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Analysis and expression of *STE13ca* gene encoding a putative X-prolyl dipeptidyl aminopeptidase from *Candida albicans*

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Abstract

Candida albicans STE13ca gene was identified by its homology to the *Saccharomyces cerevisiae STE13* gene that encodes for the dipeptidyl aminopeptidase A (DAP A) involved in the maturation of α-factor mating pheromone. Our study revealed that *C. albicans* ATCC 10231 depicts dipeptidyl aminopeptidase activity. We also analyzed the expression of the *STE13ca* gene homologue from this pathogenic yeast. This gene of 2793 pb is homozygotic and encodes for a predicted protein of 930 amino acids with a molecular weight of 107,035 Da. The predicted protein displays significant sequence similarity to *S. cerevisiae* Ste13p. This *C. albicans* gene is located in chromosome R. *STE13ca* gene increases its levels of expression in conditions of nutritional stress (proline as nitrogen source) and during formation of the germinal tube, suggesting a basic biological function for the *STE13ca* in this yeast.
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1. Introduction

The yeast *Candida albicans* is carried in the microflora of most healthy individuals as a commensal [1]. It is also the most common human fungal pathogen. When the defense mechanisms of an individual are compromised, this opportunistic pathogen can increase in number and penetrate tissues at one or more body locations, causing a variety of yeast-related diseases, such as mucosal and systemic infections [2,3]. The factors that contribute to the pathogenesis of *C. albicans* are not known completely, despite more than a decade of molecular genetic analyses. *C. albicans* was thought to be asexual until the sequencing of its genome revealed that it contains homologous genes to those in the *MAT* locus in *Saccharomyces cerevisiae* designated *MTL* (matig type-like) locus (http://www.sequence.stanford.edu/group/candida).

Sequencing of *C. albicans* genome and a comparative genome analysis between *C. albicans* and *S. cerevisiae* showed that, in addition to the *MTL* locus, this yeast contains homologues of many of the genes involved in the mating process in *S. cerevisiae* [4]. Recent reports have demonstrated mating between *MTL**a*** and *MTLα C. albicans* strains [5–8]. *STE13ca* gene has been identified by its homology to the *S. cerevisiae STE13* gene from the *Candida albicans* Genome Sequencing Project. The *S. cerevisiae STE13* gene encodes the dipeptidyl aminopeptidase A (Ste13p), which is a membrane-bound enzyme, involved in α-factor precursor processing by removing dipeptides from the N-terminus of the pheromone precursor [9]. Mutants lacking Ste13p activity (*ste13*) were sterile when their mating type was *MATα* because cells secreted incompletely processed forms of the α-factor pheromone [9,10]. Recently, Bennett et al. [11] described a mating

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pheromone encoded by the *MF*α gene in *C. albicans* α cells. This pheromone was required to mate by α cells, but not by **a** cells. They also identified that the *STE2* gene encodes the receptor for this mating pheromone and showed that this receptor is required for the mating of **a** cells, but not for α cells. Cells of the **a** mating-type respond to the **α** mating pheromone by producing long polarized projections similar to those observed in mating mixtures of *C. albicans* a and α cells [11]. This paper reports the dipeptidyl aminopeptidase activity and the bio-informatic and molecular analysis of *STE13ca* gene homologue from this pathogenic yeast.

2. Materials and methods

2.1. Strains and culture media

Strains used in this study were *Candida albicans* ATCC 10231, *Candida dubliniensis* CD36 [12], and *Candida tropicalis* CTR10 [13]. Yeast forms were routinely maintained on YEPD plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) at 37 °C. The DAP activity of *C. albicans* ATCC 10231 membrane fraction was measured growing yeasts at 37 °C in YEPD and Minimal medium broths containing 0.17% yeast nitrogen base without amino acids neither ammonium sulfate, but adding 2% glucose without or with 5% nitrogen source (proline, peptone, and ammonium sulfate) according to manufacturer's instructions. Induction of hyphae formation in yeast cells was performed according to the protocol of Lee et al. [14]. To evaluate cell growth, absorbances (A_{600}) of culture samples were measured in a Perkin–Elmer Lambda 1A spectrophotometer.

2.2. Preparation of crude extracts and differential centrifugation

Crude extracts and membrane fractions were prepared according to Suarez-Rendueles et al. [15,16].

2.3. Enzyme assays and protein determination

The enzymatic activity of ycaDAP was determined using L-alanil-prolyl-4-nitroanilide (Ala-pro-4-NA) as substrate. One unit of X-prolyl-dipeptidyl aminopeptidase was defined as the amount of enzyme that liberates 1 μmol *p*-nitroaniline from the substrate in 1 min at 37 °C in our assay conditions [15].

Protein was estimated according to method of Lowry using crystalline bovine serum albumin as standard [17].

2.4. Bioinformatic analysis

C. albicans STE13ca gene was identified by similarity to the *S. cerevisiae STE13* gene. Sequence data for *C. albicans* were obtained from the Stanford Genome Technology Center's website at http://www-sequence. stanford.edu/group/candida. Sequencing of *C. albicans* was accomplished with the support of the NIDR and the Burroughs Wellcome Fund. The Bioinformatic analysis was performed using several softwares. Search of ORF, reverse complementary sequence, restriction map, translation using alternative yeast nuclear genetic code, molecular weight, and codons usage frequency were performed with the DNAMAN version **v**. 3.0 (Lynnon BioSoft, 1994–1997). Prediction of regulatory regions was searched by means of MatInspector version 2.2 [18] (http://www.gsf.de/biodv/matinspector.html); codon adaptation index (CAI) was calculated with data from NGFN (Nationales Genomforschungsnetz) at http://ngfnblast.gbf.de/cgi-bin/emboss.pl_action=manual&_app=cai. Isoelectric point, prediction of hydrophobicity, and helical membranous regions were searched with Antheprot 2000 version 5.2. Prediction of motif was searched with Prosite [19] at http://www.expasy.org. Prediction of subcellular localization was searched with PSORT at http://www.psort.org/. *STE13ca* sequence was aligned with DAP's nucleotide sequences from *S. cerevisiae*, *S. pombe*, and other organisms available at NCBI (http://www.ncbi.nlm.nih.gov). Multiple alignments of deduced amino acid and nucleotide sequences were performed with CLUSTAL X version 1.8 [20]. A similarity tree was constructed with MEGA (molecular evolutionary genetics analysis) version 2.1 [21], using the neighbor-joining grouping method and P (Poisson) index. Statistical evaluation included 1000 bootstrap re-samplings. Percentage similitude was calculated using MEGA.

2.5. DNA Extraction and PCR to specific amplification of STE13ca gene

Total DNA was extracted according to Lehmann et al. [22]. Southern blot of *C. albicans* genomic DNA was performed with DNA digested with *Alu*I, *Cla*I, *Dde*I, *Dra*I, *Eco*RI, *Hin*cI, and *Hin*dII restriction enzymes to detect the number of copies of the *STE13ca* gene.

PCR was used to prepare hybridization probes for Southern blotting and chromosome assignment. A fragment of 466 bp in the ORF of *STE13ca* was amplified from *C. albicans* ATCC 10231. Pair of primer designed was: STE13sense: 5′-ATTTGGCTACTGGCG and STE13antisense: AAATAC-3′and 5′-CTGGTTCTGT-CTGATTGTTTC-3′. The PCR was performed with 10 ng total DNA and conditions of amplification included a hot start at 94 °C (5 min); 40 cycles of denaturation (1 min), annealing at 57 °C (1 min) and polymerization at 72 °C (1 min); a final polymerization time was applied at 72 °C (5 min).

2.6. Pulsed-field gel electrophoresis (PFGE) and chromosome assignment

Cell preparation and separation of chromosomes were performed essentially as described by Wickes et al. [23] and run in a Bio-Rad CHEF-DRII apparatus. Each chromosome was eluted and purified as described by Sambrook et al. [24]. The localization of *STE13ca* gene was determined by specific PCR of *STE13ca* gene (see above) using each DNA chromosomes as template.

2.7. Southern hybridization

DNA was transferred to a Nylon membrane positively charged (Amersham Pharmacia Biotech, UK) as described by Sambrook et al. [24]. Hybridization was performed at 65 °C with a *STE13ca*-specific probe labeled with digoxigenin-dUTP (2′-deoxyuridine 5′-triphosphate) using a random-primed digoxigenin (DIG) DNA labeling detection kit (Roche, Mannheim, Germany). Hybridization and immunological detection were performed as recommended by the supplier.

2.8. Denaturing gradient gel electrophoresis (DGGE)

A fragment of 466 bp *STE13ca* gene amplified from *C. albicans* ATCC 10231 was used in search of a homozygotic/heterozygotic character for this gene by DGGE (Dcode System for DGGE, Bio-Rad Laboratories, USA) according to the manufacturer. The denaturing gradient (40–65%) was formed parallel to the direction of the electrophoresis.

2.9. RT-PCR

Three expression conditions were chosen: (a) growth on YEPD medium during logarithmic, early stationary and late stationary phases; (b) growth on YNB medium without nitrogen and YNB medium supplemented with different nitrogen sources (ammonium, proline and peptone); (c) during the dimorphism from yeast-like to mycelium on Lee's medium. Total RNA was extracted and purified from *C. albicans* ATCC 10231 using the protocol Tripure isolation reagent (Roche, CA, USA) according to the manufacturer. To minimize the risk of contaminating DNA, RNAs (10 μg total) were digested with 10 U of DNase I (Invitrogen, CA, USA) in accordance to manufacturer's instructions. RT-PCR was performed with Thermoscript™ RT-PCR System (Invitrogen, CA, USA) from 2 μg of total RNA by using STE13sense/STE13antisense-specific primers for *STE13ca* gene (expected product size, 466 bp) and 18S rDNA-universal primers (LVT-1: 5′-CCTGCCAGT-AGTCATATGCTTGTCT-3′ and LV-2: 5′-CACCTA-CGGAAACCTTGTTACGACT-3′) for 18S rDNA gene (expected product size, 1694 bp). Expression of *STE13ca* was normalized against levels of 18S rDNA cDNA.

3. Results

3.1. DAP activity determination of C. albicans *ATCC 10231 membrane fraction*

C. albicans ATCC 10231 presented ycDAP intracellular activity in the membrane fraction. The highest level of total and specific ycaDAP activity was reached in the YNB medium with proline or peptone as nitrogen source in the yeast-like phase and during early stationary growth (Table 1a). During dimorphism, the levels of ycaDAP activity were on the increase during formation of the germinal tube (1 and 2 h) and pseudomyce-

Table 1
Total and specific activity of DAP in *C. albicans* ATCC 10231 (membrane fraction)

Growth phases	YEDP	YNB	YNB–proline	YNB–NH_2	YNB–peptone
(a) *Yeast-like stage*					
Logarithmic, 6 h	27.23[a] (1.0)[b]	0 (0)	42.24 (1.41)	21.93 (0.732)	32.34 (1.12)
Early stationary, 12 h	34.76 (1.084)	0 (0)	60.57 (1.84)	36.60 (1.44)	52.36 (2.10)
Late stationary, 24 h	29.04 (0.88)	0 (0)	20.24 (0.451)	5.43 (0.366)	44.88 (1.62)

Stages during the dimorphism from yeast-like to mycelium	Total activity (mU ml^{-1})	Specific activity (mU mg^{-1})
(b) *Dimorphism in Lee's medium*		
Phase G1	0	0
Germinal tube formation 1 h	11.9	0.780
Germinal tube formation 2 h	11.9	0.550
Pseudomycelium formation 3 h	19.07	0.805
Pseudomycelium formation 5 h	2.2	0.109
Mycelium formation 24 h	22	0.868

Cellular fractions were prepared from cells growing in the corresponding medium. Biomass from the culture medium was recovered by centrifugation at 5 °C (2000*g*), washed twice with 0.1 M Tris buffer, pH 7.0, and disrupted with glass beads to produce a cell homogenate. This fraction was centrifuged at 23,000*g*, and the resulting supernatant was centrifuged at 100,000*g* to separate the soluble fraction (cytoplasmic) from the membrane fraction. The 100,000*g* fraction (membranes) was used to determine DAP enzymatic activity against Ala-pro-pNA as substrate.

[a] Total activity (mU ml^{-1}).

[b] Specific activity (mU mg $protein^{-1}$).

lium formation (3 h). However, the highest levels of yca-DAP activity were reached during mycelium formation (Table 1b). No activity was detected in the extracellular fraction (supernatant of the culture medium) in any of the assayed conditions (data not shown).

3.2. Sequence analysis of C. albicans *STE13ca* gene

The nucleotide sequences from Contig19-10235 and 20235 of 28191 and 28192 pb, respectively, were analyzed. An ORF of 2793 bp in the reverse complementary sequence that showed homology with *S. cerevisiae STE13* gene was found. ORFs from both contigs were homozygotic. The deduced amino acid sequence showed that this gene encodes for a protein of 931 amino acid residues, molecular weight of 107,035 Da, and p*I* of 5.195. Inspection of the deduced amino acid sequence of *C. albicans* Ste13 revealed a single hydrophobic domain, and a helical membranous region, beginning 87 residues from the NH_2 terminus that could potentially span a lipid

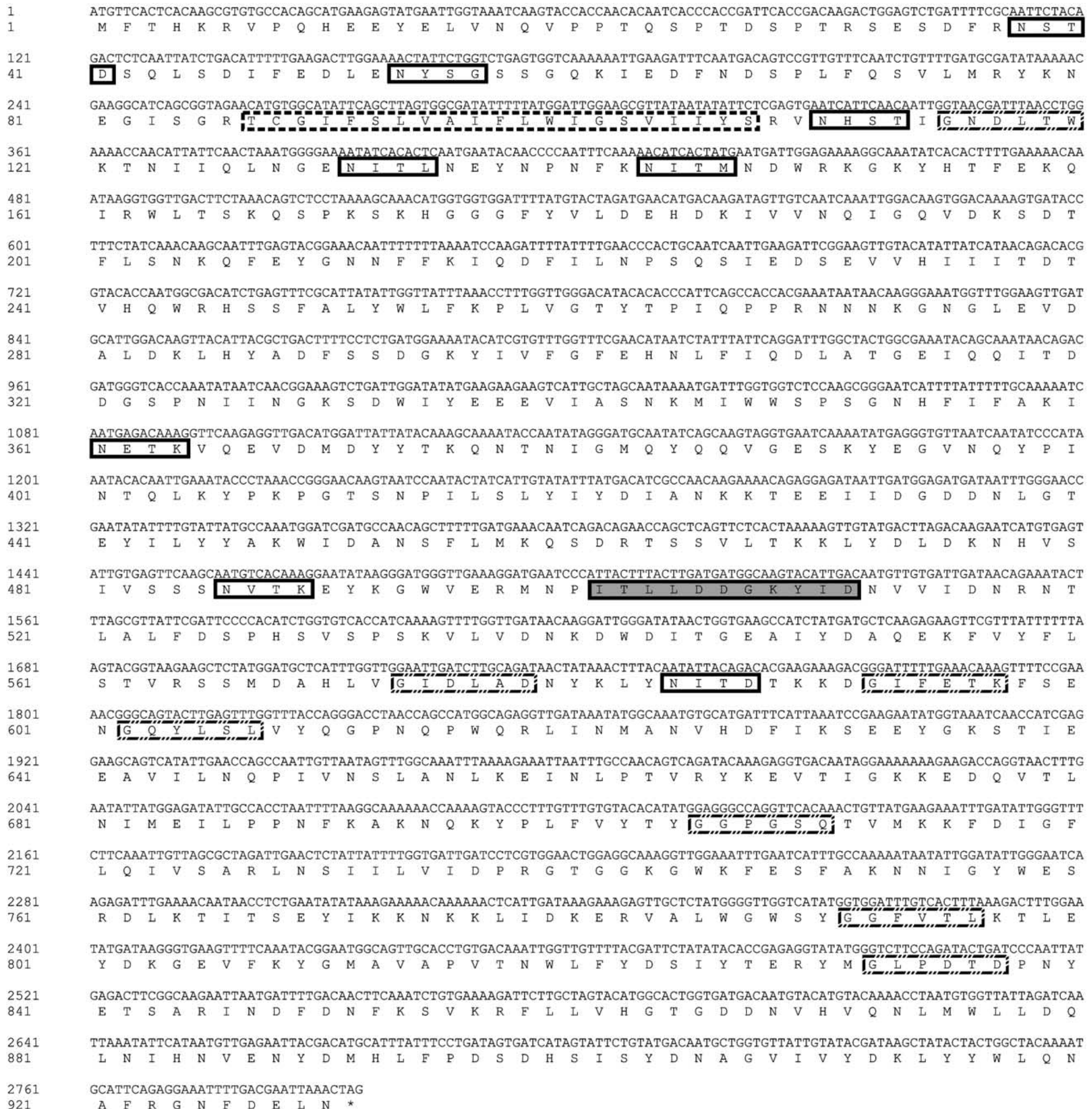

Fig. 1. Nucleotide and deduced amino acid sequences of the *C. albicans STE13ca* gene. The deduced amino acid sequence is given beneath the nucleotide sequence of the *STE13ca* coding region. Nucleotide residues are numbered in the 5′-to-3′ direction, with the *A* of the predicted ATG; initiating methionine given the number 1. The amino acid sequence comprises the putative membrane-spanning domain (marked with dotted box), the potential sites of N-linked glycosylation (open box), N-myristoylation (dashed box) and serine proteases active site (shaded box).

Table 2
Possible binding sites to transcription factors of gene *STE13ca* from *C. albicans*

Transcription factor	Further information	Position from-to	S	Core sim.	Matriz sim.	Sequence[a]
MATA1	Mating factor a1	−39 to −31	(+)	1.00	0.929	**tGATG**caac
NIT2	Activator of nitrogen-regulated genes	−304 to −298	(−)	1.00	0.994	**TATC**tct
MCM1	Yeast factor *MCM*1 cooperating with *MAT*α factors	−501 to −485	(+)	1.00	0.913	atg**CCTAa**att**ggg**aaa
STRE	*MSN2*/*MSN4*, *STRE* element, *S. cerevisiae*	−841 to −833	(+)	1.00	0.980	cc**AGGGg**at
NIT2	Activator of nitrogen-regulated genes	−946 to −940	(+)	1.00	0.994	**TATC**tcc
MATA1	Mating factor a1	−1196 to −1188	(−)	1.00	0.947	**tGATG**tgga
NIT2	Activator of nitrogen-regulated genes	−1217 to −1211	(−)	1.00	0.994	**TATC**tca
NIT2	activator of nitrogen-regulated genes	−1344 to 1338	(+)	1.00	0.994	**TATC**tct
MAT1MC	HMG-BOX protein interacts with M-box site, cooperativity with HMG-Box *STE11* protein	−1669 to 1659	(−)	1.00	0.896	**gtcaTTGT**ttt
RAP1	*RAP1* (*TUF1*), activator or repressor depending on context	−1732 to −1718	(−)	1.00	0.860	aaa**ACCCa**t**ata**aaa

2000 pb upstream of the starting codon (ATG) were analyzed.
S: Positive/negative strand.
Basepairs marked in bold show high information content, i.e., the matrix exhibits a high conservation at this position.
The "core sequence" of a matrix is defined as the (usually 4) consecutive highest conserved positions of the matrix.
The analysis was performed with a "core similarity" of 0.9. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence. More important than the core similarity is the matrix similarity, which takes into account all bases over the whole matrix length. A perfect match to the matrix gets a score of 1.00 (each sequence position corresponds to the highest conserved nucleotide at that position in the matriz), a good match to the matriz usually has a similarity of >0.80.
[a] Basepairs in capital letters denote the core sequence used by MatInspector.

bilayer. In addition, eight probably sites for asparagine-linked (N-linked) glycosylation; seven probably sites for N-myristoylation and an active site of serine proteases belonging to subtilases family with one allowed mismatch in the Lys (k) amino acid were recognized (Fig. 1). The possible site of the subcellular localization of the predicted protein of gene *STE1ca* could be the Golgi apparatus with a 0.9 certainty factor. Codon usage frequency and CAI (0.239) of *STE13ca* indicated that this gene belongs to the low-expression genes (data not shown) [25]. A region of 2000 pb upstream of the start codon ATG was analyzed to recognize regulatory regions. Transcription factor binding sites for *STRE*, *NIT-2*,*MAT1-MC*, *MATa1*, *MCM1*, *RAP1* were detected (Table 2). The BLAST program [26] was used to identify other homologous genes in GenBank database at NCBI (http://www.ncbi.nlm.nih.gov). The protein encoded by this ORF exhibits a high similarity with Ste13 from *S. cerevisiae*. The nucleotide and amino acid sequences of *C. albicans* and *S. cerevisiae* genes shared 50.70% and 31.83% of similarity, respectively. The constructed similarity tree revealed that the predicted amino acid sequence of *STE13ca* is more related to the Ste13 protease from *S. cerevisiae* than with those from other organisms (Fig. 2). Because of the similarity between *C. albicans* and *S. cerevisiae* *STE13*, this gene was also designated *STE13ca*.

3.3. Determination of the number of copies of gene STE13ca

Southern analysis revealed that gene *STE13ca* from*C. albicans* ATCC 10231 presents as a single copy, since the number and size of the hybridization bands of the genome digested with enzymes: *Alu*I, *Dde*I, *Dra*I, *Hinc* II, *Eco*RI corresponded to that expected according to the restriction map (Fig. 3(a)). Digestion of the genome with enzyme *Cla*I presented a larger size hybridization band, and the digestion with enzyme *Hin*dIII depicted, besides the expected band, and extra band of larger size (Fig. 3(b)). *C. dubliniensis* CD36 and *C. tropicalis* (CTR10) presented Southern hybridization bands with the probe of the gene *STE13ca* (data not shown).

3.4. Determination of the homo/heterozygotic character and chromosome location of gene STE13ca

The DNA fragment of the PCR amplified gene *STE13ca* of 466 bp and subjected to DGGE depicted one band only, confirming the homozygosity of this gene in *C. albicans* (Fig. 3(c)). The eight chromosome pairs of *C. albicans* ATCC 10231 were separated by PFGE (Fig. 3(d)). The gene *STE13ca* was located in chromosome R by *STE13ca*-specific PCR. No PCR

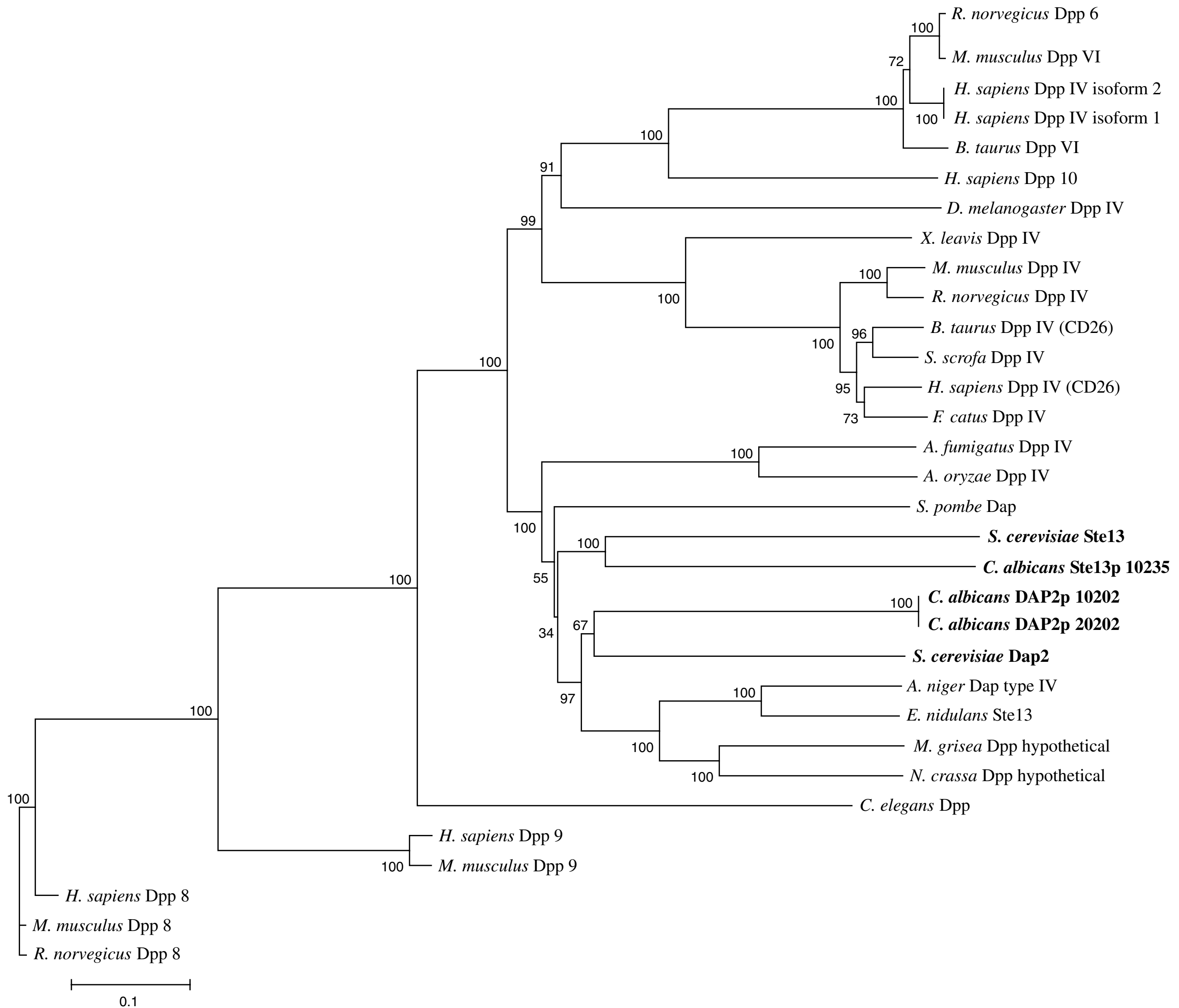

Fig. 2. Similarity tree of deduced amino acid sequence of *STE13ca* gene of *C. albicans*, and other DAPs decribed in *A. fumigatus*, *A. oryzae*, *A. niger*, *B. taurus*, *C. elegans*, *D. melanogaster*, *F. catus*, *H. sapiens*, *M. musculus*, *M. grisea*, *N. crassa*, *R. norvegicus*, *S. cerevisiae*, *Schizosaccharomyces pombe*, *S. scrofa*, and *X. leavis*. *DAP2* from *C. albicans* is another gene identified to encode the putative DAP B. The tree was defined by MEGA version 2.2 software [21]. Analysis was based on multiple alignment (data no shown) using neighbor-joining grouping method and *P* (Poisson) index. Statistical evaluation included 1000 bootstrap resamplings. The bar indicates number of nucleotide changes per 100 nucleotides.

product was obtained when other chromosomes DNA were used as template (Fig. 3(e)).

3.5. *Differential expression of gene* STE13ca

Three expression conditions were chosen: growth phases, different nitrogen sources and during the dimorphism from yeast-like to mycelium (Fig. 4). RT-PCR results indicated that this gene expresses in both the yeast-like stage and the mycelial stage in the diploid *C. albicans* ATCC 10231 strain. During the different growth phases, it expresses preferentially at 6 and 12 h, which correspond, respectively, to the logarithmic and early stationary phases of the cell growth cycle, but is not expressed during the late stationary phase (Fig. 4(d)-I). The gene *STE13ca* did not express in either YNB medium without nitrogen source or in YNB with peptone; maximal expression levels were obtained in the YNB medium with proline. Although a moderate expression was obtained in the YNB medium with ammonium, *STE13ca* is a gene mainly induced by proline (Fig. 4(d)-II). In stage G1, expression levels of gene *STE13ca* were nil. During germinal tube formation induction (1 h), levels of expression increased until the germinal tube had been formed (2–3 h); thereafter, levels decreased slightly during formation of the pseudomycelium (5 h), and increased anew to maximal levels during formation of the true mycelium (24 h) (Fig. 4(d)-III).

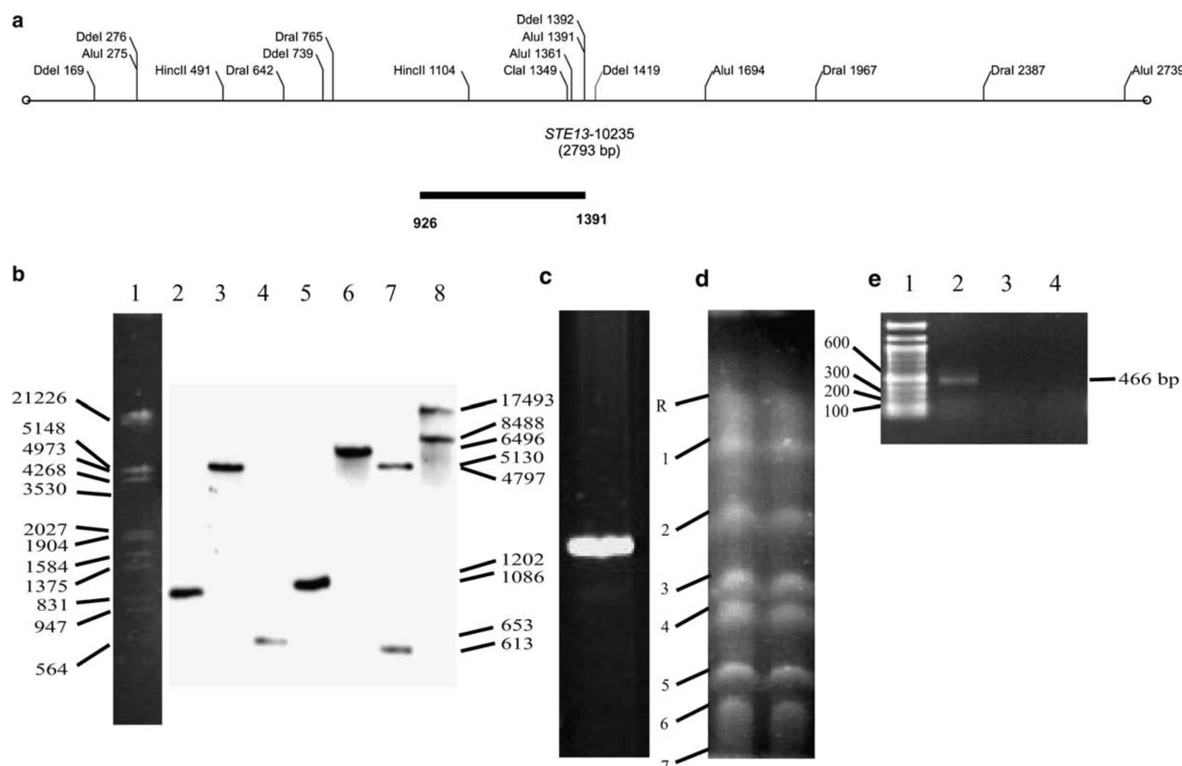

Fig. 3. Southern blot of total DNA, DGGE analysis, and electrophoretic karyotype of *STE13ca* gene from *C. albicans* ATCC 10231. (a) Restriction map of *STE13ca* gene. (b) Southern blot of total DNA digested with several restriction enzymes and probed with *STE13ca*-specific probe: (1) DNA marker, (2) *Alu*I, (3) *Cla*I, (4) *Dde*I, (5) *Dra*I, (6) *Eco*RI, (7) *Hinc*II, (8) *Hin*dII. (c) DGGE analysis of the fragment of 466 bp STE13 probe amplified by PCR. A single band indicates absence of nucleotide changes and homozygotic character of *STE13ca*. (d) Electrophoretic karyotype. Electrophoretic conditions were 120, 240, and 360 s of initial and final pulsed time per 24 h each, all at 150 V, 11 °C, and TBE buffer 0.5×. Numbers refer to chromosomes. (e) PCR of the chromosomes: (1) DNA marker, (2) Chromosome R, (3) chromosome 1, and (4) chromosome 2. *STE13ca* amplified on chromosome R.

4. Discussion

In *S. cerevisiae*, a membrane-bound X-prolyl dipeptidyl aminopeptidase activity has been described [15,27], which is associated to two different enzymes: yscDAP A, located in the membrane of the Golgi apparatus and coded by gene *STE13*, [28,29]; and yscDAP B, detected on the vacuolar membrane and coded by gene *DAP2* [30]. The present results show that *C. albicans* ATCC 10231 has ycaDAP membrane activity in both the yeast-like and the mycelial stages. Similarly to *S. cerevisiae*, this activity was intracellular in all the assayed conditions, since the activity could not be detected in the supernatants of liquid cultures. During dimorphism, the highest levels of specific ycaDAP activity were encountered during formation of the germinal tube. These results could suggest the participation of this enzyme during dimorphism. During the yeast-like stage, the highest ycaDAP activity levels were found with proline as sole nitrogen source, an amino acid considered as a non-preferential nitrogen source [31].

Although it has been proven that *C. albicans* possesses a diploid genome (http://www.sequence.stanford.edu/group/candida), the gene *STE13ca* resulted homozygotic. Our DGGE results with the amplified fragment of this gene coincided with the homozygosity of the sequence. This result supports the hypothesis of Hull and Johnson about heterozygosity of the *MTL* locus of *C. albicans*, and the homozygosity of the rest of the genome [5]. The ORF of the gene *STE13ca* (2793 pb) encodes a predicted 930 amino acids protein homologous to the Ste13p of *S. cerevisiae* (2796 pb and a 931 amino acids protein) [32,33].

The search for binding sites to the transcription factors of gene *STE13ca* suggested a possible regulatory action promoted by stress, nitrogen source, and processes related with mating. Thus, a binding site to Stre (stress response element) encoded by *STRE*, which regulates stress response genes in *S. cerevisiae* was located. STREs regulate the induction of transcription due to thermal shock, nitrogen inanition, oxidative and osmotic stress, low external pH, sorbate, benzoate, or ethanol induced stress [34,35]. As in *S. cerevisiae*, this could indicate that expression of *STE13ca* in *C. albicans* is induced when the cell is subjected to diverse stress conditions. Also, four binding sites to Nit-2, which activates the nitrogen-regulated genes, were found. *NIT-2* is the major positive-acting regulator of nitrogen in *Neurospora*

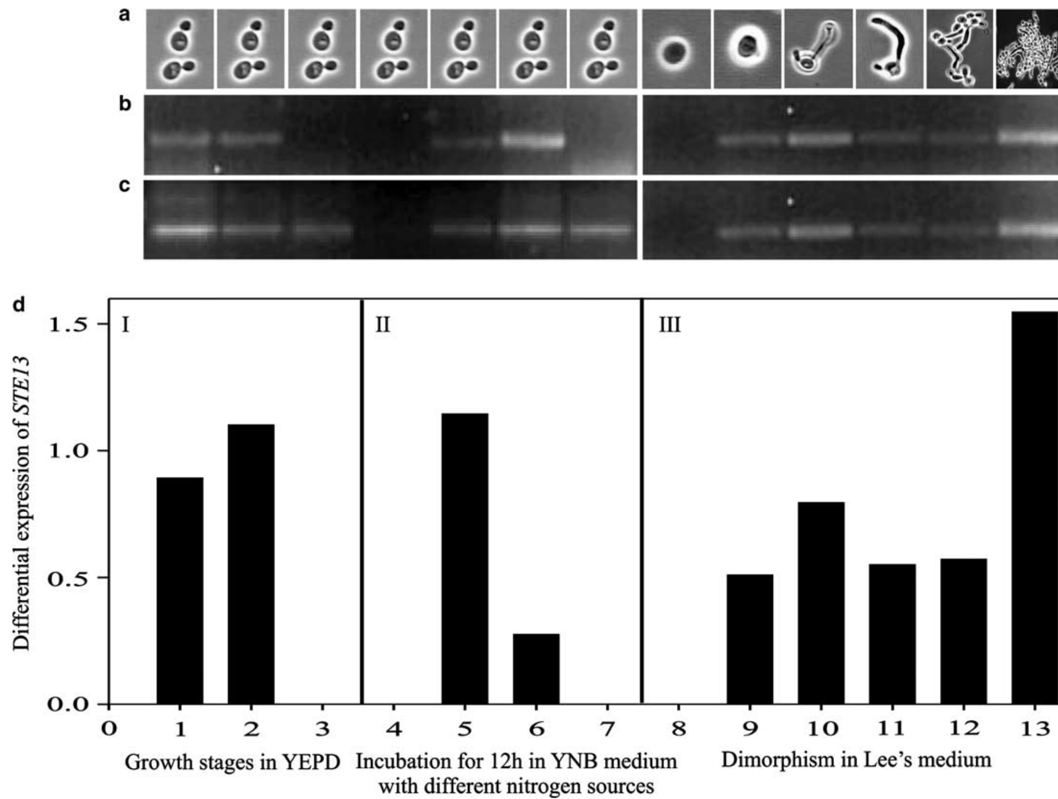

Fig. 4. Differential expression of *STE13ca* gene in *C. albicans* ATCC 10231 during different growth phases, nitrogen sources and during dimorphism using RT-PCR. (a) Digital imagery of cellular morphology, (b) cDNA *STE13ca* gene, (c) 18S rDNA cDNA, and (d) *STE13ca* gene expression. (d)-I Growth phases in YEPD: (1) logarithmic, 6 h; (2) early stationary, 12 h; (3) late stationary, 24 h. (d)-II Incubation for 12 h in YNB medium with different nitrogen sources: (4) YNB, (5) YNB–proline, (6) YNB–NH_2, (7) YNB–peptone. (d)-III Dimorphism in Lee's medium: (8) phase G1, (9–10) germinal tube formation, (11–12) pseudomycelium formation, (13) mycelium formation. The intensity of bands *STE13ca* cDNA was normalized to the intensity of 18S rDNA cDNA.

crassa. This regulator turns on genes when primary sources of nitrogen (ammonium or glutamine) are not available [36]. The present study on *STE13ca* gene expression revealed higher expression levels (at mRNA level) in the presence of proline as nitrogen source, suggesting that gene *STE13ca* could be under the transcriptional control of Nit-2. Other binding sites to the transcription factors MAT1-MC and MATa1 were located. These genes act in mating determination in *S. pombe* and *S. cerevisiae*, respectively [37–39]. These binding sites suggest that the gene *STE13ca* could be involved in *C. albicans* mating. As well a binding site to *MCM1*, which together with *MAT*α2 (α2/Mcm1p) represses aspecific genes in the a/α diploid of *S. cerevisiae*, was detected [40]. Upstream sequence of *STE13ca* gene possesses a binding site to Rap1 (Tuf1), which displays a transcriptional activation and silencing of *MAT* genes, among other functions. The *rap1* strains are also deficient in α-pheromone production. Elimination of the binding site for *RAP1* in the *HMR* locus (silent mating-type) has an opposed effect on this locus, which becomes partially depressed [41]. Rap1 activates and represses *MAT* genes according to the situation, allowing us to infer that gene *STE13ca* from *C. albicans* might be activated by the action of Rap1.

Codons usage has been characterized in *S. cerevisae*, where highly expressed genes depict a use of synonym codons strongly directed to those codons efficiently translated by the most abundant tRNA species [42]. The CAI value for *STE13ca* was 0.239, which when was compared with values of already reported genes [25], was placing among the low-expression genes.

Analysis of the hydrophobicity profile and the prediction of transmembrane regions of the predicted Ste13 protein from *C. albicans* indicate that this protein could be associated to membranes, a prediction that was proven by detecting yscDAP activity in the membrane fraction of *C. albicans* ATCC 10231. Similarly, in *S. cerevisiae*, Ste13p contains a transmembrane segment close to the amino acid terminus [33]. Analysis of the possible subcellular site indicated that this predicted protein from *C. albicans,* just as in *S. cerevisiae,* might be located in the Golgi apparatus membrane. Prediction of "motif" sequences revealed that similarly to the Ste13p from *S. cerevisiae*, the Ste13p from *C. albicans* possesses an active site of serine proteases. This predic-

tion was also confirmed since the ycaDAP membrane activity was inhibited by the presence of PMSF, an inhibitor of serine proteases (data not shown).

As can be observed in the tree showing the similarity relations between the amino acid sequences of proteins with DAP activity of diverse organisms, the ORF deduced from the gene *STE13ca* showed a high similarity with the amino acid sequence of DAP A, from *S. cerevisiae*. As mentioned before, in *S. cerevisiae*, the function of the gene *STE13* is to process the precursor of the α sexual factor by removing the dipeptide from the end of the amino terminal of the pheromone precursor. Since, the mating process has been described in *C. albicans*, the *STE13ca* gene could perform the same function in this yeast, where a possible homologous of α sexual factor has been described [11].

We also investigated the presence of homologous genes to *STE13* in other yeasts of the *Candida* genus. In this way, gene *STE13* was PCR-amplified in *C. dubliniensis*. The Southern analysis confirmed the presence of a homologous gene to *STE13* in *C. dubliniensis* and *C. tropicalis* (data not shown). This is not surprising, since *C. dubliniensis* is a species phylogenetically related to *C. albicans* [12,43,44].

Southern analysis indicated that there is probably only one copy of the *STE13ca* gene in each *C. albicans* chromosome R, discarding the possibility of genes originated by duplication events (paralogous genes) as in the secreted aspartyl proteinases genes family (*SAP1–SAP10*) [45].

The *STE13ca* gene of *C. albicans* is located in chromosome R. This chromosome varies in its electrophoretic motility among *C. albicans* strains; in general, it runs equally, slower or faster than chromosome 1 [23]. Therefore, chromosome R was PCR identified based on the location of the *SAP2* gene [13], located in chromosome R [45,46].

We found that expression of the gene *STE13ca* is regulated by the growth phase, the nitrogen source, and the dimorphism from yeast to mycelium. The highest mRNA levels of gene *STE13ca* were encountered during the logarithmic and early stationary growth phases, in the presence of proline as sole nitrogen source, and during formation of the germinal tube. It must be mentioned that proline has been classified as a hard to assimilate nitrogen source, and has been used as an inductor of nutritional stress [31,47]. The increase in gene *STE13ca* expression could be related with several stress conditions.

In early stationary phase, it was detected high ycaDAP activity in YNB + peptone medium, although no mRNA was produced for the same conditions, perhaps the measured ycaDAP activity corresponds to the protein encoded by *DAP2ca* gene that is able to attack Ala-pro-4-NA substrate. *C. albicans DAP2ca* gene was identified by similarity to the *S. cerevisiae DAP2* gene and it is expressed in early stationary phase in YNB + peptone medium, it is not expressed in YNB + proline, and *DAP2* mRNA does not increase during dimorphism (data not shown).

Aimed at assigning a possible function to the product of gene *STE13ca* from *C. albicans*, we decided to analyze the amino acids sequence of the peptide proposed as α sexual factor, encoded by the *MFα1* gene. The α-sexual factor of *S. cerevisiae* contains four equally spaced repeats of the peptidic sequence of the mature α-sexual factor, this protein is processed and the four peptides are released [48]. The first three are flanked in their carboxyl terminus by spacing peptides, each one starting with the processing site by protease Kex2 KR (Lys-Arg)$_1$, followed by the processing sites for protease Ste13p EA (Glu-Ala)$_2$, EA (Glu-Ala)$_3$ or -E-A-D-E-A-sequence or (-Glu-Ala-Asp-Ala-Glu-Ala-sequence). The last copy ends in an amino acid before the terminus of the deduced protein [49]. In *C. albicans*, *MFα1* encodes a precursor protein containing three equally spaced repeats of the α-pheromone sexual factor [50]. Although the number and sequence of amino acids differ between the pheromones (α-sexual factors) of *C. albicans* and *S. cerevisiae*, the general arrangement of the amino acid sequences is similar in both peptides. Fig. 5 depicts the possible cutting sites proposed here of the DAP activity of *C. albicans* for the maturation of this factor. Future studies will allow testing this hypothesis. On the other hand, disruption in *KEX2* (another gene involved in processing the *S. cerevisiae*

a *Saccharomyces cerevisiae MFa1*

```
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIP   34
AEAVIGYLDLEGDFDVAVLPFSNSTNNGLLFIN   67
TTIASIAAKEEGVSLDKREAEAWHWLQLKPGQ   99
PMYKREAEAEAWHWLQLKPGQPMYKREADA 129
EAWHWLQLKPGQPMYKREADAEAWHWLQL 158
KPGQPMY                             165
```

Kex1 Kex2 Ste13 Ste13 Ste13

b *Candida albicans MFa1*

```
MKFSLTLLTATIATIVAAAPAQYTGQAIDSNQVVE  35
IPESAVEAYFPIDDELTPVFGEIDNKPVILIVNGTTL  72
TSGANNEKREAKSKGGFRLTNFGYFEPGKRDAN 105
ADAGFRLTNFGYFEPGKRDANAEAGFRLTNFGY 138
FEPGK                                143
```

Kex2 Ste13 Ste13 Ste13

Fig. 5. Potential maturation sites of α-pheromone by protease Ste13 from *C. albicans*. Deduced amino acid sequence of *MFα1* gene from (a) *S. cerevisiae*, and (b) *C. albicans*. The *C. albicans* α-pheromone contains three equally spaced sequences encoding α-pheromone peptides: two encoding 13-mers and one encoding a 14-mer. The arrows indicate sites of α-pheromone maturation by Kex1, Kex2 and Ste13 in *S. cerevisiae*, and potential maturation sites of α-pheromone by Ste13 in *C. albicans*.

pheromone Mfα) prevented mating in *MTL*α but not in *MTLa* cells [51] and diminished *C. albicans* virulence [52]. Therefore, the functional complementation of a *ste13*Δ mutation in *S. cerevisiae* with *STE13ca* and/or disruption in *STE13ca* is very important to demonstrate the function of this gene in *C. albicans*: its possible role in α-sexual factor maturation, maturation of another proteins, as well as its role in nitrogen metabolism under nutritional stress conditions.

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