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TUNG CHEE HONG

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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DEDICATION

I dedicate this thesis to the special one, Crystale Lim Siew Ying, who is with me through good and bad times.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

EFFECT OF *EFG1* ANTISENSE RNA EXPRESSION ON HYPHAE FORMATION IN *CANDIDA ALBICANS*

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Chairman: Associate Professor Chong Pei Pei, PhD

Faculty: Medicine and Health Sciences

Candida albicans is a fungus that lives as a commensal in healthy humans. The ability of *C. albicans* to switch from the yeast to the hyphal form is thought to be crucial in its pathogenesis. Efg1p is one of a number of transcriptional regulators involved in *C. albicans* yeast to hyphae transition and *EFG1* gene null mutants are unable to form hyphae under several hyphae-inducing conditions. Antisense RNA is a relatively new method in *C. albicans* gene functional studies and was established in a study screening for *C. albicans* essential genes. Based on the success of the antisense RNA gene silencing method in other fungi, this method has the potential to become a more efficient and effective alternative to the widely used gene deletion method in *C. albicans*. In this study, an *EFG1* antisense RNA expression system was created by cloning the antisense RNA of the *EFG1* gene into an expression vector, pGAL1PNiST-1, after a series of restriction enzyme digestion, purification and ligation processes. This recombinant plasmid was designated as pGEFG1, in which the antisense RNA fragment was under the regulation of a *GAL1* promoter. Transformation of this plasmid into an auxotrophic *C. albicans* strain, CAI4,

generated a mutant designated as pGCAI4. Microscopic observation comparisons of the pGCAI4 mutants which were incubated under hyphae-inducting conditions in antisense-inducing or antisense-repressing media showed that the hyphae switching ability of the pGCAI4 mutants had not been impaired by the expression of *EFG1* antisense RNA. The addition of lithium acetate as an agent to improve the efficacy of the antisense RNA was not feasible due to the interaction between galactose (the antisense-inducing agent for the *GAL1* promoter) and lithium acetate, which resulted in the inhibition of hyphae formation. Further analysis using real-time PCR indicated that levels of *EFG1* antisense RNA was 4.12-fold higher in the antisense-inducing medium than in the antisense-repressing medium. Hence, this strongly suggests that the *EFG1* antisense RNA expression system was functioning in all the conditions tested, albeit not at a sufficient level of expression. Therefore, in an attempt to generate more antisense RNA transcripts in antisense-inducing medium, a stronger regulated promoter, *PCK1*, was used to replace the original *GAL1* promoter in the expression vector. By replacing *GAL1* with the new *PCK1* promoter, the hyphae switching ability of the newly generated pGPCKCAI4 mutants was found to be impaired, even in hyphae-inducing conditions, after one hr of incubation in antisense-inducing medium, compared to those in antisense-repressing medium. This observation implies that antisense RNA transcript levels could be an influencing factor in knocking down a target gene. However, microscopic observations of the mutant morphologies at two and three hrs after incubation found that the populations of hyphae cells in both media were similar. This observation could be due to three possibilities. Firstly, it has been suggested that Efg1p may only be important in the initiation of hyphae formation, not in later events, as *EFG1* is

highly expressed during hyphae initiation. Therefore if the level of expressed *EFG1* antisense RNA is unable to effectively knock down these endogenous *EFG1* transcripts, over time free mRNA may generate sufficient amounts of protein to trigger hyphae growth. Secondly, it is possible that the structures observed at the later time points were pseudohyphae, not true hyphae, as previous studies have found that the morphological forms generated by *EFG1* mutants were highly similar to pseudohyphae. Thus, further investigations using electron microscopy and other techniques are required to determine and analyze these structures. Lastly, the serum-induced hyphagenesis pathway may be regulated by several different regulators which appear to act in a cumulative fashion, whereby inactivation of *EFG1*, depending on a particular set of environmental conditions, may be compensated by alternative pathways. In conclusion, an *EFG1* antisense RNA expression system was successfully developed in this study, where an improved *PCK1*-regulated system successfully expressed sufficient levels of *EFG1* antisense RNA to impair the ability of *C. albicans* to form germ tubes during early hyphae development, thereby delaying the formation of hyphae. This suggests the possible application of this method as an alternative to existing gene knockout methods in gene functional studies of *C. albicans* morphology switching genes.

Abstract tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN PENGEXPRESI RNA ANTISENSE *EFG1*PADA PEMBENTUKAN HIFA DI *CANDIDA ALBICANS*

Oleh

TUNG CHEE HONG

MAC 2011

Pengerusi: Profesor Madya Chong Pei Pei, PhD

Fakulti: Perubatan dan Sains Kesihatan

Candida albicans merupakan kulat yang hidup secara komensal dalam manusia sihat. Keupayaan *C. albicans* untuk bertukar daripada bentuk yis ke bentuk hifa dianggap penting dalam patogenesis kulat ini. Efg1p merupakan salah satu pengatur yang terlibat dalam penukaran yis ke hifa *C. albicans* dan mutan sifar gen *EFG1* tidak berupaya membentuk hifa dalam beberapa keadaan penginduksi hifa. RNA 'Antisense' merupakan satu teknik yang agak baru dalam kajian fungsi gen *C. albicans* dan telah diwujudkan dalam kajian sebelum ini untuk menyaring gen-gen *C. albicans* yang penting dalam kebolehidupan kulat ini. Berdasarkan kejayaan kaedah 'silencing' gen RNA 'antisense' dalam kulat-kulat lain, kaedah ini berpotensi sebagai alternatif kepada kaedah pemadaman gen yang luas digunakan dalam *C. albicans*. Dalam kajian ini, satu sistem ekspresi RNA 'antisense' *EFG1* telah diwujudkan setelah pengklonan 'antisense' gen *EFG1* ke dalam satu vektor expresi, pGAL1PNiST-1, selepas proses bersiri yang melibatkan penghadaman enzim sekatan, penulenan and cantuman. Plasmid rekombinan yang terhasil ini digelar pGEFG1, di mana fragmen 'antisense' diletak di bawah kawalan satu

promoter, *GAL1*. Transformasi plasmid ini ke dalam satu strain *C. albicans* auksotrofik, CAI4, menjana satu mutan yang digelar pGCAI4. Bandingan pemerhatian mikroskopik mutan-mutan pGCAI4 yang telah dikultur di bawah keadaan penginduksi hifa dalam medium penggalak atau penghalang RNA 'antisense' menunjukkan bahawa kebolehan penukaran hifa mutan-mutan tersebut (pGCAI4) tidak dihalang oleh ekspresi RNA 'antisense'. Penambahan litium asetat sebagai agen untuk menambahbaik efisiensi RNA 'antisense' juga tidak munasabah, disebabkan oleh interaksi antara galaktosa (agen penggalak RNA 'antisense' untuk mengaktifkan promoter *GAL1*) dan litium asetat, yang menghalang pembentukan hifa. Analisis seterusnya dengan menggunakan PCR masa-nyata menunjukkan bahawa paras RNA 'antisense' *EFG1* adalah 4.12 kali lebih tinggi dalam medium penggalak RNA 'antisense' berbanding dengan paras dalam medium penghalang RNA 'antisense'. Maka, keputusan ini mencadangkan bahawa sistem ekspresi RNA 'antisense' *EFG1* berfungsi dalam semua keadaan yang telah dikaji, cuma pada paras penghasilan RNA 'antisense' yang tidak cukup tinggi. Dengan itu, untuk menghasilkan lebih banyak transkrip 'antisense' dalam medium penggalak 'antisense' B, satu promoter boleh-kawal yang lebih kuat, *PCK1*, telah digunakan untuk menggantikan promoter *GAL1* asal dalam vektor ekspresi. Dengan menggunakan promoter *PCK1* baru ini untuk mengawal ekspresi 'antisense', keupayaan mutan-mutan baru pGPCKCAI4 ini didapati terhalang, walaupun dalam keadaan penginduksi hifa, selepas satu jam dalam medium penginduksi 'antisense' berbanding dengan mutan-mutan dalam medium penghalang 'antisense'. Pemerhatian ini mencadangkan bahawa paras transkrip RNA 'antisense' mungkin merupakan faktor yang mempengaruhi pengurangan transkript gen sasaran. Namun

demikian, pemerhatian morfologi mutan-mutan melalui mikroskop selepas 2 dan 3 jam inkubasi mendapati bahawa populasi sel-sel hifa dalam kedua-dua medium adalah sama. Tiga penjelasan adalah berkemungkinan untuk pemerhatian ini. Untuk penjelasan pertama, adalah dicadangkan bahawa Efg1p mungkin hanya penting dalam permulaan pembentukan hifa. Maka jika paras ekspresi RNA 'antisense' *EFG1* tidak mencukupi untuk 'knock down' ekspresi transkrip-transkrip *EFG1* asal ini, dengan masa berpanjangan mRNA mungkin menghasilkan paras protin secukupnya untuk memulakan pertunbuhan hifa. Kedua, berkemungkinan bahawa struktur-struktur yang telah diperhatikan selepas 2 dan 3 jam dalam kajian ini bukanlah sel hifa, tetapi adalah sel pseudohifa, kerana kajian-kajian sebelum ini telah memdapati bahawa bentuk morfologi yang telah dihasilkan oleh mutan-mutan *EFG1* sangat menyerupai pseudohifa. Dengan itu, kajian seterusnya dengan menggunakan mikroskop elektron serta teknik-teknik lain diperlukan untuk mengenalpasti dan menganalisa struktur-struktur tersebut. Untuk penjelasan terakhir, laluan hifagenesis yang diinduksi oleh serum mungkin dikawal oleh beberapa pengawal yang beraksi secara kumulatif, di mana penyahaktifan *EFG1*, berdasarkan satu set keadaan persekitaran, mungkin digantikan oleh lalun alternatif. Kesimpulannya, sistem ekspresi RNA 'antisense' *EFG1* telah berjaya dihasilkan dalam kajian ini, di mana satu sistem yang dikawal oleh *PCK1* berjaya mengekspres paras RNA 'antisense' *EFG1* yang mencukupi untuk mencacatkan kebolehan *C. albicans* membentuk 'germ tube' semasa pertumbuhan hifa awal lalu melewatkan pembentukan hifa. Ini mencadangkan bahawa kaedah ini boleh diaplikasikan sebagai alternatif kepada kaedah-kaedah 'knockout' gen yang sedia ada dalam kajian fungsi gen-gen penukaran morfologi *C. albicans*.

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I certify that an Examination Committee has met on _____ to conduct the final examination of **Tung Chee Hong** on his **Master of Science** thesis entitled "Effect of *EFG1* antisense RNA expression on hyphae formation in *Candida albicans***"** in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Examination Committee were as follows:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of **Master of Science**. The members of the Supervisory Committee were as follows:

Chong Pei Pei, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Seow Heng Fong, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Syahrilnizam Abdullah, D. Phil
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD. GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malayia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____

TUNG CHEE HONG

Date: 11 MARCH 2011

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