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Search for Genes Potentially Related to Germ Tube Formation in *Candida albicans* by Differential-Display Reverse Transcription Polymerase Chain Reaction

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Candida albicans exhibits the ability to grow in either a yeast or a mycelia form in response to different environmental factors. The mycelia form, found in infected tissues, is important as a virulence factor in the adherence of the organism to the host epithelium. *In vitro*, the morphological transition can be induced by environmental shifts in the growing conditions, or by a variety of exogenous factors, including ambient pH, nutritional status and temperature. The differential-display reverse transcription polymerase chain reaction (DDRT-PCR) is a powerful technique for comparing gene expression between cell types, stages of development or differentiation. Hyphae related genes were identified and characterized using a PCR-based differential display. *Candida albicans* formed a germ tube when cultured in rabbit serum, RPMI 1640 medium or 39℃-YPD medium. We gained 21 cDNA bands showing a different expression pattern from that of the uninduced culture. DNA was extracted from the same location of the isolated bands, and PCR was performed under the same conditions, which reamplified the PCR product, showing the specific expression patterns according to the culture conditions. We cloned 18 germ tube-related cDNA clones (inserts average size is 80 - 700 bp) and sequenced them. The nucleotide sequences of the 18 clones were identified through in the present study from GenBank, and were found to have the accession number (AF405213-AF405230). We could not find any nucleotide sequence having a high homology with these clones. This study could form a part of the projects in the search for genes related to the germ tube formation of *C. albicans*.

Key Words: *Candida albicans*, germ tube formation, DDRT-PCR, gene sequence

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INTRODUCTION

Candidiasis refers to all the clinical manifestations of primary or secondary infections due to the species belonging to the genus *Candida*, but most of infections are caused by *Candida albicans*. The main infection route is opportunistic, through the gastrointestinal tract. *Candida* sp. are isolated from the mouth, intestinal tract, vaginal mucosa, and skin in about 10 - 30% of healthy individuals. They are isolated at a high rate in oral mucosa, in elderly people, and in the vaginal mucosa of pregnant women.[1] The incidence of candidiasis is becoming increasing significantly. This is due to a number of factors: the drastic increase in the usage of antibiotics, immunosuppressive agents and cytotoxic agents; the increase in the number of elderly people, due to improved living standards and increased life expectancy; the increase in the number of people with compromised immune systems, including patients with chronic consumptive diseases, malignant cancers, and acquired immune deficiency syndrome (AIDS).[2-4]

The virulence factors of *Candida* sp. currently determined include proteinase and coenzymes produced by *Candida* sp.,[5-10] the affinity between organisms and the surface during the adhesion process to the host epithelium and interactions between adhesin-receptors,[11,12] hydrophobicity of cell surfaces,[13] the ability to produce germ tubes and to transform into pseudohyphae.[14] Proteinases and phospholipases are produced and secreted from the blastoconidia of *Candida* sp. and the lower part of the germ tube that has penetrated through the epithelial surface.[15] The ability to adhere to epithelial cells differs according to each species, and *C. albicans* showed a higher ability than other *Candida* sp.,[16] and cells that formed germ tubes showed a higher ability than the yeast forms.[17] *C. albicans* is usually present in the yeast or mycelial forms. Infections occur in different parts of the body with the mycelial form, which has a very close relationship with the pathogenicity.[18] It grows into the mycelial form, with the production of germ tubes, when cultured in rabbit serum,[19] a YPD medium at 39℃,[20,21] and in media lacking glucose.[22,23] Until recently, studies on the pathogenicity of *C. albicans* were limited to the external changes, affinity with host cells, hydrophobic property of cell surface, and exoenzyme secretion. However, studies on the genes relating to the pathogenicity from a molecular biological approach are rare. Therefore, we believed we could modify the virulence of *C. albicans* as long as we could control the expression of pathogenic factors, through the search of genes relating to the expression of each pathogenic factor. DDRT-PCR is a technique used to compare the pattern and intensity of different gene expressions that occur under given conditions.[24] In order to search for the genes presumed to participate in germ tube formation, we used DDRT-PCR to clone parts of genes expressed during the process of germ tube formation, and to determine the sequence of each cDNA clones.

MATERIALS AND METHODS

Strains and plasmids

The *C. albicans* strains ATCC 10231 and ATCC 36801 were used for the germ tube formation. The *E. coli* strain JM109 was used in the transformation, and proliferation of the recombinant plasmids. To insert the PCR product into the plasmids, the pGEM-T vector (Amp^r, *lacZ*; Promega, Madison, WI, USA) was used.

Germ tube formation

The *C. albicans* was cultured in Sabouraud's dextrose agar media, and the colonies collected, washed 3 times with sterile saline solution and used for the study. The candida was inoculated in rabbit serum, RPMI 1640 media or YPD (1% yeast extract, 2% peptone and 2% dextrose) media, to a final concentration of 1×10^5 cells/ml. After culturing each inoculation at 37℃ for 2 h, or at 39℃ for 1 h, the cells were collected and washed twice with PBS. After confirming the germ tube formation from the observation of 10㎕ of cells under an optical microscope (× 400), the total RNA was extracted, and used for this study.

Total RNA isolation

The *C. albicans* that formed germ tubes were isolated from rabbit serum, RPMI 1640 media and 39℃-YPD media, and the total RNA was isolated using a RNeasy Mini Kit (QIAGEN, Hilden, Germany). After the cell pellets, with germ tube formation, had been suspended in 500㎕ yeast lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl and 1 mM EDTA; at pH 8), the same volume of phenol, and 0.1 g of glass beads (Ø, 0.5 mm), were added; the mixture was reacted in a bead beater for 50 sec. After centrifugation at 12,000 rpm for 5 min, the supernatant was purified.[25] The isolated RNA was quantified after dissolving in 50㎕ of RNase free sterile distilled water, and was kept at -70℃ until use.

Differential-display reverse transcription polymerase chain reaction

A GenHunter's RNAimage kit (GenHunter Co., Nashville, TA, USA) was used for the DDRT-PCR. After synthesizing the cDNA by reverse transcription using 3 types of oligo-dT primer (Table 1) according to the instruction of the manufacturer.

Table 1. Arbitrary Primers in Differential Display RT-PCR

Primer	Name	Sequence (5 → 3)
Antisense (16 mer)	HT11G	AAGCTTTTTTTTTTTG
	HT11C	AAGCTTTTTTTTTTTC
	HT11A	AAGCTTTTTTTTTTTA
Sense (13 mer)	HAP1	AAGCTTGATTGCC
	HAP2	AAGCTTCGACTGT
	HAP3	AAGCTTTGGTCAG
	HAP4	AAGCTTCTCAACG
	HAP5	AAGCTTAGTAGGC
	HAP6	AAGCTTGCACCAT
	HAP7	AAGCTTAACGAGG
	HAP8	AAGCTTTTACCGC

AAGCTT : *HindIII* siter.

The 3 oligo-dT primers, and 8 types of arbitrary primer (Table 1), were combined for each polymerase chain reaction. PCR was performed in 40 cycles at 94 ℃ for 30 sec, at 40℃ for 2 min, and at 72 ℃ for 30 sec; and finished at 72 ℃ for 5 min. The amplified cDNA underwent electrophoresis on 6% denaturing polyacrylamide gel; after the gel was dried, it was exposed to X-ray film for 24 h to select the bands showing the locations with the specific expression of interest. The same location of each gel was cut out, for comparison with the isolated band, placed in 100 μl of TE buffer, boiled for 15 min, and used as the template for the second amplification. After the addition of 4 μl of the extracted cDNA, 20 μM dNTP, 0.2 μM arbitrary primer, 0.2 μM oligo-dT primer and 0.4 μl Taq DNA polymerase (5 U/μl; Takara Bio Inc., Shiga, Japan), the final volume was adjusted to 40 μl. The second amplification was performed under the same PCR conditions.

Cloning and analysis of nucleotide sequences

The second PCR amplification product was placed in a pGEM-T vector (Promega, USA) and transformed into the prepared *E. coli* JM109 strain, using the $CaCl_2$ method. After selecting the transformants showing antibiotic resistance, the insertion of the PCR products was confirmed from the digestion pattern of restriction enzymes. The nucleotide sequence of each clone was determined using an Auto-sequencer (LONG READIR 4200, LI-COR, Lincoln, NE, USA). The analysis of the confirmed nucleotide sequences were performed by a comparison with those nucleotide sequences registered in GenBank, using the BLAST Network Service provided by the NCBI (National Center for Biotechnology Information, Bethesda, MD, USA), and with those registered for the Stanford's Candida database.

RESULTS

Differential-Display Reverse Transcription Polymerase Chain Reaction

The *C. albicans* was cultured in condition to encourage the germ tube formation, including rabbit serum, and RPMI 1640 and 39℃-YPD media for 2 h. The formation of germ tubes was observed with all three on the above conditions, but not when cultured in YPD at 37℃ for 2 h (Fig. 1). Using 100 ng of the isolated RNA as a template, we compared the differential display of the mRNA using a RNAimageTM Kit (GenHunter, USA). The comparison of the mRNA was performed using 3 oligo-dT primers and 8 arbitrary primers, in 24 total combinations, and the products obtained from the DDRT-PCR underwent electrophoresis in sequencing gel. One cDNA band was equally detected in the rabbit serum and the RPMI 1640 and 39℃-YPD media, but was not detected in the 37℃-YPD medium. Sometimes only one band was detected in the uninduced germ tube cultures. We obtained 21 differential expressed cDNA bands inform the rabbit serum, and the RPMI 1640 and 39℃-YPD media, compared to the uninduced culture (Fig. 2).

Subcloning of the band with differential display

We isolated the cDNA bands showing different expression patterns from those of the uninduced culture (Fig. 2). We isolated the transformants showing ampicillin resistance, and obtained 21 clones with about an 80 - 700 bp nucleotide insert (A1, A2, A3, A4, A6, A7, A8, A9, A10, G1, G2, G3, G4, G5, G6, G7, G8, G11, G12, G14 and G16) (Fig. 3).

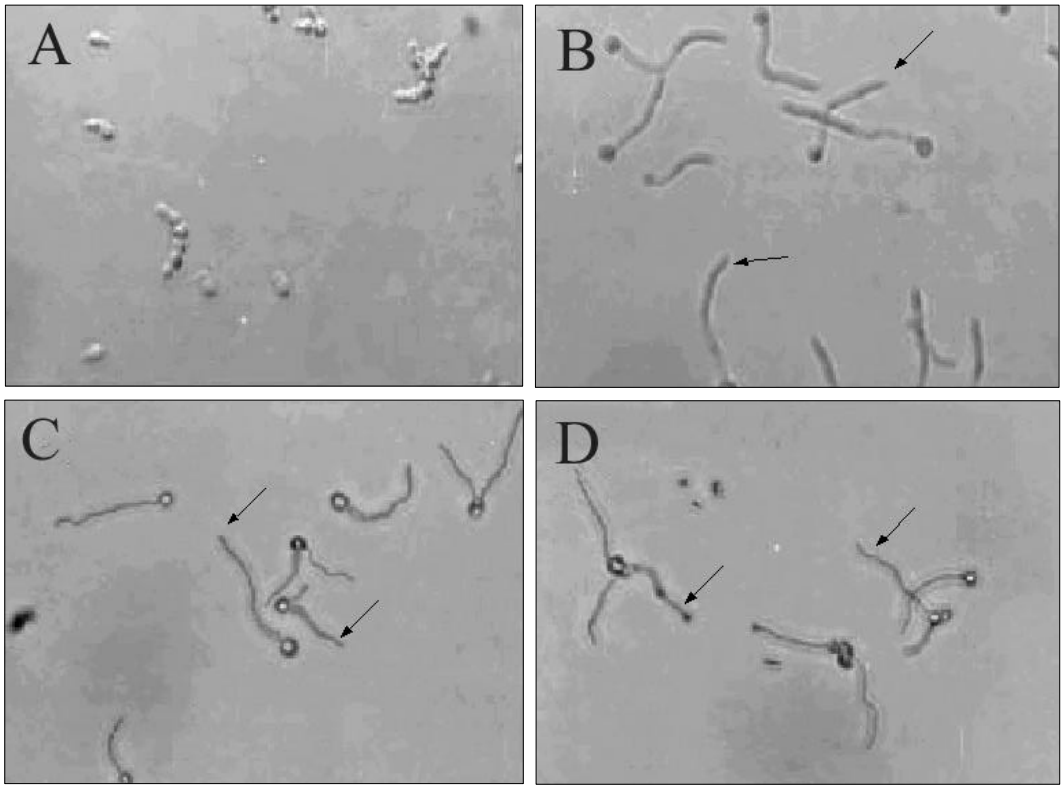

Fig. 1. Photograph of the germ tube induced under the selected culture conditions. A; YPD at 37℃ for 2 h. B; Rabbit serum at 37℃ for 2 h. C; RPMI 1640 at 37℃ for 2 h. D; YPD at 39℃ for 2 h. (magnification, × 400). At an initial cell concentration of 2×10^7 cells/ml. Arrows indicate the germ tube.

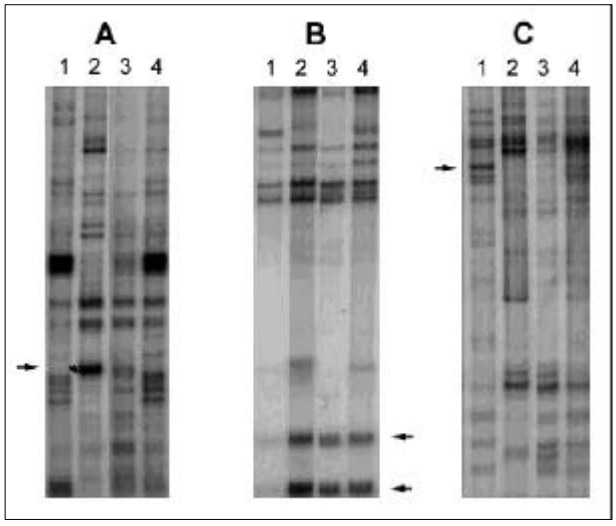

Fig. 2. An autoradiogram of the cDNA bands of the DDRT-PCR for the *C. albicans* ATCC 10231. Arrows indicate the differentially expressed cDNA bands. Lane 1, normal culture in YPD media (37℃); lane 2, Germ tube induced by serum (37℃); lane 3, Germ tube induced by RPMI 1640 media (37℃); lane 4, Germ tube induced in YPD at 39℃. A, HT11G-HAP1 primer; B, HT11G-HAP2 primer; C, HTG-HAP3 primer.

Analysis of nucleotide sequences

We isolated and purified the plasmid DNA from the 21 clones to confirm the DNA nucleotide sequences. The nucleotide sequence of each of the 21 clones was confirmed by referring to a center specializing in nucleotide sequence analysis (Table 2). Of these, A2 and G3, A3 and A9, A6 and G12 were confirmed to be the same clones. Each clone with a confirmed nucleotide sequence was compared for homology with the nucleotide sequences in the GenBank of the NCBI. The A6, G2 and G14 showed homology (20 - 30%) with some parts of the *S. cerevisiae* chromosome XIII, the *C. albicans* cosmid Ca38F10, and the *S. cerevisiae* chromosome II genes. However, we could not confirm any nucleotide sequence having a high homology. Thus, we could not determine the gene function of these clones. Conversely, those clones with confirmed nucleotide sequences were compared, and analyzed, with the data on nucleotide sequences registered at the Stanford's DNA

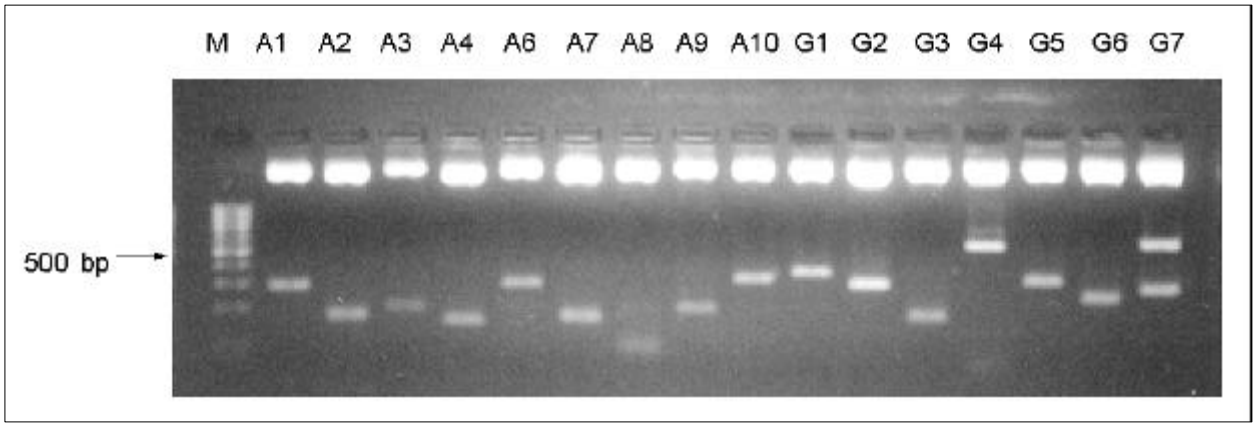

Fig. 3. Agarose gel electrophoresis of *HindIII* digested cloned plasmid DNAs from the *E. coli* strain JM109 transformants. M; 100 bp ladders.

Sequencing and Technology Center (in the Candida Genomic Project). The results showed that the DNA nucleotide sequences obtained in our study were present in Contig,[5] with 97 - 100% homology. However, we have not yet determined the function of these clones, even after comparing them with the database from the Stanford's DNA Sequencing and Technology Center (Table 3). This study would form part of the projects for the identification of genes relating to germ tube formation in *C. albicans*.

DISCUSSION

An understanding of how the pathologic factors function, whether through an active or a passive immunity, during the pathogenesis in an infectious disease would be helpful in the diagnosis and treatment of the disease. The pathogenic mechanism of *C. albicans* is affected by many factors, rather than just one specific factor. In recent years, many studies, mainly on the genetics, have been conducted on the pathogenicity of *C. albicans*. Of the pathogenic factors of *C. albicans*, genes related to phospholipase and proteinase were cloned, and studies were performed to see if these genes are related with the pathogenicity.[26,27] However, apart from the pathogenicity, studies have reported not just one or two genes, but several, participating in the germ tube formation. Recent studies have reported that a *C. albicans* integrin-like protein (INT1),[28,29] agglutinin-like proteins (ALS gene family; ALS1, ALS2, ALS4, ALS5),[30,31] and a hypha-specific gene (HWP1),[32] might participate in the germ tube formation. An adenylate cyclase-associated protein gene (CAP1),[33] and a *C. albicans* RAS-related gene (CaRSR1)[34] are involved in the hypha development and filamentous growth. However, we think that these genes are only some of the genes related with the germ tube formation, and that many more genes might participate in the germ tube and hyphae formation. Furthermore, these genes might function through complex interactions with one another.

The present study was conducted to confirm the expressions of the genes relating to germ tube formation, using differential display RT-PCR at the mRNA level. To reduce false-positive results due to intersample variability, it is recommended that two uninduced and two induced populations should be run simultaneously.[35] However, we ran one uninduced and three differently induced populations simultaneously, as we were focusing on the search for genes related to germ tube formation. The product was selected only if it was differentially expressed in all three of the induced populations compared to uninduced population. We analyzed the differences in the expression of each gene presumed to participate in germ tube formation, through the germ tube formation ability, and the nucleotide sequence, by cloning the parts of the DNA showing differences in their expression patterns. Of these clones, the A2, A3 and A6 were found to be the same as clones G3,

Table 2. Sequence of cDNA Clones

Clone (bp)	Sequence
A1 (260)	ataggggctgggagatgtggaataaaagctctaaaaatagctgacactttatatcatttgattatggaaggatcttttccattttaaccgaaattaccataactaatacactgattgcatgaactat tacaattaacaagatacagacacacacacacacagactcttctgaaaatagtataaaagctactagggtcactgcaaatcgaaaaacagaaaattggttgattccaatcttcaattcattctaaa ctgtattt
A2 (146)	caagatgatgacaataatgatgacggaagtaaccagtcatcagagtctctgcaattagagagtgaaccttctgttgacaagcatgctaaatcttttactatagacactccaatggctcgtcatca agctgttaattattcagtccc
A3 (172)	aataaggcagcaatgacaccaactacacctgctgccaagagatcatttactactggtctctcagcgccattaccttgaggagcagtagaagccatactagaagaagtgttgactgcactttgag atgatgatgaagatgagtttgacatagttgtgttagcattgccgtgtc
A6 (268)	tttaacagcacattatattcaacaatgggtaaatcatccggtaaaggaaaaggaggtaaaaatagaagaagaggaaagaatgtcaacagtggacaaaaaagagaattaatctataaagaa gagggccaagaatatgctcaaatcactaaaatgttaggtaatggtagaatcgaagtaagttgtttcgatggtattaaaagaatgggtcacattagaggaaaattgagaaagaaggtgtggat gggacaaggtgatattattttggtgt
A7 (142)	tgataagttaaagaaaacaacaaaacaaaaatcaaactacgaactgaggagtgtaaagtacaaagctctgtgaaagtgaaatggtatttatgtacgagagaaagaaaaaaaaaaacatta acaaagtaagctaggtcgtatg
A8 (62)	cataatcgcgttatacatatatacgtcataagggaaggaggaaagaggagattgtctatata
A9 (172)	atcaaggcagcaatgacaccaactacacctgctgccaagagatcatttactactggtctctcagcgccattaccttgaggagcagtagaagccatactagaagaagtgttgactgcactttgag atgatgatgaagatgagtttgacatagttgtgttagcattgccgtgtc
A10 (299)	gatacgagggaactaataactcagggtcaaaagtttcgtcatcttcaacagttccgccatcaccactaacaaagaaagatcttttcttatgaatcgaattatcgattttattggcagcaaaggact gggctctggtgaaagaacttgcaaagttgtgtaatgagttgggtcctttgaaagagcctattggagtgtcaagaaagctgtgagacggacgtctagaaagtaaagaagtattttcgtcttcctc aatcatgatatactaaagaagatgtggagaagtagtttggcaagttcgt
G1 (330)	ccgcctgtctttttctattgacaaatatatttttaaatattaaaattttgagaccagatataatggtggtggtggtgataacattttctctcgaattaaagaaggaatttaaatatcaaagtacaaaat caaaaatctaatcgtgacactaatctctataatttagtaattgatgtgtaaaatattattcatattctaatattgaagtgaactgtagtaataatttgattggttaattcatttcaaatattgaatgattg aagtgactgaccaactacatgacggaagtaatcattgttgttgttattcgttgatttgtagacgatta
G2 (270)	catttagagaagtaacccattggaagtctattgctagttgtagttatatagaatttaatctttactactataatacatgttcatagtcggtagtataatatagacactactgtagtttgtcagaactag atcattcttattctactagtaactcaggaaacactttcatctgtcgttgtccactgaactaggtctaagattctgattttacttgctattttagttccataaagtttttatggtgtgccactttatgcggatg tctacgtc
G3 (146)	caagatgatgacaataatgatgacggaagtaaccagtcatcagagtctctgcaattagagagtgaaccttctgttgacaagcatgctaaatcttttactatagacactccaatggctcgtcatca agctgttaattattcagtccc
G4 (580)	gaatgtactaacaaaattgaagagtaatttttgcccgtatcttgatagtaaagatcaagtcagataaagagcggctgatagcaggatcataagattgaaaatttcacttgaatggattattttaa aacttatgacagtacctaccggcgtcccgataaatattgtcatgttttaaaattgctatcggcaagtccctttgctttccgacggaatcccaacggaatgcaatgctgtgataaagccatatgcagt ctccaatgaatttttgttatcaacaataaactagagcatgacaaactataaatagataggttgtccctgtcttgaattatgattttattttcattttcaaataaatctacacataatacccactattacatc aaagataacataactacaatgcacaagattctttagttggtactcctttanctgacgctccattttggctggtcaaattgaattacccaagctnttgncaaaacgatcccctcttnttttgaatgatg aaaaattttgcccaagcntggatgaatgcttggtccaaaggcattggggntnggttcnttattccc
G5 (282)	attaggggtttctttgtaattttctgtctttgcgtggaggaaggaaggatcaagacaaagaatgtatgtatgggggaaagaaagaaaaaaaaggcggacttctacagttattccagcatggtt ttatttggtctgagtaagtaattcattttagaaataatatgattgggtccggcaaaaagactgtctccttaacaacaaccaacaacgaacaatcaacaaccaacaaatacacaagagttgctgatt cacaacggaattgcaaattccaaatggccctg
G6 (205)	gtaattcaatgattaattcttgagtggcagggtcatagactttaaaagtggaggttgatttggagtcgtaccattgaccattaataaatggtttatttttgaaataaatttgggttatttaatttagga gccatgttgaattagtcaaatggtaagtgatttgttagaagtagttagttattatactatagaagaagaatcaaatg
G11 (185)	aaacatataaatagcaatttggtgaaattgaatgaaatcgcttcgagtgcagacaacgcacaaattatcagacaacacatcaaactattgaatgagttaatgcggcaattgagttaatggaaa aggttgtgtgtatagatatgtatagtcaacttcgacataatatagcatttagtattcttctc
G12 (267)	tttaacagcacattatattcaacaatgggtaaatcatccggtaaaggaaaaggaggtaaaaatagaagaagaggaaagaatgtcaacagtggacaaaaaagagaattaatctataaagaa gagggccaagaatatgctcaaatcactaaaatgttaggtaatggtagaatcgaagtaagttgtttcgatggtattaaaagaatgggtcacattagaggaaaattgagaaagaaggtgtggat gggacaaggtgatattattttggtg
G14 (425)	aagattagtcagaatcggtgtcttgtctgaagacaaatgaaattggattatgtcttggctttgaaaccagaagatttcttggaaagaagattacaaacccaagttttcaaattaggtttagctaga tctatccaccacgccagagttttgatcacccaaagacacattgctgttggtaaacaaattgttaacatcccgtcattcactgtcagattggactctcaaaaacacattgactttgcccacaactctcc atacggtggtggtagagccggtagagttaagagaaagaaccaaggtaaaggtggtgaagaaggtgccgaagaagaagaataaatgcattagcattcaaaaacaacctgtnatttatata aacataaataaaactttttctttttaatatcaatatattggtttacaaccttc
G16 (805)	caaaaaaaacactgaaacaaacaaggtaggcgattcagggaatacccaaatgaacaacattagtaacaagttgacgtaataacacaaatcagctaaataatattggaaggacagtttaaag taagtgtaaaaacgaattggcattaacaccacaaacaacgcagtatggaaataaggaaagtaaattggatatttaccaatggtaaacccggcacaaaaaatcaaaaagaccgaagtagcat aaaacgccttttccgtcgctgaactttcaagttgtttatggactcgtttatcaaaattctggagtctttgattgattatttcctgtaacttgtccaattccaagtttt

Table 3. Homology Search of NCBI and Stanford's Database

Clone	Blast search results	Sequence of Stanford's	Accession number	Function
A1	nd	Contig5-2721	AF405213	Unknown
A2	nd	Contig5-3235	AF405214	Unknown
A3	nd	Contig5-3257	AF405215	Unknown
A6	*S. cerevisiae* chr. XIII	Contig5-3262	AF405216	Unknown
A7	nd	Contig5-3235	AF405217	Unknown
A8	nd	not Contig	AF405218	Unknown
A9	nd	Contig5-3257	AF405219	Unknown
A10	nd	Contig5-3264	AF405220	Unknown
G1	nd	Contig5-3103	AF405221	Unknown
G2	*C. albicans* cosmid Ca38F10	Contig5-3162	AF405222	Unknown
G3	nd	Contig5-3235	AF405223	Unknown
G4	nd	Contig5-3121	AF405224	Unknown
G5	nd	Contig5-1969	AF405225	Unknown
G6	nd	Contig5-3253	AF405226	Unknown
G11	nd	Contig5-3250	AF405227	Unknown
G12	*S. cerevisiae* chr. XIII	Contig5-3262	AF405228	Unknown
G14	*S. cerevisiae* chr. II	Contig5-3215	AF405229	Unknown
G16	nd	Contig5-2900	AF405230	Unknown

nd, no data.

A9 and G12, respectively. The nucleotide sequences of 15 of the obtained clones were analyzed for their similarity with those from the GenBank and the Stanford's DNA Sequencing and Technology Center, but we could not confirm homology between the clones obtained in our study and reference clones. Although, the clones A6, G12 and G14 showed similarity with parts of the *S. cerevisiae* chromosome XIII and the chromosome II genes, and the *C. albicans* cosmid, Ca38 F10, although this similarity was less than 30%, indicating no homology in the DNA nucleotide sequence. Hence, this means the DNA nucleotide sequences of the our obtained clones are different from those of others that have been reported to date, mainly due to lack of information on the genes related with *Candida*. We registered the nucleotide sequences of the 18 clones we identified during our study in the GenBank, and obtained the accession number (AF405213-AF405230). In conclusion, as the isolated clones in our study showed high levels of expression for the germ tube formation, although we could not determine the expression pattern and function of these clones, they have a direct or an indirect effect on germ tube formation. Conversely, when we compared the nucleotide sequences of these clones, with those of *Candida* from Stanford's DNA Sequencing and Technology Center, 97-100% homology was seen. We confirmed that both the A2 and A7 were present on contig5-3235 of the Stanford's database, and that these two genes affect the mono-cistronically due to a regulating factor. Further studies on the cloning of the complete gene of each clone, and on the functional aspects of the genes related with the pathogenicity, will be required.

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