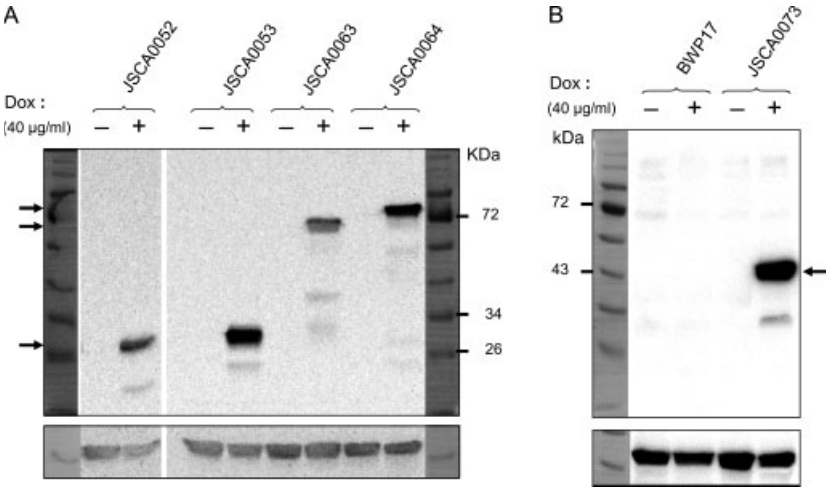

Figure 3. Western blotting analysis to assess protein expression in *C. albicans* strains based on the cassettes of plasmids derived from pTET25M. (A) Assessment of ability of the JSCA0063 and JSCA0064 strains to express N-terminal GFP-tagged Cdc10 and C-terminal GFP-tagged Cdc11, respectively. The strains JSCA0052 and JSCA0053 carrying cassettes from plasmids pTET25M-N and pTET25M-NC, respectively, from which the plasmids for the strains JSCA0063 and JSCA0064 were derived were also assessed. (B) Assessment of the JSCA0073 strain in expressing C-terminal 6×His epitope-tagged Cdc10, along with the wild-type strain BWP17. The cells were grown in YEPD to log phase, and protein expression was induced by adding 40 μg/ml doxycycline 3 h before western blot analysis. Arrows indicate predicted positions to which the relevant proteins migrated

ADH1 locus were confirmed by colony PCR with specific primers. Significantly, the expression of both the N- and C-terminal GFP fusion proteins was doxycycline-dependent (Figure 3A).

To assess the preservation of doxycycline-inducible expression of GFP fusion proteins in cells with *URA3* removed from the Ura-blaster cassette of pTET25M-NC, the JSCA0063F and JSCA0064F strains, which had *URA3* removed, were obtained from the JSCA0063 and JSCA 0064 strains by treatment with 5-FOA and were verified by yeast colony PCR. The ability of doxycycline-inducible expression of GFP fusion proteins in the JSCA0063F and JSCA0064F strains was verified and was comparable to that of the parental strains with *URA3* (data not shown). This suggests that the doxycycline-inducible expression of either N-terminal or C-terminal GFP fusion proteins is not affected by the absence of *URA3*.

To extend the application of pTET25M, the vector system expressing an epitope-tagged protein rather than a *GFP* fusion protein was developed. The *GFP* coding sequence on pTET25M was replaced by DNA sequence encoding Cdc10 with a 6×His tag at the C-terminus to obtain pTET25M-CDC10-CH (Figure 1). Several restriction sites, in addition to the cloning sites of *Sal*I and *Bgl*II, were also introduced at either end of *CDC10* (Figure 1) for future application to other genes. The pTET25M-CDC10-CH plasmid was linearized by *Sac*II and *Kpn*I and introduced into the *C. albicans* strain BWP17 for Ura^+ prototrophs. The stable integrant with pTET25M-CDC10-CH targeting the *ADH1* locus (JSCA0073) was confirmed by colony PCR with specific primers. The ability of doxycycline-inducible expression of Cdc10 with a C-terminal 6×His tag in JSCA0073 cells was confirmed by western blot (Figure 3B). Hence, we concluded that the modified pTET25M was capable of expressing a C-terminal 6×His protein.

To assess the functionality of the GFP fusion proteins, the localization of the N-terminal GFP fusion of Cdc10 (GFP-Cdc10) in JSCA0063 cells and C-terminal GFP fusion of Cdc11 (Cdc11-GFP) in JSCA0064 cells was examined by fluorescence microscopy. Green fluorescence was observed in the cytoplasm of JSCA0051 cells expressing GFP alone (Figure 4). In contrast, green fluorescence was clearly present at the septum between two cells and in the cytoplasm of JSCA0063 and JSCA0064 cells expressing GFP-Cdc10 and Cdc11-GFP, respectively (Figure 4). In addition, the absence of *URA3* in JSCA0063F and JSCA0064F cells did not significantly alter the

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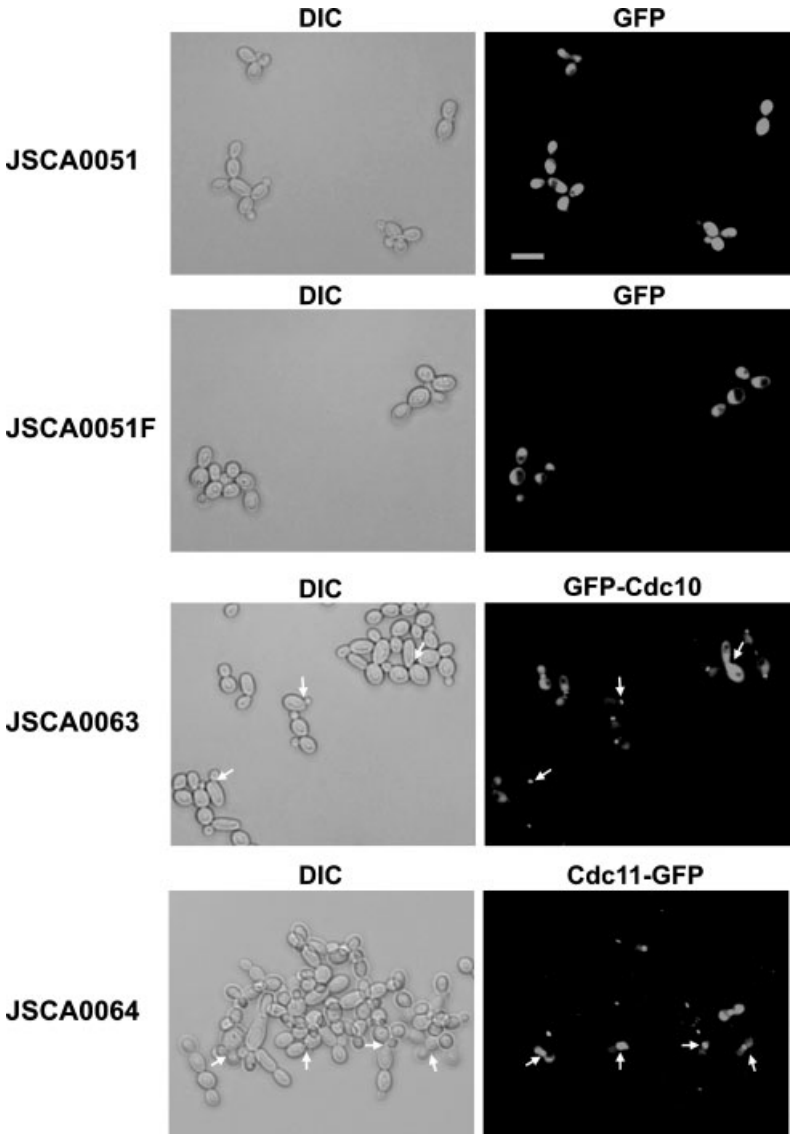

Figure 4. Assessment of protein expression of the representative *C. albicans* strains by fluorescence microscopy. Cells of the strain expressing N-terminal GFP fusion Cdc10 (JSCA0063) and C-terminal GFP fusion Cdc11 (JSCA0064) with those expressing GFP with or without *URA3* (JSCA0051 or JSCA0051F) were grown in YEPD to exponential phase before adding 40 μg/ml doxycycline to induce protein expression for 3 h. The emission of green fluorescence alongside the DIC images was examined microscopically. The representative cells in which the GFP fusion proteins are localized to the septin complex at the mother–daughter junctions are indicated with white arrows. Bar = 10 μm

distribution and intensity of the green fluorescence (data not shown). Therefore, we concluded that pTET25M-NC enables doxycycline-inducible expression of functional GFP fusion proteins.

Potential applications

The pTET25M-NC vector allows the construction of strains expressing either N- or C-terminal fluorescence fusion proteins for purification, detection and localization studies. The ability to express both N- and C-terminal GFP fusion proteins can be critical. In many cases, GFP fusion at one terminus disrupts proper localization of the protein of interest, while a GFP tag at the other terminus maintains proper localization. In addition, numerous restriction sites at either end

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of *GFP* on the pTET25M-NC vector provide multiple options for gene cloning. Furthermore, the doxycycline-inducible system can overexpress protein at a higher level compared to other regulatory expression systems (Park and Morschhauser, 2005). Hence, this newly-developed doxycycline-inducible system enables visualization of the fusion protein when protein expression levels from the native promoter are too low to be detected. One extension of this system is that the *GFP* can be replaced by the *C. albicans*-adapted *CFP* and *YFP* (Gola *et al*., 2003), mCherry (Keppler-Ross *et al*., 2008), DsRFP (Gerami-Nejad *et al*., 2009) or other fluorescent protein-encoding genes developed in the future.

To fully explore the application of this newly developed system, pTET25M-CDC10-CH was constructed, in which Cdc10 is expressed as a C-terminal 6×His-tagged protein when the vector is introduced into *C. albicans* with doxycycline induction. The presence of several cloning sites flanking the *CDC10* coding sequence on pTET25M-CDC10-CH allows *CDC10* to be replaced by other genes. Such tagged proteins can also be used for isolation, detection and localization studies in concert with the GFP fusion proteins. The epitope-tagged proteins may reflect the function of the non-tagged native proteins more accurately than GFP fusion proteins, because the relatively larger size of GFP compared to the 6×His epitope makes it more likely to interfere with the protein function. Moreover, the system allows titration of doxycycline to monitor protein expression in conjunction with the phenotypes of either the physiological behaviour of proteins when expressed near the native level or the perturbed performance of proteins when significantly overexpressed. Furthermore, the system includes a recyclable mini Ura-blaster cassette (Wilson *et al*., 2000). The presence of the Ura-blaster cassette in the plasmid allows it to be introduced into *C. albicans* consecutively with another plasmid carrying a *URA3* selection marker, facilitating the functional assessment of protein–protein interactions.

We believe that there is room for further improvement in this system. For example, an N-terminal 6×His epitope and other epitope tags on either the N- or C-terminus should be introduced to make the system universally applicable in terms of assessing protein–protein interactions. Moreover, in the current system, the *ADH1* locus is the sole integration site from which constitutive rtTA expression is driven. It is desirable to have an additional gene locus for integration and for constitutively driving rtTA expression. A system capable of targeting two loci combined with the doxycycline-inducible expression of two proteins that are fused with different fluorescent markers or epitope tags could be used to reveal protein–protein interactions. Furthermore, because the Ura-blaster cassette can only be used to recycle the *URA3* marker, it is worth introducing more common systems, such as *FLP*/*FRT* (Reuss *et al*., 2004) or Cre/*loxP* (Dennison *et al*., 2005), into the current plasmid with different selection markers to allow excision of different selection markers for the functional analysis of several gene products simultaneously.

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國科會補助計畫衍生研發成果推廣資料表

日期:2011/10/24

國科會補助計畫	計畫名稱: 解析白色念珠菌CaCDC7在型態形成上的新穎功能
	計畫主持人: 謝家慶
	計畫編號: 97-2320-B-040-014-MY3　　學門領域: 醫學之生化及分子生物

無研發成果推廣資料

97 年度專題研究計畫研究成果彙整表

計畫主持人：謝家慶	計畫編號：97-2320-B-040-014-MY3
計畫名稱：解析白色念珠菌 CaCDC7 在型態形成上的新穎功能	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	5	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	2	0	100%	人次	
		博士生	1	0	20%		
		博士後研究員	0	0	100%		
		專任助理	2	0	70%		
國外	論文著作	期刊論文	1	3	100%	篇	研究過程已發表一篇雖無直接相關但間接有應用價值之研究用工具能經由去氧羥四環素-誘導蛋白質以時間及劑量依賴表現(Yeast 2010；28:253-263)。此本載體系統已經要求提供給給包括英國、瑞士、美國及大陸四個研究團隊，顯示其應用價值。
		研究報告/技術報告	0	0	100%		
		研討會論文	2	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	

	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	本計劃目標已完成確認 Dbf4-dependent Cdc7 kinase (DDK)在調節白色念珠菌菌絲生長之負調節者的新穎功能，刻正整理撰寫研究論文，並嘗試以兩篇併稿方式先以 Nature 為目標。由於 DDK 在真核生物啟動 DNA 複製功能上具高度保留性，在白色念珠菌卻控制菌絲形成是新發現之功能，而菌絲生長又與白色念珠菌致病力有關，若能持續尋找及研究其受質及調節者，抑制此重要且能致死的白色念珠菌真菌病菌之新穎藥物可得以發展。 此計劃三年來且有至少 10 位大學部學生參與，其中已有三位今年獲得全額獎學金赴美國深造，另有至少五位推薦申請至各國立大學研究所碩士班。另有三名部份或主要參與之碩士班學生，分別申請赴應國，國內國防大學及中興大學博士班。 氧羥四環素載體系統之未來發展性上，則是已根據本載體系統延展開發進行整基因體型態形成基因鑑定之研究並已提出國家衛生研究院整合性醫藥衛生科技研究計畫創新研究計劃之申請，研究題目為－系統化雙向基因干擾及發展新穎工具以闡明調節白色念珠菌型態形成之基因。

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
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	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

與計劃直接相關部份已確認 Dbf4-dependent Cdc7 kinase (DDK)在調節白色念珠菌菌絲生長之負調節者的新穎功能，刻正整理撰寫研究論文，並嘗試以兩篇併稿方式先以 Nature 為目標，預計 2011 年底前完成投稿。由於 DDK 在真核生物啟動 DNA 複製功能上具高度保留性，在白色念珠菌卻控制菌絲形成是新發現之功能，而菌絲生長又與白色念珠菌致病力有關，若能持續尋找及研究其受質及調節者，抑制此重要且能致死的白色念珠菌真菌病菌之新穎藥物可得以發展。

研究過程發表一篇雖無直接相關但間接有應用價值之研究用工具能經由去氧羥四環素-誘導蛋白質以時間及劑量依賴表現(Yeast 2010； 28:253-263)。此本載體系統已經要求提供給給包括英國、瑞士、美國及大陸四個研究團隊，顯示其應用價值。未來發展性上則已根據本載體系統延展開發進行整基因體型態形成基因鑑定之研究並已提出國家衛生研究院整合性醫藥衛生科技研究計畫創新研究計劃之申請，研究題目為－系統化雙向基因干擾及發展新穎工具以闡明調節白色念珠菌型態形成之基因。