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Original Article

Transcriptional Changes in *Candida albicans* Genes by Both Farnesol and High Cell Density at an Early Stage of Morphogenesis in *N*-acetyl-D-glucosamine Medium

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Abstract

Quorum sensing through farnesol, a quorum sensing molecule, regulates virulence and morphogenesis in *Candida albicans*. Farnesol and high cell density of *C. albicans* repress hyphal formation in a minimal medium containing *N*-acetyl-D-glucosamine. Global transcription profiling at an early stage of quorum sensing by *C. albicans* in the *N*-acetyl-D-glucosamine medium was analyzed. Twenty-two of a total of 53 genes responded to both farnesol and high cell density. From *in silico* analysis and previous published data, nine of these genes including those encoding amino acid biosynthesis were controlled by the Gcn4p regulator. Nine other genes which included genes encoding central carbon metabolism were controlled by negative regulators including Nrg1p, Tup1p, Ssn6p, and/or Mig1p. Other genes not controlled by these regulators included genes related to oxidative stress, glucose metabolism, and agglutination. Expression of genes related to amino acid biosynthesis and central carbon metabolism in this study is similar to a previous report of transcription profiling in *C. albicans* following its internalization by phagocyte cells and adaptation to host challenges.

Key words: *Candida albicans*, farnesol, cell density, quorum sensing, morphogenesis, N-acetyl-D-glucosamine

Introduction

Candida albicans is an opportunistic fungal pathogen found in the normal flora of healthy humans. During infection, the organism forms biofilms in tissues or on indwelling catheters[1, 2]. Biofilms contain yeast, pseudohyphae, and hyphal forms. Moreover, this polymorphic transition is linked to a quorum sensing system in which hyphal growth of the organism is regulated by inoculum size. Cell density is one of several factors that influence hyphal growth[3]. In 2001, a quorum sensing molecule of *C. albicans* (farnesol: $C_{15}H_{26}O$) was identified[4]. Farnesol is a metabolic product of mevaronate/sterol synthesis in eukaryotes[5]. Generally this molecule functions endogenously; however, in *C. albicans*, farnesol acts in an autocrine manner. It is not clear how farnesol regulates virulence and the yeast-to-hyphal transition in *C. albicans*.

Two laboratories have demonstrated different expression patterns in *C. albicans* treated with

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farnesol. Farnesol accumulates in a growth medium of stationary-phase cells in *C. albicans*[4]. Enjalbert and Whiteway[6] focused on hyphal repression in stationary-phase cells in buffered yeast extract-peptone-dextrose (YPD) growth medium to investigate the relationship between quorum sensing molecules and hyphal growth. They analyzed transcription profiling of stationary-phase cells at several time points in fresh YPD medium with/without farnesol at 30°C or 37°C. They reported that farnesol delayed and reduced the induction of hypha-related genes and enhanced the expression of genes encoding drug efflux components. The other laboratory studied gene expression in biofilm exposed to farnesol at 37°C for 24h in RPMI 1640 medium in *C. albicans*[7]. Their results showed that especially farnesol enhanced *TUP1* expression and reduced *CSH1* (cell surface hydrophobicity) expression.

During the initial stages of morphological transition in *C. albicans*, the organism has the capacity to resume growth either as budding yeast or as hyphae[8]. Mosel *et al.*[9] reported that a limited time point (an early stage) of the yeast-to-hyphal conversion is linked to quorum sensing system. We previously reported a minimal liquid medium (*N*-acetyl-D-glucosamine: GlcNAc medium) in order to analyze the mechanism of the yeast-to-hyphal conversion[10]. In this paper, we used the GlcNAc medium to understand the mechanism by which during early growth quorum sensing connects to the morphogenetic switch in *C. albicans*.

We focused here on global transcription profiling of cells in the initial stage in the GlcNAc medium to which farnesol was added or in which the medium was inoculated at a high cell density. Transcription profiling revealed that *C. albicans* genes were mainly regulated by two types of regulators: Gcn4p or Mig1p, Tup1p, Nrg1p, and/or Ssn6 (except for unknown genes). These regulated genes are required for amino acid biosynthesis or central carbon metabolism functions.

Material and Methods

Strain and growth conditions

C. albicans strain JCM 9061 (serotype B) was maintained as frozen glycerol stocks at −80°C. Cultures of the organism were grown on yeast extract-peptone-dextrose (YPD) (1% yeast extract, 2% Bacto peptone, 2% glucose) agar plates at 37°C for 24 h. Cell suspension in a YPD broth medium was adjusted from a colony on a YPD plate, then inoculated at 6×10^3 cells into 300-m*l* of YPD broth medium. Cells were cultured at 37°C with orbital shaking (150 rpm) for approximately 17.5 h to logarithmic phase ($1-3\times10^5$ cells ml^{-1}).

Germination studies

Germ tubes were induced in GlcNAc medium (L-thiazolidine-4-carboxylic acid, L-proline, sodium bicarbonate, sodium phosphate monobasic, sodium acetate, and N-acetyl-D-glucosamine, pH 6.65)[10]. The final concentration of each medium component was 0.01%. Trans-trans farnesol (Sigma-Aldrich, Missouri, USA) was prepared as a 25 mM stock solution in dimethyl sulfoxide under nitrogen, and then diluted to obtain a final concentration of 25 μM in the medium. Logarithmic phase cells that were counted in a Burker-Turk counting chamber were collected by filtration on cellulose acetate membranes (0.8 μm pore size) *in vacuo*. The filters were transferred to 50-m*l* beakers (15-m*l*), 1-L beakers (70-m*l*), or about 30 cm diameter Petri-dishes (600-m*l*) containing the GlcNAc medium needed. Cultures for determining the % of cells with germ tubes were grown at 37°C for 180 min without shaking, but for extracting total RNA, cells were incubated at 37°C for only 30 min without shaking. The germination of strain JCM 9061 cells (3×10^4 cells ml^{-1}) was regulated by a lower inoculum size than strain CAI4 (5×10^5 cells ml^{-1})[11].

RNA extraction

Total RNA was extracted from cells either grown to logarithmic phase in YPD medium at 37°C or incubated in GlcNAc medium at 37°C for 30 min without shaking. All cultures were quickly collected by filtration on cellulose acetate membranes *in vacuo*, then quick-frozen and stored at −80°C. Total RNA was extracted using the RNeasy Mini (QIAGEN GmbH, Germany) according to the kit instructions. Cells on the frozen membrane filters were transferred to microtubes containing 0.3 g of zirconia/silica disruption beads and the first solution of the kit, and then shaken vigorously in a Disruptor Genie (Scientific Industries, Inc., NY, USA) for 5×45 s with periodic chilling on ice-water.

DNA microarray hybridization and data analysis

The 6,165 *C. albicans* mRNA sequences from the National Center for Biotechnology Information (NCBI) were used to design probe sets on the oligo-based microarrays. The probe sets consisted of 15 pairs of 24 mer perfect match and mismatch probes per gene. The probes were

synthesized on a glass slide, using a Maskless Array Synthesizer that was manufactured by NimbleGen Systems Inc. (USA). The glass slides were SuperClean slides from TeleChem International Inc. The Superscript Choice system (Invitrogen, Tokyo, Japan) was used for double-stranded cDNA synthesis, and then cRNA samples with biotinylated nucleotides were synthesized from cDNA templates using T7 RNA polymerase with T7 promoter oligo-dT primers. The biotinylated cRNA samples were fragmented to an average of 200 bases with 5× 200 mM Tris-acetate, pH 8.1, 500 mM potassium acetate and 150 mM magnesium acetate tetrahydrate solution at 95°C for 35 min. The fragmented cRNA samples were applied to the glass slides and then the hybridization was performed with NimbleGen's Hybriwheel Hybridization chambers at 45°C for 16-20 h. The DNA microarray slides were scanned with an Axon 4000B 5-μm scanner (Molecular Devices Corp. CA, USA). The scanned images were initially captured with Axon GenePix 4.0 and then analyzed using NimbleScan Software (NimbleGen Systems Inc., USA). The expression data from the arrays was normalized using the Robust Multi-chip Analysis algorithm[12] and filtered to remove any genes with expression that did not indicate more than 3-fold increase relative to that of logarithmic phase yeast cells. The quality of the arrays was examined by performing two independent hybridizations for the logarithmic phase yeast cells. Replicate hybridizations showed correlation coefficients of $>$0.9979.

Results and Discussion

Quorum sensing during germination of *C. albicans* strain JCM 9061

The effects of farnesol and cell density on the germination of strain JCM 9061 in GlcNAc medium at 37°C for 3 h are shown in Fig. 1. When the inoculum size of cells was 3×10^4 cells ml^{-1}, about 75% of the cells germinated. However, 25 μM farnesol completely inhibited germination at this inoculum density. When the inoculum was increased to 3×10^5 cells ml^{-1}, 3×10^6 cells ml^{-1}, or 3×10^7 cells ml^{-1}, germination was reduced to less than 20%. Our GlcNAc medium does not include serum, as the concentration of farnesol was enough to block germination. Mosel *et al.*[9] reported that the concentration of farnesol needed to reduce germination to 50% was ~1 μM for strains SC5314 and A72. However, at such a low concentration of farnesol, a high percentage of the cells finally formed germ tubes (data not shown), but we needed a higher concentration of farnesol to completely block germination. Strain JCM 9061 germinated in GlcNAc medium at a lower inoculum than strain CAI4[11]. Even at an inoculum of 5×10^5 cells/m*l*, strain JCM 9061 cells budded instead.

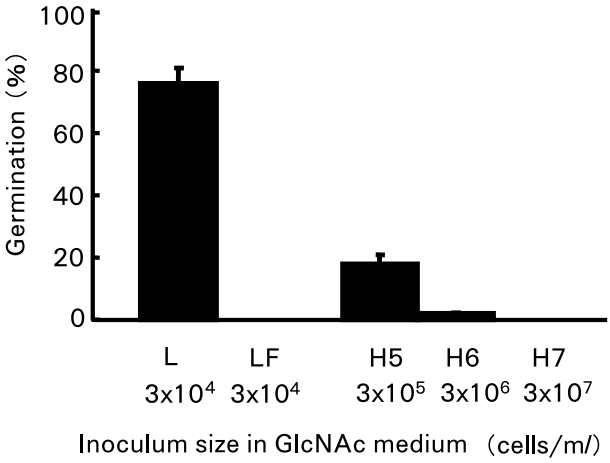

Fig. 1 Effect of farnesol and high cell density on germination of *C. albicans* JCM 9061 in N-acetyl-D-glucosamine (GlcNAc) medium.
C. albicans JCM 9061 was grown to logarithmic-phase in a YPD medium at 37°C. The cells were transferred into the GlcNAc medium with/without farnesol (25 μM), then cultured for 3 hrs at 37°C. L, H5, H6, and H7, represent inocula of 30×10^4, 3×10^5, 3×10^6, and 3×10^7 cells/m*l*, respectively; the LF inoculum was 3×10^4 cells/m*l* in the medium with farnesol. Percentages of germination are expressed as the means ± standard deviations of three independent replicates.

Genes regulated by farnesol and high cell density

We previously focused on genes transcribed during the early stage of the yeast-to-hyphal transition in *C. albicans*; 2 genes were identified, including *CGR1* and *MSI3*. *CGR1* gene expression increased when the growth of logarithmic phase cells was stopped by keeping cells in water for 20 min at 37°C[13]. Growth cessation of logarithmic phase cells induced a high percentage of germination in the glucose medium[14]. *MSI3* was identified as a gene encoding an interaction protein with Cgr1p by the yeast-two hybrid method[15]. We focused on the initial stages of quorum sensing which links yeast-to-hyphal transition in *C. albicans*[4] and provides a system to study the regulation of the morphological transition. In this paper, expression of common genes in both farnesol and high cell density growth was analyzed using DNA microarrays.

In Fig. 2, the number of genes with more than a 3-fold increase relative to gene expression in logarithmic-phase yeast cells in each experiment is shown using a Venn diagram. Fifty-three genes

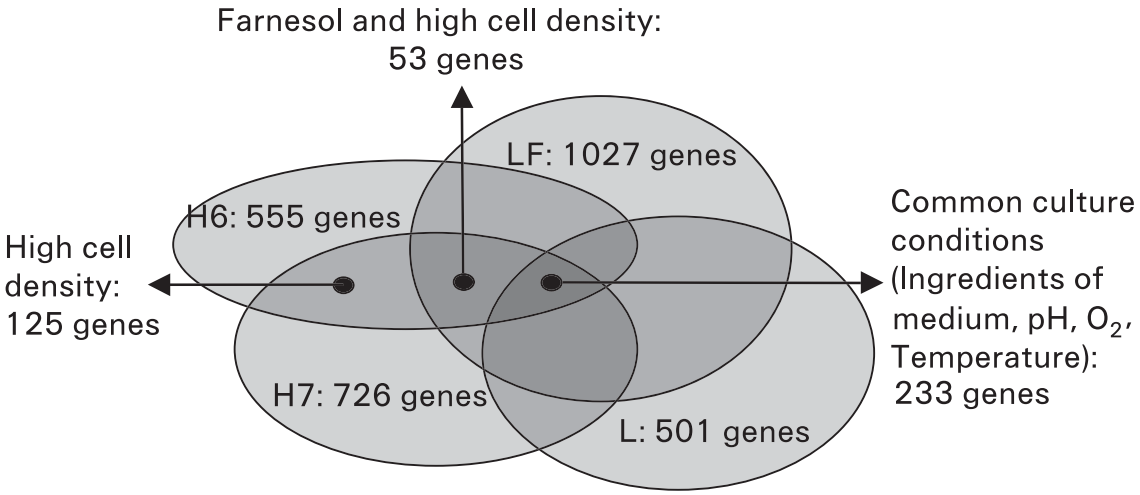

Fig. 2. A Venn diagram showing numbers of *C. albicans* genes that were up-regulated over three-fold in cells treated with farnesol (25 μM) and grown at a high cell density at 30 min in GlcNAc medium, relative to the logarithmic phase cells. Abbreviations are the same as in Fig. 1.

Table 1. Categorization of the genes controlled by regulators

	Orf. 19	Name	Function	Regulator
A	56	*ARG2*	Ornithine biosynthesis	Gcn4p*
	4788	*ARG5, 6*	Ornithine biosynthesis	Gcn4p, Tup1p*
	183	*HIS3*	Histidine biosynthesis	Gcn4p, Gcn2p
	3099	*TRP4*	Tryptophane biosynthesis	Gcn4p, Gcn2p
	1613	*ILV2*	Valine biosynthesis	Gcn4p
	88	*ILV5*	Valine biosynthesis	Gcn4p
	1789	*LYS1*	Lysine biosynthesis	Gcn4p*
	3912	IPF10021 (SGD nema: *GLN1*)	Nitrogen metabolism	Gcn4p, Gcn2p
	882	*HSP78*	Mitochondrial translocation/ Protein folding/ Response to stress	Gcn4p, Gcn2p, Nrg1p, Mig1p
B	744	IPF9740 (SGD nema: *GDB1*)	Glycogen catabolism	Nrg1p, Tup1p
	6178	*FBP1*	GIuconeogenesis	Ssn6p, Efg1p
	1743	*ACS1*	Acetyl-CoA biosynthesis	Nrg1p, Mig1p, Ssn6p
	3733	*IDP2*	Glutamate biosynthesis/ Isocitrate metabolism	Ssn6p
	7481	*MDH11*	Malate metabolism/ TCA cycle	Tup1p, Mig1p
	2437	*ARC35*	Cell growth, maintenance	Ssn6p
	2722	*CGR1*	Senescence marker protein	Nrg1p*
	4788	*ARG5, 6*	Ornithine biosynthesis	Tup1p*, Gcn4p
	882	*HSP78*	Mitochondrial translocation/ Protein folding/ Response to stress	Nrg1p, Mig1p, Gcn4p, Gcn2p
C	5736	*ALS5*	Agglutination	
	6511	*TRL1*	tRNA splicing	
	4235	*CNA1*	cAMP-mediated signaling	
	2747	*RGT1*	Glucose metabolism	
	3308	*STB5*	GIycolysis regulation/ Oxidative stress	
	6848	IPF3518 (SGD name: *VPS16*)	Vacuole organization	

The functions were noted by annotation of Assembly 20. A group of genes are regulated by Gcn4p. B group genes are regulated by Mig1p, Nrg1p, Ssn6p, and/or Tup1p. C group genes are not classified by these transcription factors. Regulators with an asterisk are from published data [16, 17], while regulators without an asterisk are from annotation of Assembly 20.

were selected that responded to both farnesol and high cell density. Genes (233) expressed in all samples responded to culture conditions including components of GlcNAc medium, pH, O_2, and temperature. Fifty-three genes were categorized by annotation of Assembly 20 and information from other papers[16, 17]. Probe sets for a microarray were designed by using Assembly 19; however, three genes were deleted from Assembly 20.

Twenty-two known genes of a total of 53 responded to both farnesol and high cell density. The total number of genes controlled by Gcn4p was 9 (Table 1A). Genes controlled by Gcn4 were related to amino acid biosynthesis (*ARG2*, *ARG5, 6*, *HIS3*, *TRP4*, *ILV2*, *ILV5*, *LYS1*), and nitrogen metabolism (IPF10021: *GLN3*). *C. albicans GCN4* isolated by Tripathi *et al.*[18] is a functional homologue of *Saccharomyces cerevisiae* which regulates control of general amino acid biosynthesis during starvation for any one of several amino acids[16]. Gcn4p is degraded in a rich medium, but is stabilized under amino acid starvation conditions[19]. The GlcNAc medium used in this study is not a rich medium. The expressions of *GCN4* gene were greater than 3-fold compared to logarithmic phase cells in all tests of L, LF, H6, and H7 (abbreviations indicated in Fig. 1). Therefore, *GCN4* appears to be activated by amino acid starvation in the GlcNAc medium and results in the expression of genes related to amino acid biosynthesis. However, greater stimulation of the genes shown in Table 1A was achieved with farnesol or high cell density. The genes related to amino acid biosynthesis are shown in Fig. 3. IPF10021

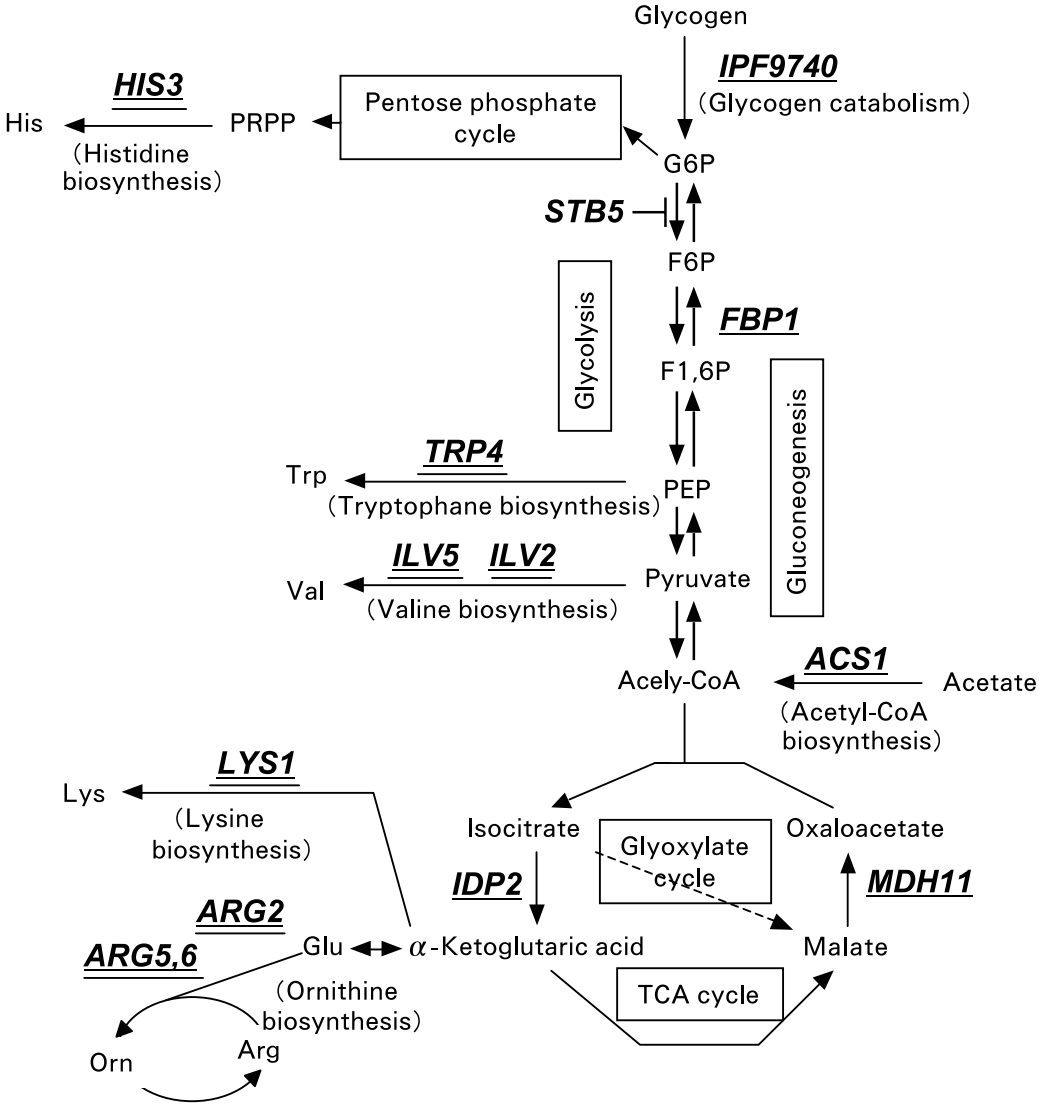

Fig. 3. Schematic drawing of amino acid biosynthesis and central carbon metabolism with the *C. albicans* genes selected in Table 1. The genes were selected that respond to both farnesol and high cell density. The double underlined genes were regulated by Gcn4p. The genes represented with an underline were regulated by negative regulators including Mig1p, Nrg1p, Ssn6p, and/or Tup1p. All other genes shown (*STB5*) were not similarly regulated.

(SGD: *GLN3*) in *C. albicans* encodes a GATA transcription factor of the ammonium permease (Mep2p)[20]. Also, Gln3p acts as a regulator of nitrogen starvation-induced filamentous growth[20]. *HSP78* (encoding heat shock protein) and *ARG5, 6* are controlled by both Gcn4p and negative repressors (Table 1A & 1B).

A total of 9 genes were regulated by repressors including Mig1p, Nrg1p, Ssn6p, and /or Tup1p (Table 1B). Five of the nine genes were related to central carbon metabolism (IPF9740: *GDB1*, *FBP1*, *ACS1*, *IDP2*, *MDH11*), illustrated in Fig. 3. Over 15% of the Tup1-, Mig1-, and Nrg1-regulated gene sets in *C. albicans* control genes involved in metabolism[17]. These 5 genes are not glycolysis related genes that are activated by Efg1p[21]. Although *PCK1* involved in gluconeogenesis was not among those in Table 1, its expression was more stimulated in LF and H7 than L. IPF9740 (SGD: *GDB1*) is a gene involved in glycogen metabolism that is expressed in response to stress and growth conditions of *S. cerevisiae*[22]. *ARC35*, which is regulated by Ssn6, is a gene required late in G1 for its cell cycle function in *S. cerevisiae*[23]. Arc35p functions with calmodulin (a major calcium sensor) in the organization of the actin and microtubule cytoskeleton[24]. The *CGR1* that we identified at the early stage of morphogenesis in *C. albicans*[13] was interestingly selected here. Six genes were not classified by those transcription factors (Table 1C). The *ALS5* encoded cell surface glycoprotein of *C. albicans* binds to mammalian ligands and functions fungal cell-cell interactions[25, 26]. *TRL1* in *S. cerevisiae* encodes a yeast tRNA ligase that executes the end-healing and end-sealing steps of tRNA splicing[27]. *CNA1* (SGD: *PDE1*) in *C. albicans* is a gene encoding a low-affinity cyclic AMP (cAMP) phosphodiesterase[28]. *PDE1* in *C. albicans* is less well characterized, whereas *PDE2* is involved in hypha production, nutrient sensing, and entry into stationary phase[29]. The *PDE1* gene in *S. cerevisiae* functions in controlling cAMP signaling induced by glucose and intracellular acidification[30]. *RGT1* in *C. albicans* represses expression of several *HXT* (encoding high-affinity glucose transporters) in the absence of glucose[31, 32]. The *C. albicans* glucose-sensors including Rgt1 are involved in growth on fermentable carbon sources such as glucose and filamentation[31, 32]. Rgt1 in *S. cerevisiae* is phosphorylated by the cAMP-dependent protein kinase A[33]. Genes related to the cAMP phosphodiesterase and the glucose-sensors are included in Fig. 4. *STB5* in *S. cerevisiae* encodes one of the zinc cluster activators of multi-drug resistance genes[34]. Stb5 is an activator of genes involved in NADPH production for oxidative stress resistance[35]. Moreover, its other

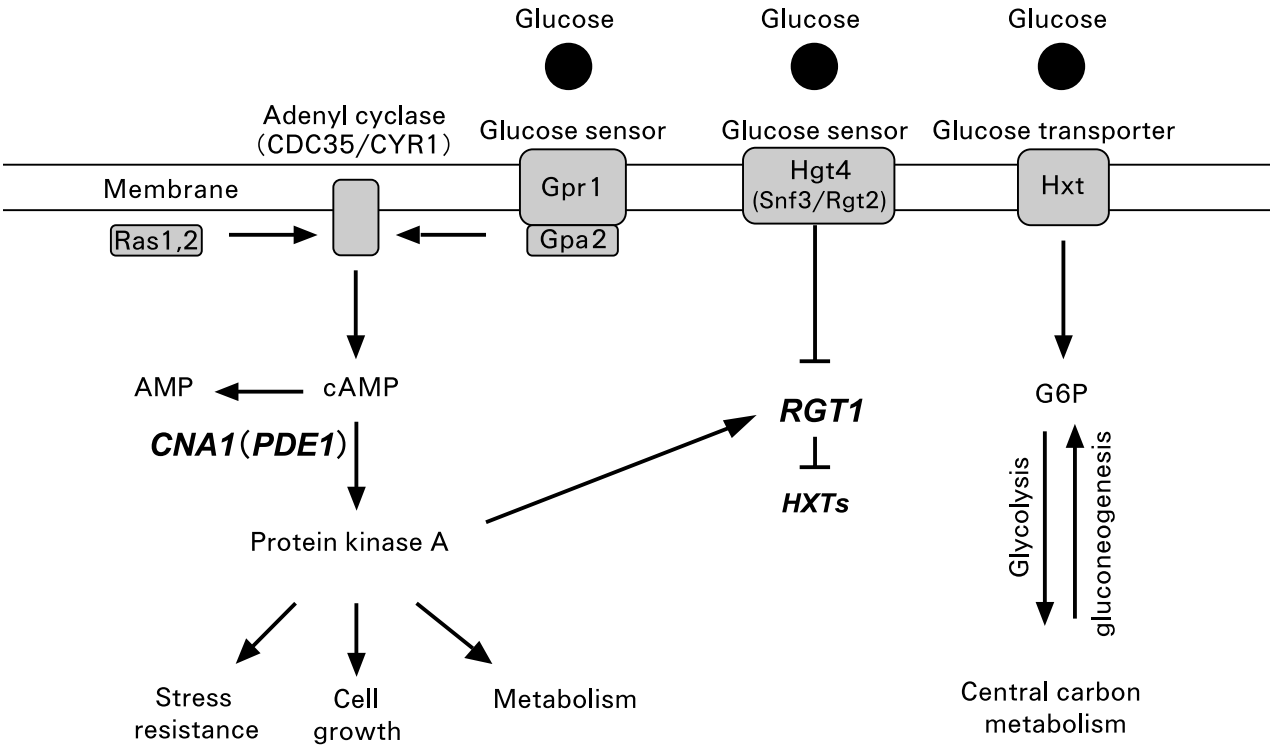

Fig. 4. Schematic drawing of the cAMP signaling and glucose sensing pathways with *CNA1*(*PDE1*) and *RGT1* selected from Table 1. The pathways were illustrated based on the previous information of *S. cerevisiae* and/or *C. albicans*[28–33].

function is a repressor of genes related to the normal glycolysis pathway[35]. IPF3518 (SGD: *VPS16*) is a gene belonging to the class C vacuolar protein sorting that is related to vacuole biogenesis in *S. cerevisiae*[36, 37], although there are three classes (A,B, and C) of vacuole proteins in the organism. Incidentally, the class A genes have a role in vacuolar pH in protein sorting and the class B genes are related to a fragmented vacuole morphology[38]. In *C. albicans*, the class C *VPS11* was investigated and reported to be required for vacuole biogenesis and germ tube formation[39].

Two laboratories have reported the affect of farnesol on hyphal growth or on biofilm formation using DNA microarray analysis[6, 7]. Our data are much different from these results, perhaps because of strain differences, the growth phase of cells and temperature, the medium for germination, the time of incubation in farnesol, or inoculum size. Nickerson *et al.*[40] pointed out that the condition of cells exposed to farnesol is especially important in establishing a farnesol function. Our results instead show a pattern similar to previous transcription profiling in *C. albicans* using genome microarray following internalization of the organism by phagocyte cells[41-44]. From the results of co-cultures of phagocytes and fungal cells, Rubin-Bejerano *et al.*[41] suggested that the human neutrophil phagosome is amino acid-deficient, and *S. cerevisiae* and *C. albicans* phagocytosed by human neutrophils respond by inducing genes of the methionine and arginine pathways. Lorenz *et al.*[42] reported that *C. albicans* cells phagocytosed by cultured-macrophages shifted to a starvation mode including gluconeogenic growth as the first step, then resumed glycolytic growth to escape from the macrophage by forming hyphae. Also, they reported that there were oxidative stress responses in the early phase of internalization by macrophages[42]. Fradin *et al.*[43] also confirmed that the presence of human neutrophils arrested *C. albicans* growth, stimulated gene expression to overcome nitrogen and carbohydrate starvation, and induced the expression of genes involved in the oxidative stress response. Barelle *et al.*[44] also confirmed that *C. albicans* cells induced genes involved in gluconeogenesis and the glyoxylate cycle at an early phase after phagocytosis but did not induce glycolytic genes following phagocytosis. Those transcription profiles suggested that the human neutrophil phagosomes are glucose- and amino acid-deficient, and exert an oxidative stress response in the phagocytized organism. Moreover, Lorenz *et al.*[42] suggested that the early response of *C. albicans* during phagocytosis by macrophages contributes to survival, not to the morphogenetic switch from the yeast form to the hyphal form, since activation of glycolysis occurred in the late response in which *C. albicans* showed the morphogenetic response[42]. On the other hand, our GlcNAc medium lacks glucose and has very few amino acids. Expression of genes involved in gluconeogenesis and amino acid/nitrogen metabolism would reflect a starvation response to the nutrient-poor medium. However, farnesol and high cell density still increased the expression of genes related to starvation (Table 1A & 1B). In a previous paper[6], farnesol delayed the expression of hypha-specific genes. Therefore, we speculate that addition of farnesol and a high cell density inoculum in GlcNAc medium would temporally prolong starvation conditions for the organism and its adaptation to environmental changes; a consequence of this is the postponement of the morphogenetic switch. However, it is not known yet about overlapping mechanisms of the initial quorum sensing system and the morphogenetic switch in *C. albicans*. Our speculation could be confirmed with more array analyses of mRNA at other stages during quorum sensing.

Acknowledgements

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