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ORIGINAL ARTICLE

The Goldilocks effect of respiration on canavanine tolerance in *Saccharomyces cerevisiae*

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Abstract

When glucose is available, *Saccharomyces cerevisiae* prefers fermentation to respiration. In fact, it can live without respiration at all. Here, we study the role of respiration in stress tolerance in yeast. We found that colony growth of respiratory-deficient yeast (petite) is greatly inhibited by canavanine, the toxic analog of arginine that causes proteotoxic stress. We found lower amounts of the amino acids involved in arginine biosynthesis in petites compared with WT. This finding may be explained by the fact that petite cells exposed to canavanine show reduction in the efficiency of targeting of proteins required for arginine biosynthesis. The retrograde (RTG) pathway signals mitochondrial stress. It positively controls production of arginine precursors. We show that canavanine abrogates RTG signaling especially in petite cells, and mutants in the RTG pathway are extremely sensitive to canavanine. We suggest that petite cells are naturally ineffective in production of some amino acids; combination of this fact with the effect of canavanine on the RTG pathway is the simplest explanation why petite cells are inhibited by canavanine. Surprisingly, we found that canavanine greatly inhibits colony formation when WT cells are forced to respire. Our research proposes a novel connection between respiration and proteotoxic stress.

Keywords Respiration · Mitochondria · Arginine biosynthesis · Yeast · Canavanine · Petite · Amino acids

Introduction

In eukaryotic cells, the citric acid cycle generates NADH and $FADH_2$, electron carriers used in oxidative phosphorylation of the electron transport chain (ETC) to create ATP, a process collectively known as respiration (Woelders 1989). This occurs in the mitochondrial matrix and inner mitochondrial membrane (Larosa and Remacle 2018). While mitochondria are well known for their role in ATP production and regeneration of reducing power via respiration, this work aims to contribute to the growing body of research on the importance of respiration outside of energy production (Jambhekar and Amon 2008; Knupp et al. 2019; Peng et al. 2012; Vlahakis et al. 2017).

Saccharomyces cerevisiae, the common baker's yeast, is a valuable tool for exploring the alternate roles of respiration due to its ease of genetic manipulation and versatile metabolism. *S. cerevisiae* is able to respire yet prefers to create ATP through glycolysis even under aerobic conditions (de Alteriis et al. 2018; de Deken 1966). In fact, in the presence of sufficient glucose, transcription of genes involved in respiration and gluconeogenesis are repressed (Carlson 1999; Gancedo 1998). Furthermore, yeasts with mutations in proteins of the ETC are able to survive despite their inability to respire, a class of mutants known as petites due to their small colony size (a result of reduced ATP production) (Chen and Clark-Walker 2000; Day 2013), yet yeasts cannot survive without mitochondria (Contamine and Picard 2000; Evans and Neuman 2016).

Besides respiration, mitochondria carry out a variety of essential cellular tasks, including cell signaling, amino acid biosynthesis (Jauniaux et al. 1978;

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Zelenaya-Troitskaya et al. 1995), and nitrogen metabolism (Evans and Neuman 2016; Magasanik and Kaiser 2002). The amino acids partially or completely synthesized in the mitochondria are arginine, ornithine, proline, citrulline, and glutamate, which can be easily converted to glutamine. Glutamate and glutamine are the sole nitrogen source for all biosynthetic reactions (Guillamon et al. 2001; Magasanik and Kaiser 2002). Generation of these two amino acids is proposed to be one of the primary functions of the retrograde pathway (Liu and Butow 2006).

The retrograde (RTG) pathway is a feedback mechanism wherein mitochondria communicate with the nucleus under both normal and stressful conditions (Chelstowska and Butow 1995; Liao and Butow 1993; Liu and Butow 1999). Although mitochondria have their own DNA (mtDNA), mtDNA encodes for a small number of proteins (compared to nuclear DNA), all of which encode for translational machinery and components of the ETC (Anderson et al. 1981). However, because the mitochondria are responsible for a variety of cellular tasks that depend on input from the nuclear genome, they must be able to alert the nucleus when a metabolic shift or protein under nuclear control is needed. Genome-wide transcriptional profiling of yeast cells undergoing mitochondrial dysfunction has identified key players involved in the RTG response (Wodicka et al. 1997). *DLD3* and *CIT2* are two genes under the regulation of the RTG pathway. In petites, these genes have elevated expression levels (Burns et al. 1994; Chelstowska et al. 1999; Liao et al. 1991). *DLD3* converts D-2-hydroxyglutarate (D-2HG) to α-ketoglutarate, a citric acid cycle intermediate, with simultaneous reduction of pyruvate to D-lactate (Becker-Kettern et al. 2016). *CIT2* encodes for citrate synthase, a peroxisomal isozyme that catalyzes the production of citrate, another citric acid cycle intermediate occurring upstream of α-ketoglutarate, from acetyl-coA and oxaloacetate (Lewin et al. 1990). α-Ketoglutarate combines with ammonia to form glutamate. Glutamate can then form glutamine from glutamine synthetase and ammonia.

We use canavanine, a toxic amino acid analog of arginine, to examine mechanisms underlying proteotoxic stress in yeast. Canavanine can be mistakenly incorporated into proteins during translation, causing protein misfolding and aggregation (Rosenthal 1977). However, the cellular processes governing canavanine tolerance once it enters the cell are unknown. Through serendipity we found that cells lacking respiration capacity (petites) are highly susceptible to canavanine. By working with sublethal doses of canavanine we can elucidate the role of respiration in the tolerance of proteotoxic stress, as well as identify the genetic networks involved in tolerance to canavanine-induced toxicity. This work will demonstrate two novel effects of canavanine: (1) canavanine affects the RTG pathway, and (2) canavanine causes a reduction in the efficiency of mitochondrially targeted arginine biosynthesis proteins.

Materials and methods

Yeast strains

BY4741 was used as our WT laboratory strain. *pif1*Δ, *mgm1*Δ, *mip1*Δ, *mks1*Δ, *rtg2*Δ, and *rtg3*Δ of the BY4741 background were of the library constructed by Giaever et al. (Giaever et al. 2002). CS47, a complete arginine auxotroph, is a derivative of S288C strains with an *arg4* mutation. C-terminally tagged-GFP strains were from the library constructed at Professor Erin O'Shea's lab at UCSF (Huh et al. 2003). Arg5,6 C-terminally tagged-GFP was transformed with a plasmid expressing N-terminally tagged cherry-Tom22 under TEF2 promoter from URA Cen/Ars plasmid using the *delitto perfetto* approach (Storici and Resnick 2006). Petite strains used for microscopy were generated from fluorescently labeled parent strains exposed to ethidium bromide for 1 h in liquid YPD at 250 RPM (Goldring et al. 1970).

Media

Unless otherwise stated, all experiments with canavanine were carried out in complete synthetic media (CSM) without arginine [(−) arginine]. Similarly, experiments using thialysine were carried out on media lacking lysine. YPD solid media contains 2% Bacto Agar, 2% Bacto Peptone, 1% Bacto Yeast Extract (Difco, Sparks, MD, USA), and 2% anhydrous dextrose (Avantor Performance Materials, Center Valley, PA, USA) dissolved in DDW. All other media (complete, YPG, or media lacking a specific amino acid) contain 2% Bacto Agar, 0.67% Yeast Nitrogen Base without Amino Acids (Difco Laboratories), 0.082% Complete Supplement Mixture (no amino acid drop-out) or 0.074% Complete Supplement Mixture Drop-out: ARG or LYS (Formedium), respectively. All media are brought to pH 5.8 using NaOH pearls (Bio-Lab LTD, Jerusalem, IL, USA), autoclaved, and the desired carbon source (2% for glucose and 3% for non-fermentable carbon sources such as lactic acid, ethanol, and glycerol), is added after autoclaving. For nitrogen-starvation experiments, 0.17% Yeast Nitrogen Base lacking nitrogen and amino acids was used, with 0.067% ammonium sulfate and 0.074% Complete Supplement Mixture Drop-out: ARG (Formedium) added. For glutamate addition experiments, L-glutamic acid (Formedium) was added to autoclaved media at a final concentration of 38 mg/l and filter-sterilized. For α-ketoglutarate addition, α-ketoglutaric acid disodium salt dihydrate was added to autoclaved media at a final concentration of 10 mM and filter-sterilized. Liquid media were

prepared in the same fashion as solid media with the exclusion of Bacto Agar.

Growth assays on solid media

Using a 96-well tissue culture plate (Jet Biofil), single colony undergoes a tenfold serial dilution. If there were great discrepancies between colony sizes of various isolates, multiple colonies were used to obtain comparable cell numbers per well. Using a 6-by-8-column metal prong, approximately 1 µl from each well is pronged onto desired media. After allowing suspended cells to fully absorb into the plate, the plate is put into 30 °C and growth followed for 1–2 weeks.

Growth assays in liquid media

A single colony was put into 10 ml of media of choice in a breathable Erlenmeyer flask and grown overnight in 30 °C at 250 RPM. The following day, the culture was diluted 50-fold and OD600 was read at various time points. For colony-forming unit (CFU) counts, 100 ul was removed, diluted as needed, and 100 µl spread onto YPD medium. After 2 days, CFUs were counted.

Real-time PCR

A single colony was patched onto CSM (−) arginine or CSM (−) arginine 1 µg/ml canavanine solid media and grown for 4 days in 30 °C. Cells were removed from solid media, resuspended in DDW, and total RNA was isolated using the RNeasy kit (Qiagen) and the MasterPure™ Yeast RNA Purification Kit (Epicentre). cDNAs were synthesized with the FastQuant RT Kit (with gDNase) according to the manufacturer's protocol. Real-time PCRs were done in a 10 µl volume using the Absolute quantitative PCR SYBR Green mix (Sigma) in a 96-well plate. Triplicates for each sample were included for each reaction. The real-time PCR primers used are forward 5′–3′ primer for *ACT1*: TCCAAG CCGTTTTGTCCTTG; reverse 5′–3′ primer for *ACT1*: AAG ATTGAGCAGCGGTTTGC; forward 5′–3′ primer for *CIT2*: CGGAACTACCTAGTCATGTCGTTCA; reverse 5′–3′ primer for *CIT2*: CATCCTTAGAACCAATCAAGTTGA CCAG; forward 5′–3′ primer for *DLD3*: ACGTCAGGGTCC AATAAGAGACAC; reverse 5′–3′ primer for *DLD3*: CAA ACCGGCTGCGTTTAATCTCTC; forward 5′–3′ primer for *ACO1*: AGTAACTGCGTTCGCCATTG; reverse 5′–3′ primer for *ACO1*: AGGCAAACCATCACCATGTG; forward 5′–3′ primer for *IDH1*: TTGTCGACAATGCCTCCA TG; reverse 5′–3′ primer for *IDH1*: TCAAAGCAGCGC CAATGTTG; forward 5′–3′ primer for *IDH2*: TTGCCG GTCAAGATAAAGCG; reverse 5′–3′ primer for *IDH2*: TGT TTTCTGGACCTGATGCG; forward 5′–3′ primer for *CIT1*: TGGTCGTGCCAATCAAGAAG; reverse 5′–3′ primer for *CIT1*: AAAACCGCATGGCCATAACC. *ACT1* was used as the internal control, with RNA levels of the genes of interest normalized to *ACT1* levels. PCRs were initiated at 95 °C for 20 s in the holding stage. The cycling stage consisted of 3 s at 95 °C, 30 s at 60 °C, and 15 s at 95 °C, repeated for 40 cycles. Melt curve stage consisted of 15 s at 95 °C followed by 1 min at 60 °C and finally 15 s at 95 °C.

Microscopy

For each strain, a single colony was put into glucose (−) arginine media for 3 h, then divided evenly into two separate cultures with one culture inoculated with 1 µg/ml canavanine. Cultures grew overnight in 30 °C at 250 RPM. OD600 was read the following morning and adjusted as needed to a final OD600 of 0.6 for all samples. 50 µl of sample was placed in a glass-bottom microplate (MGB101-1-2-LG-L, Brooks). Cells were allowed to settle for 20 min, followed by three washes with 50 µl DDW. A fourth and final addition of 50 µl DDW was added for imaging of the wells in the microscope. Analysis of GFP fluorescence was done using ImageJ. Fluorescence strength and area were calculated by converting GFP images to a "Thresholded" image (thresholding method: default; threshold color: B&W; color space: HSB), followed by "Analyze Particles" [Size (pixel^2): 30-infinity; Circularity: 0.00–1.00; excluded on edges and not including holes] (https://imagej.nih.gov/ij/docs/menus/analyze.html). Reasoning that strong localization to the mitochondria results in a smaller area and poor localization results in a larger area, density was calculated as fluorescence/area.

Membrane potential

Arg5,6 C-terminally tagged-GFP and its petite derivative were grown overnight as described above in *Microscopy* methods with OD600 adjusted to 0.6 the following day. 100 µl of cell suspension was inoculated with 1 µM TMRE (ab113852, Abcam) for a final concentration of 100 nM TMRE. Each sample was incubated for 20 min at 37 °C followed by two washes with 100 µl PBS; cells were then resuspended in 100 µl PBS. 50 µl of sample were placed in a glass-bottom microplate (MGB101-1-2-LG-L, Brooks). Cells were allowed to settle for 20 min, followed by a wash with 50 µl DDW. A final addition of 50 µl DDW was added for imaging of the wells in the microscope.

Hot methanol extraction of amino acids

A single colony of yeast was grown overnight in 5 ml of liquid media at 30 °C shaking in a 50-ml test tube. After overnight growth, 100 ml of sample is diluted and spread onto YPD to count cells. The remaining suspended cells are centrifuged at 3000 RPM for 3 min (Eppendorf Centrifuge

5810 R). The supernatant is disposed of and the remaining pellet washed three times with 3 ml of DDW via vortex, followed by centrifugation at 3000 RPM for 3 min, followed by removal of the supernatant. The remaining pellet is suspended in 1 ml DDW in a 1.5-ml Eppendorf tube and spun down at 14,000 RPM (Eppendorf Centrifuge 5418) for 2 min, followed by removal of supernatant with a pipette. 1 ml of 66% methanol was added to each sample and put into a hot water bath of 70 °C for 30 min with occasional vortexing. Sample is then spun down for 10 min at 14,000 RPM and the supernatant saved and stored at − 20 °C until analysis was done at the Interdepartmental Equipment Unit of The Robert H. Smith Faculty of Agriculture, Food and Environment.

Amino acid measurement

Free amino acid concentrations were determined using LC–MS/MS method. Samples were analyzed on LC–MS system which consisted of Dionex Ultimate 3000 RS HPLC coupled to Q Exactive Plus hybrid FT mass spectrometer equipped with heated electrospray ionization source (Thermo Fisher Scientific Inc.). The HPLC separation of compounds was carried out on Acclaim C18 column (2.1 × 150 mm, particle size 2.2 µm, Dionex) employing linear gradient of acetonitrile/water (with 1.5% acetic acid) binary solvent composition. The mass spectrometer was operated in positive ionization mode, ion source parameters were as follows: spray voltage 3 kV, capillary temperature 300 °C, ESI capillary temp. 300 °C, sheath gas rate (arb) 35, and auxiliary gas rate (arb) 10. Mass spectra were acquired in "full MS"—"all ions fragmentation" at resolving power 70,000. The LC–MS system was controlled and data were analyzed using Xcalibur and TraceFinder software, respectively (Thermo Fisher Scientific Inc.).

Sample preparation An aliquot of unknown sample methanolic solution (10 or 50 µl depending on concentration) was spiked with 10 µl of labeled amino acid internal standard mix (0.2 µg of all labeled amino acids in 10 µl) following addition of 600 µl borate buffer (0.05 M, pH 11). Ethyl carbamates of amino acids were prepared by addition of 30 µl ethylchloroformate solution in acetonitrile (1:9) to the buffer. We allowed 10–15 min for carbamates to form. The buffer is acidified with 30% HCl to pH 1–2 following extraction of amino acid derivatives with 800 µl ethyl acetate. Aqueous and organic phases are separated by centrifugation and the upper, organic phase is accurately transferred to HPLC vial. Ethyl acetate is gently evaporated upon a stream of nitrogen, residue is dissolved in 1 ml of acetonitrile/water/acetic acid (20:79:1) and filtered through 0.2-µm RC membrane filter (regenerated cellulose) prior to LC–MS analysis.

Internal standards (*IS*) The solution of IS was prepared in 0.1 N HCl from "cell free" labeled amino acid mix (20 AA, ^{13}C, ^{15}N) purchased from Cambridge Isotope (P/N CNLM-6696-1) at final concentration 0.2 mg (of all amino acids)/ml. There was separately added labeled methionine (methyl-D_3, Cambridge Isotope, P/N DLM-431-1) to the IS mixture because it was not detected in the "cell free" mix.

Calibration Standards of 24 amino acids were purchased from Sigma-Aldrich. Calibration solutions of amino acids were prepared in 0.1 N HCl at concentration ranges from 1 to 2000 ng/ml. Preparation and derivatization of calibration samples were carried out as described above with a small modification; water was evaporated from 1000 to 150–50 µl volume prior to derivatization. All raw results of amino acid measurements are found in Table S1.

Results

Canavanine-induced growth inhibition of petites on solid media

By serendipity we found that respiration-deficient colonies (petites) undergo extreme growth inhibition in the presence of sublethal doses of canavanine when grown on solid media. This observation was first identified in spontaneous petite colonies; nevertheless, the work presented here was done using lab strains that have permanently lost mitochondrial DNA due to lack of enzymes that are needed for mitochondrial DNA maintenance, such as DNA helicase (*pif1Δ*), GTPase (*mgm1Δ*), and DNA polymerase gamma (*mip1Δ*). The severe growth inhibition of petite cells by canavanine is not due to a general weakness of petites, as they are able to grow to wild-type (WT) level when exposed to other stressors, such as heat shock (not shown), fluconazole, and thialysine (Fig. 1). However, we do not see the same canavanine-induced growth inhibition when petites are grown in liquid phase (Fig. 2). Petite cells were able to grow to stationary stage in liquid media containing canavanine as determined both by OD measurements (data not shown) and colony formation (Fig. 2). We hypothesized that petite cells lack a specific metabolite required for canavanine tolerance; in liquid phase, cells have many opportunities to come into contact with the resources needed to produce or utilize this metabolite, but in solid phase, acquiring necessary resources from the environment is difficult due to low diffusion rate.

Arginine addition somewhat rescues the petite phenotype

Arginine, which is synthesized in the mitochondria, competes with canavanine for cellular uptake and incorporation into proteins (Ahmad and Bussey 1986). We asked if addition of arginine to the media could rescue the petite phenotype from canavanine-induced growth inhibition. A small

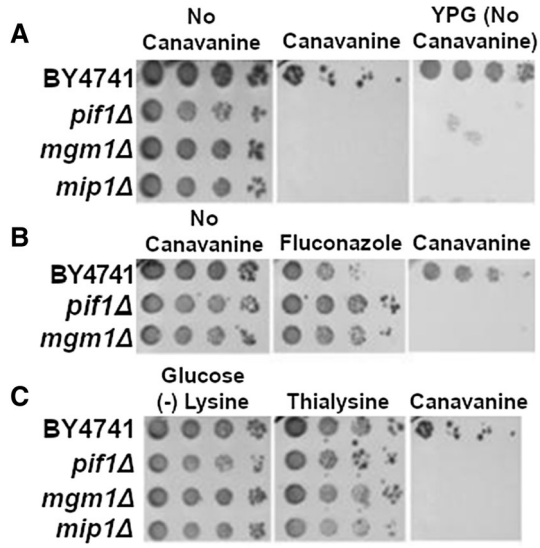


Fig. 1 Respiration-deficient yeasts are specifically sensitive to canavanine. **a** Cells were pronged onto glucose (−) arginine with no canavanine, 2 µg/ml canavanine YNB, or YPG (carbon source glycerol). Cells were grown for 1 week at 30 °C. **b** WT and petites were pronged onto glucose (−) arginine 2 µg/ml canavanine, complete media and complete media containing fluconazole. Cells were grown for 1 week at 30 °C. **c** WT and petites cells were pronged onto glucose (−) arginine 2 µg/ml canavanine, glucose (−) lysine, and glucose (−) lysine with thialysine. There was no observable difference in toxicity between WT and petites when increasing doses of fluconazole or thialysine

amount of arginine was added to the media and gradually increased. Respiratory-competent cells that are completely auxotrophic for arginine (strain CS47) were unable to form colonies under these conditions, indicating that we did not saturate the system with arginine (Fig. 3). In contrast, the viability of petites grown in the presence of canavanine was improved, but did not reach WT levels, an indication that petites do not produce enough arginine and that arginine availability alone cannot explain petites' sensitivity to canavanine.

Amino acid profile of petites is different than WT cells

The above results suggest that petite cells do not generate enough arginine. To further examine petites' availability of arginine, we grew WT and petite cells in media lacking arginine and then extracted amino acids using hot methanol. Through GC–MS analysis we were able to measure the relative amounts of free amino acids in petites versus the WT. Both WT and petites were tested under three conditions: growth in liquid media without arginine, growth on solid media without arginine, and growth on solid media with 1 µg/ml canavanine without arginine. Exclusion of arginine in the media allows us to roughly characterize the state of arginine biosynthesis in each condition.

Petite cells are not arginine auxotrophs and can in fact produce arginine, though in liquid (−) arginine media, petite cells have relatively less free arginine than WT, with *pif1Δ* cells having an average of 6% arginine versus WT 12% arginine (Fig. 4). These differences were not observed in solid media—both WT and petite cells had around 12% free arginine. The relative arginine amounts in WT were similar irrespective of whether canavanine was added to solid media or not. However, the amount of arginine was slightly lower in *pif1Δ* cells grown in canavanine solid media compared with media without canavanine (10% versus 13%, respectively, Fig. 4). Moreover, differences between WT and *pif1Δ* cells were observed for other amino acids in the arginine biosynthesis pathway, specifically glutamine, glutamate and ornithine (Fig. S1, Fig. 4). In liquid, WT has a higher amount of glutamine (26% versus 8%) and ornithine (8%

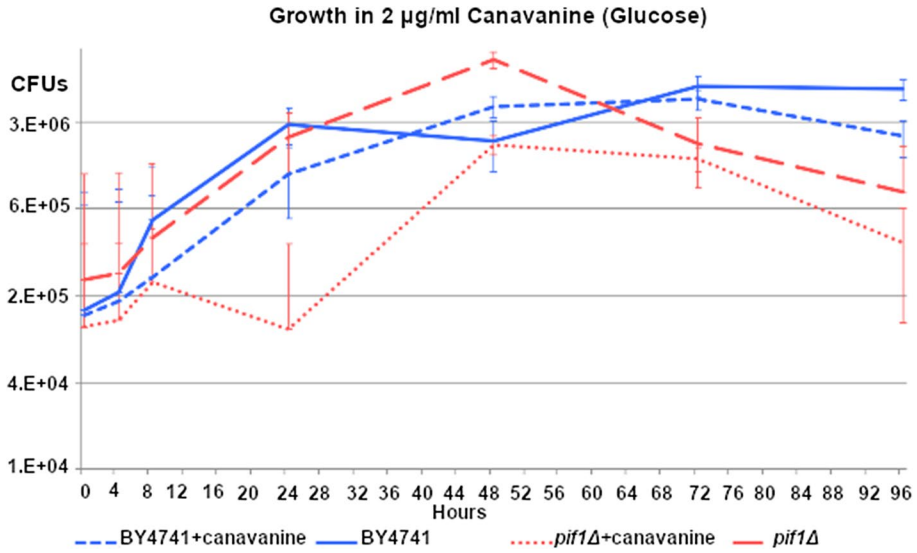


Fig. 2 Respiration-deficient yeasts can tolerate canavanine in liquid phase. A single colony of each genotype was grown overnight in 1 ml glucose (−) arginine 2 µg/ml canavanine in 30 °C, 250 RPM shaking. The following day, cultures were diluted 50-fold and continued to grow for a total of 96 h. At seven time points (marked with error bars), 100 microliter of culture was diluted and spread onto YPD plates and CFUs counted after 1-week growth in 30 °C

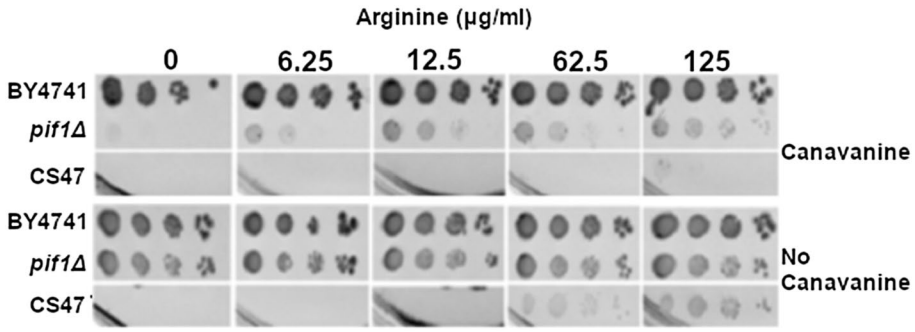


Fig. 3 Arginine addition somewhat rescues petites. Strains were grown on glucose (−) arginine and glucose with increasing amounts of arginine, either with 2 µg/ml canavanine (top) or with no canavanine (bottom). The amount of added arginine was based on the amount of arginine in complete media and the amount shown is proportional to that in complete media (from left to right: 0%, 0.5%, 1%, 5%, and 10%). CS47 is an arginine auxotroph and serves as a control to show the presence or absence of arginine in the media. Growth after 10 days. Experiment was also done in *mgm1Δ* and *mip1Δ* of the BY4741 background (Fig. S3)

versus 2%) than *pif1Δ*; in solid media, 8% versus 2% glutamine and 11% versus 4% ornithine in WT versus *pif1Δ*, respectively; in solid media with canavanine, 6% versus 4% glutamine and 13% versus 3% ornithine in WT versus *pif1Δ*, respectively. In liquid media, WT and *pif1Δ* had an equal amount of glutamic acid (19%), but in solid media, glutamic acid was depleted both with canavanine (15% in WT versus 8% in *pif1Δ*) and without canavanine (15% in WT versus 6% in *pif1Δ*) in the petite background. Citrulline, the most immediate biosynthetic precursor to arginine, comprised less than 1% under all conditions in both genotypes. While it is known that growth in media lacking a specific amino acid will increase production of that amino acid, our data suggest that petites are unable to replenish glutamine/glutamate stores during this shift to arginine production, resulting in depleted quantities of these precursor amino acids.

Interestingly, compared to growth in liquid, alanine is overrepresented in both WT and *pif1Δ* grown on solid media with and without canavanine, with the petite having double than that of WT. The increase in alanine on solid media is intriguing given the fact that pyruvate, the product of glycolysis that can enter the citric acid cycle, can be converted into alanine in a reversible reaction via alanine aminotransferase encoded by *ALT1* (α-ketoglutarate + L-alanine ⟺ L-glutamate + pyruvate).

Glutamate and alpha-ketoglutarate support canavanine tolerance

Based on the amino acid profile of petite cells we reasoned that α-ketoglutarate and glutamate availability should decrease canavanine-induced growth inhibition in petites. We added glutamate and α-ketoglutarate to (−) arginine media containing canavanine and followed growth for 9 days. Both glutamate and α-ketoglutarate had a transient effect on canavanine-induced growth inhibition. After 4 days of growth, petite colonies grown on glutamate and 1 µg/ml canavanine were clearly visible and larger in size when compared to those grown on canavanine only. Petite and WT cells grown on canavanine 2 µg/ml also showed improved growth on the glutamate background (Fig. 5a). A similar but less pronounced effect was observed with α-ketoglutarate addition (Fig. 5b). *mgm1*Δ showed the greatest improvement in growth on canavanine under both glutamate and α-ketoglutarate addition.

Canavanine affects localization of arginine biosynthesis enzymes

Our data indicate that petite cells have reduced capacity to generate intermediates in the arginine pathway. Arginine biosynthesis enzymes are encoded in nuclear DNA, yet four of these ultimately localize to the mitochondria (Arg2, Arg5,6, Arg7, and Arg8). We asked if petites' ability to transport arginine biosynthesis proteins was compromised, possibly due to decreased mitochondrial membrane potential resulting from a lack of ETC and oxidative phosphorylation. When grown in glucose (−) arginine liquid media, we did not observe a major difference between WT and petite mitochondrial localization of these enzymes. When grown in glucose (−) arginine 1 µg/ml canavanine, delocalization in both WT and petites was observed, with petites displaying a more extreme phenotype (Fig. 6a). We quantified the strength and localization of GFP in Arg5,6 and Arg8, represented as density (fluorescence/area) in Fig. 6b. Next, we studied if the delocalization was due to mistargeting or due to disruption of mitochondrial structure. On the basis of the Arg5,6-GFP strains described above, we constructed Tom22 RFP strains.

Tom22 is a central component of the TOM (translocase of outer membrane) receptor complex (Honlinger et al. 1995; Lithgow et al. 1994). It does not require the mitochondrial

membrane potential to be inserted into the mitochondrial membrane. In respiration-proficient strains with or without canavanine addition, the GFP (Arg5,6) and the RFP (Tom22) signals overlapped (Fig. 7). The same was true for respiration-deficient strains without canavanine exposure. However, respiration-deficient strains that were exposed to canavanine showed only partial overlap between the Arg5,6 and Tom22 (Fig. 7). To test if the delocalization of enzymes within the arginine biosynthesis pathway was due to reduction in the mitochondrial membrane potential, we measured mitochondrial membrane potential using TMRE (tetramethylrhodamine, ethyl ester) staining. TMRE is a red fluorescent dye that is accumulated in active mitochondria—loss of mitochondrial membrane potential is reflected in loss of the TMRE signal. Adding canavanine to respiration-proficient strains caused reduction in the relative amounts of cells with positive TMRE signal (Fig. 8). Respiration-deficient strains showed reduction in the relative amounts of cells with positive TMRE signal compared with respiration-proficient ones, a trend that was further decreased when canavanine was added (Fig. 8). The proportion of TMRE-stained mitochondria in petite cells treated with canavanine was minimal, indicating a combined effect of canavanine and loss of respiration on the mitochondrial membrane potential (Fig. 8).

Nitrogen starvation increases canavanine-induced growth inhibition more than glucose starvation

We have shown above that petite cells produce relatively less amino acids in the pathway leading to arginine. Next, we asked what resources are limiting in the production of amino acids. One such resource required for petite growth is glucose (or any other fermentable carbon source). We grew petites in increased concentrations of glucose but found no effect on canavanine tolerance (data not shown). Conversely, we grew petites on reduced concentrations of glucose in the presence of canavanine and found petites could tolerate these reduced glucose concentrations, though not as well as WT (Fig. 9a). This could be due to the relief of glucose repression—that is, under sufficient glucose levels, the transcription of genes involved in respiration and gluconeogenesis is inhibited, but under glucose-scarce conditions (like those used in this experiment), transcription of such genes can proceed.

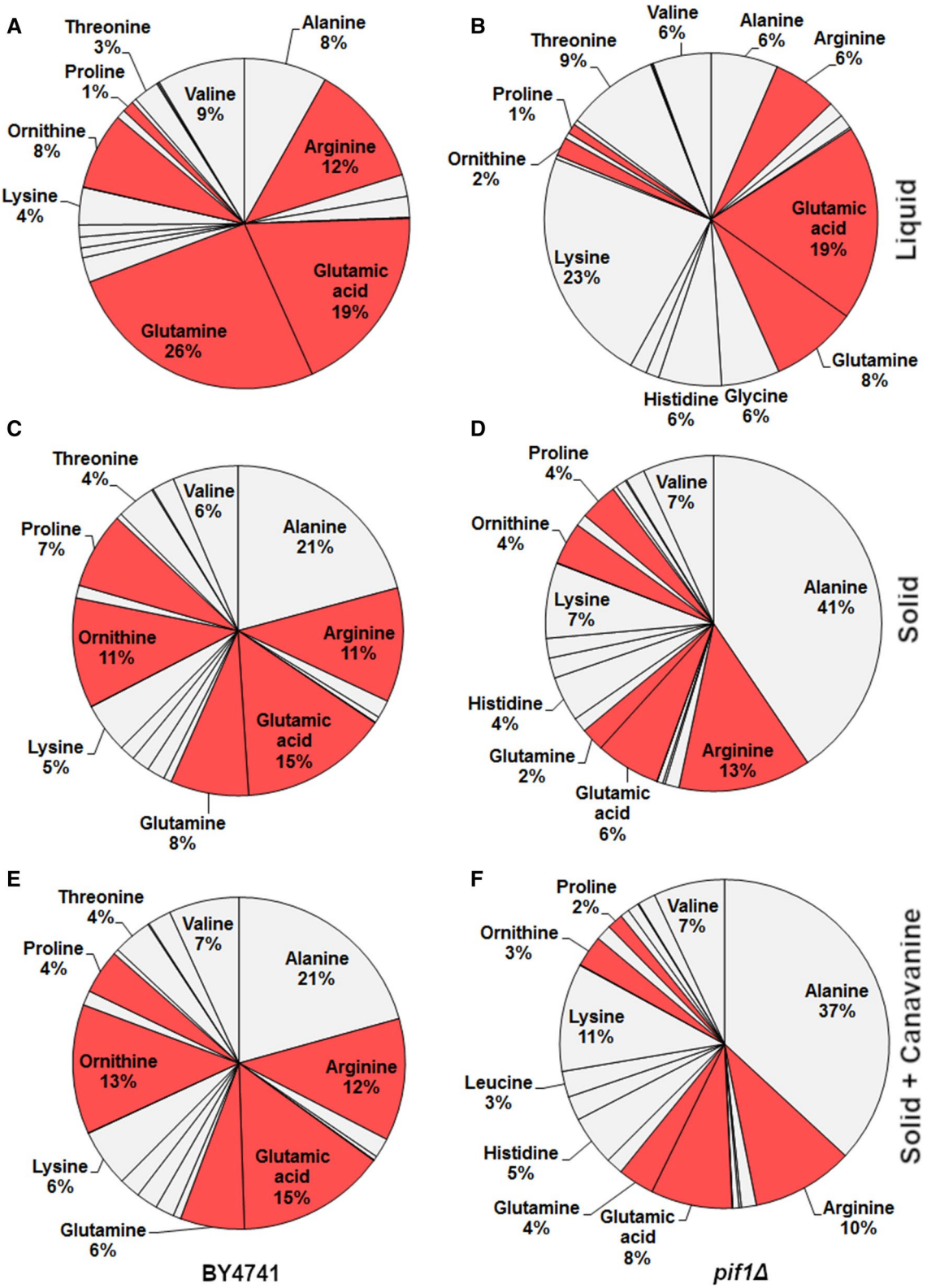
Arginine synthesis ultimately begins with the availability of nitrogen in the form of ammonia or the amino acid glutamate; glutamate and glutamine are not supplied in the growing media and, therefore, are synthesized from simple compounds. Addition of ammonium sulfate to the media had no effect on colony formation in petites, likely due to saturation of nitrogen transporters (data not shown). However, reduction of ammonium sulfate in the media caused an increase in petite cells' sensitivity to canavanine, and even the WT showed an increase in canavanine-induced growth inhibition, although to a lesser extent (Fig. 9b). This shows the importance of nitrogen availability in the presence of canavanine, especially in respiration-deficient cells.

Canavanine interferes with the RTG pathway

We have observed amino acid production defects of petite cells; some of the defects were exacerbated in the presence of canavanine, such as reduction in arginine amounts in solid media. We were able to link these differences to reduction in the mitochondrial membrane potential. The retrograde (RTG) pathway connects between mitochondrial function and regulation of nuclear gene expression (Liu and Butow 2006). Petite cells of various backgrounds have the RTG pathway turned on even under normal conditions (Wodicka et al. 1997). We asked if addition of canavanine would cause an increase in mitochondrial stress, measured by the abundance of the two prototypical RTG pathway genes in petites, *CIT2* and *DLD3*; surprisingly, we found that canavanine caused a decrease in mRNA expression levels (Fig. 10a–c). We used the Δ/ΔCt method to quantify the relative abundance of these two genes, with comparisons between four different pairs: *pif1Δ* glucose/BY4741 glucose, *pif1Δ* canavanine/BY4741 canavanine, *pif1Δ* canavanine/*pif1Δ* glucose, and BY4741 canavanine/BY4741 glucose. Under normal conditions, *pif1Δ* expresses more *CIT2* (2.45-fold expression) and *DLD3* (1.49-fold expression) than WT (Fig. 10a, blue bars), as expected. However, we see an overall trend of decreased expression upon exposure to canavanine, with *pif1Δ* expression of *CIT2* reducing from 2.45-fold to 1.15-fold, and there is a significant reduction in *DLD3* mRNA expression in *pif1Δ* compared to the WT, reducing from 1.49-fold to 0.47-fold in *pif1Δ* versus WT upon exposure to canavanine (Fig. 10a, red bars). When we compare mRNA levels of exposure to canavanine versus non-exposure within the same genotype, we continue to see this downward trend in which canavanine-exposed cells, both WT and petite, express less mRNA of both *CIT2* and *DLD3* (Fig. 10b, c). *pif1Δ* exposed to canavanine expresses *CIT2* at a level of just 0.02 when compared to mRNA expression of *CIT2* in *pif1Δ* without canavanine, and similarly, *DLD3* drops to 0.18-fold expression in the presence of canavanine (Fig. 10b). WT expression of *CIT2* mRNA drops to just 0.07-fold and *DLD3* drops to 0.59-fold expression when exposed to canavanine and compared to WT growth without canavanine (Fig. 10c).

Canavanine alters expression of genes that are under RTG control in rho^0 cells

The Hap2,3,4,5p transcription complex is needed for expression of many mitochondrial proteins involved in electron transport and the TCA cycle. Liu and Butow (1999) showed



that a decrease or loss of respiration caused expression of *CIT1*, *ACO1*, *IDH1*, and *IDH2* to switch from *HAP* control to *RTG* control. These four TCA genes encode for enzymes that synthesize the immediate precursors of α-ketoglutarate. Since expression of these four TCA genes is dependent on the *RTG* genes in rho^0 cells, induction of their expression in petites is a retrograde response (Traven et al. 2001). We again used the Δ/ΔCt method to measure the relative

◂**Fig. 4** Amino acid analysis. Only amino acids comprising greater than 2% have been included (unless they are produced in the mitochondria). Relative amounts of free amino acids were determined by nanograms of free amino acids divided by total amount (in nanograms) of free amino acids per sample. **a** BY4741 grown overnight in liquid glucose (−) arginine. **b** *pif1Δ* grown overnight in liquid glucose (−) arginine. **c** BY4741 grown for 2 days on solid glucose (−) arginine. **d** *pif1Δ* grown for 2 days on solid glucose (−) arginine. **e** BY4741 grown for 2 days on solid glucose (−) arginine with 1 µg/ml canavanine. **f** *pif1Δ* grown for 2 days on solid glucose (−) arginine with 1 µg/ml canavanine. Liquid analysis was also done in *mgm1Δ* and *mip1Δ* of the BY4741 background, as well as *mgm1Δ* from the HAY75 background (56) with similar results (not included for brevity)

abundance of these genes in petites versus WT. Under normal conditions, *pif1Δ* expresses more *CIT1* mRNA (4.93-fold), *ACO1* (14.62-fold), and *IDH1* (2.08-fold) than WT (Fig. 10a, blue bars). Taking into account the standard deviation, *IDH2* mRNA expression in *pif1Δ* was roughly equal to WT under these conditions (1.05-fold). When grown on canavanine, expression of all four genes decreases in *pif1Δ* compared to WT: *CIT1* expression drops from 4.93-fold to 0.91-fold, *ACO1* expression drops from 14.62-fold to 0.21-fold, *IDH1* expression drops from 2.08-fold to 0.36-fold, and *IDH2* expression drops from 1.05-fold to 0.02-fold (Fig. 10a, red bars). Comparing mRNA levels of exposure to canavanine versus non-exposure within the same genotype, mRNA levels in *pif1Δ* and WT had opposite responses to canavanine (Fig. 10b, c). In *pif1Δ*, canavanine caused a decrease in mRNA expression (*CIT1* = 0.64-fold, *ACO1* = 0.03-fold, *IDH1* = 0.48-fold, *IDH2* = 0.37-fold) (Fig. 10b). In WT, canavanine caused an increase in mRNA expression (*CIT1* = 3.47-fold, *ACO1* = 2.05-fold, *IDH1* = 2.79-fold, *IDH2* = 15.59-fold) (Fig. 10c).

RTG2, the central regulator of the RTG pathway, is required for canavanine tolerance

We found that canavanine exposure correlates with decreased expression of the prototypical RTG genes mRNA in both petites and WT, with petites displaying a more severe phenotype. Thus, we hypothesized that a switching off of the RTG pathway should induce petite-like canavanine sensitivity in a respiration-proficient cell. To test this, we used *rtg2Δ*; Rtg2p is a sensor of mitochondrial dysfunction and a central regulator of the RTG pathway (Liu et al. 2003). In agreement with our RT-qPCR findings, *rtg2Δ* is unable to grow on canavanine (Fig. 11). *rtg3Δ* and *mks1Δ* were also unable to grow on canavanine (Fig. 11). All three knockout strains grew to WT level on YPD media, but grew slowly on (−) arginine media, suggesting they are deficient in arginine production. Rtg3p is a transcription factor that forms a complex with Rtg1p to activate the RTG response. Mks1p is a negative transcriptional regulator involved in RTG signaling.

The Goldilocks effect: enforcing respiration causes severe inhibition by canavanine

Based on our work with petites, we see respiration is a requirement for growth in the presence of canavanine, so we examined how respiration as the sole means of ATP production would affect canavanine tolerance. Unlike petites (Fig. 2), canavanine has a fungistatic effect on WT cells grown in a liquid non-fermentable carbon source (Fig. 12a). When grown on a solid non-fermentable carbon source, WT cells experience canavanine-induced growth inhibition at a level similar to that of petites (Fig. 12b). This leads us to hypothesize that different mechanisms govern canavanine-induced growth inhibition among respiratory-proficient cells grown on a non-fermentable carbon source and respiratory-deficient cells grown on a fermentable carbon source, especially considering that a *can1*Δ mutant is also sensitive to the combination of a non-fermentable carbon source and canavanine (Fig. S2).

In conclusion, our data show an important role of respiration and fermentation in facilitating growth in the presence of canavanine. The system presented here can model the effect of varying respiration capacities on stress tolerance, a topic at the heart of cancer biology research.

Discussion

Saccharomyces cerevisiae is an elegant model to study how different respiration capacities affect the cell's ability to deal with stress, because, unlike many eukaryotic organisms, *S. cerevisiae* is petite positive and exhibits the Crabtree effect—fermentation in the presence of oxygen (Merico, et al. 2007). Despite their lack of respiration, and thus decreased ATP production (Rodrigues et al. 2006; Williamson et al. 1971), petite cells are not generally weak. This is supported by our work (Fig. 1), as well as prior work showing petite cells are more resistant to heat shock and certain drugs, as they exhibit pleiotropic drug resistance via upregulation of *PDR5* (Moye-Rowley 2005; Traven et al. 2001). In the past, *pif1Δ* was identified as a mutant conferring increased resistance to canavanine in a high-throughput screen of a BY4741 knockout library (Shi et al. 2011); however, our data show that petite cells, and specifically *pif1Δ*, are sensitive to canavanine. This false positive is likely due to the gain of a *can1* mutation since absence of Pif1 increases mutation rates, especially in telomere proximal genes such as *CAN1* (Myung et al. 2001; Zhang and Singh 2014).

We hypothesize that even low rates of respiration contribute to overall mitochondrial integrity and, therefore, is necessary for tolerance of canavanine. Our investigation points to two factors: first, petite cells are deficient in producing amino acids within the arginine biosynthesis pathway

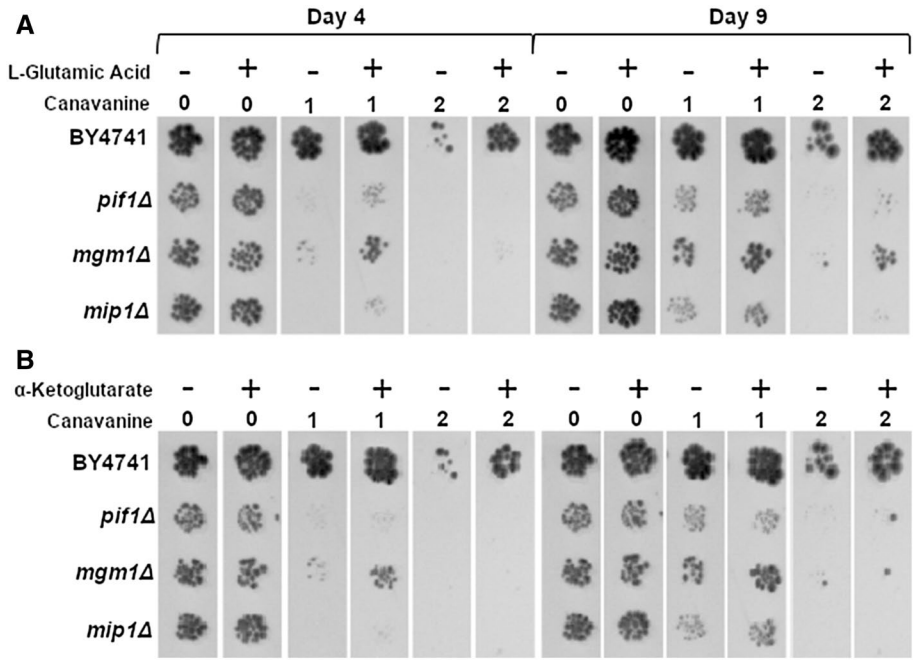


Fig. 5 Glutamate and α-ketoglutarate effect on canavanine-induced growth inhibition. **a** Glutamic acid addition. A single colony was placed in 200 µl DDW, serial dilutions performed, and pronged onto glucose (−) arginine media with 0, 1, or 2 µg/ml canavanine, with or without addition of L-glutamic acid. **b** α-Ketoglutarate addition. A single colony was placed in 200 µl DDW, serial dilutions were performed, and pronged onto glucose (−) arginine media with 0, 1, or 2 µg/ml canavanine, with or without addition of α-ketoglutarate. Images show growth after 4 and 9 days

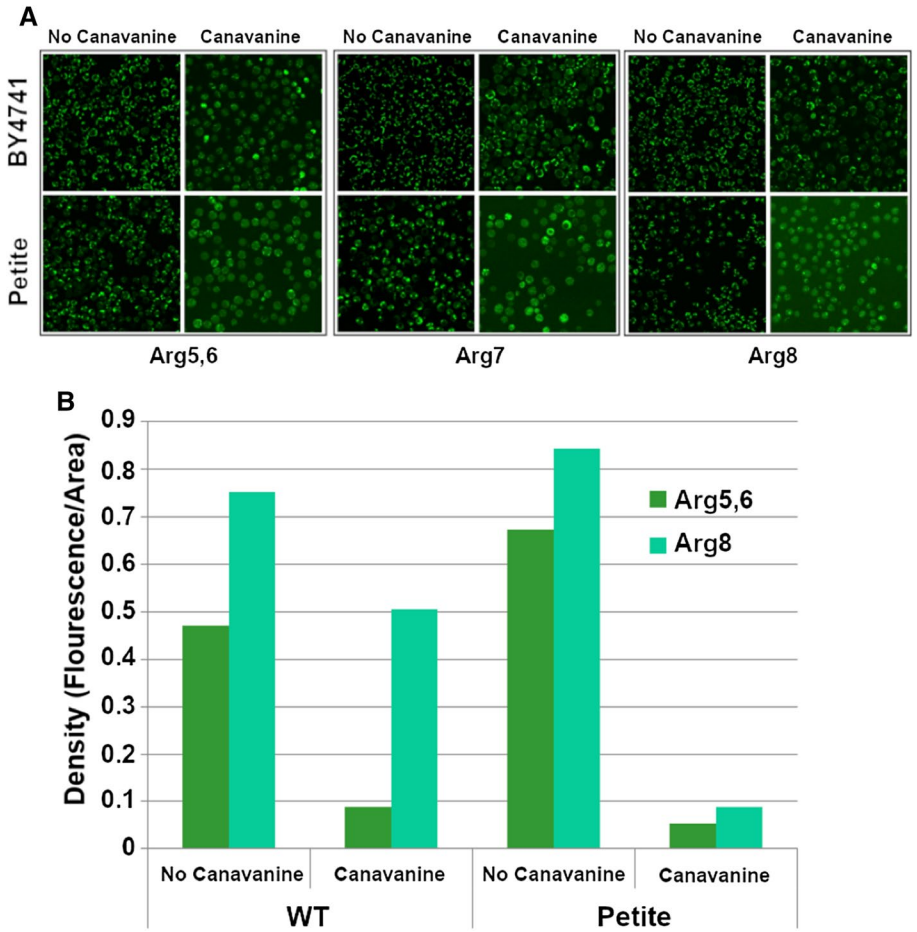


Fig. 6 Canavanine affects localization of arginine biosynthesis genes. A single colony was grown in glucose (−) arginine for 3 h and then divided into two separate cultures with one culture inoculated with 1 µg/ml canavanine. Cultures grew overnight in 30 °C at 250 RPM and pictures were taken with a fluorescent microscope. **a** Microscope images showing localization of GFP-labeled enzymes. A representative 704 × 704-pixel selection is shown for each of the total 2048 × 2048 pixel images. Arg2 not included due to poor imaging. **b** Bar graph showing the density of two GFP-labeled enzymes. Quantification is of the total 2048 × 2048 pixel images

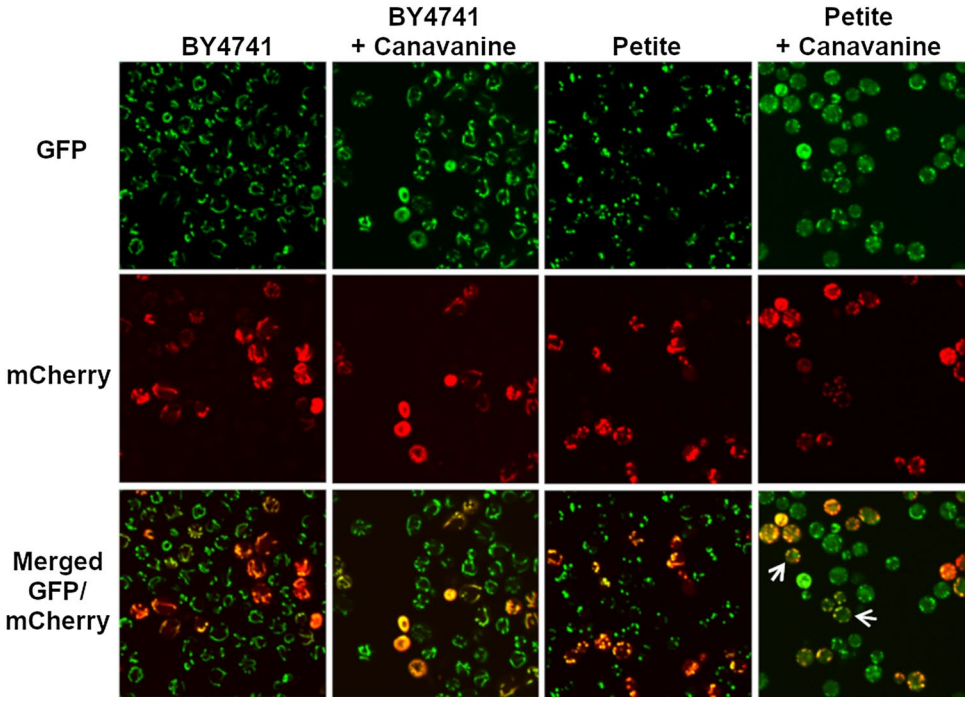


Fig. 7 Canavanine-affected cells have functioning mitochondria. Arg5,6-GFP Tom22-RFP respiration-proficient and -deficient strains were grown in glucose (−) arginine for 3 h, then divided into two separate cultures with one culture inoculated with 1 µg/ml canavanine. Cultures grew overnight in 30 °C at 250 RPM and pictures taken with a fluorescent microscope, green and red signals were overlaid. White arrows in the bottom-right image point to examples of mislocalization of Arg5,6

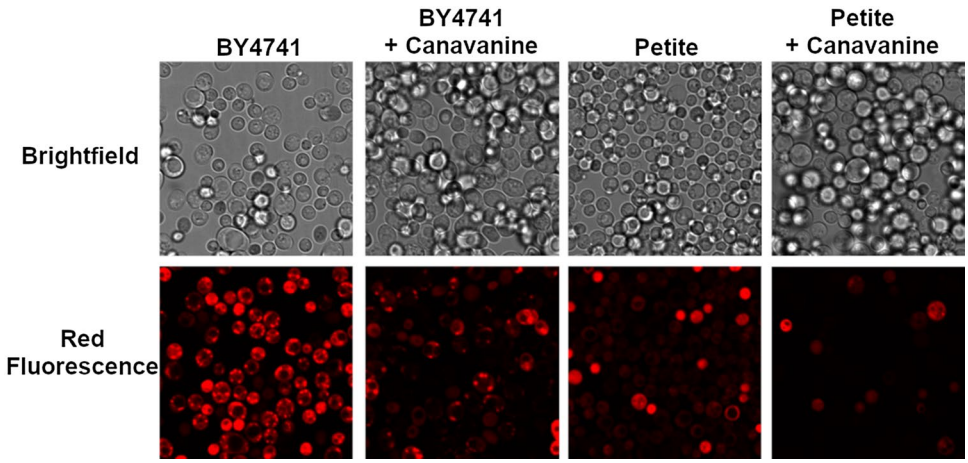


Fig. 8 Canavanine reduces mitochondria membrane potential. Respiration-proficient and -deficient strains were grown in glucose (−) arginine for 3 h, then divided into two separate cultures with one culture inoculated with 1 µg/ml canavanine. Cultures grew overnight in 30 °C at 250 RPM and pictures taken with a fluorescent microscope after 20 min incubation with 100 nM TMRE as described in the manufacturer protocol

(Figs. 4, 13), and canavanine reduces the efficiency of targeting to the mitochondria of enzymes in the arginine biosynthesis pathway, probably through reducing mitochondrial membrane potential (Figs. 6, 7, 8). Second, we show for the first time that canavanine interferes with the RTG pathway in yeast cells. This interference can be tolerated in respiration-proficient yeast, but respiration-deficient ones rely more on the retrograde pathway (Liao et al. 1991; Liu and Butow 1999) and are thus more susceptible to canavanine interference. Moreover, the expression of *CIT1*, *ACO1*, *IDH1* and *IDH2*, all part of the citric acid cycle, is induced in WT cells following canavanine exposure (Fig. 10c). In contrast, the expression of these genes is suppressed in petite cells following canavanine exposure (Fig. 10b). This difference can be partly explained by these four genes being under different regulation in WT and petite (*HAP* in WT (Forsburg and Guarente 1989), *RTG* in petite (Liu and Butow (1999); discussed further below). We think that the differential effect of canavanine on petite cells from an arginine biosynthesis standpoint and activation of the RTG pathway are major determinants in the sensitivity of petite cells to canavanine. However, other explanations are valid and may contribute significantly to this phenomenon. For example, enhanced permeability of petite cells to canavanine or more efficient uptake of the toxic analog into proteins in petite is possible.

To the best of our knowledge, the amount of information regarding the effect of respiration capacity on amino acid biosynthesis is limited (Martiez-Force and Benitez 1992).

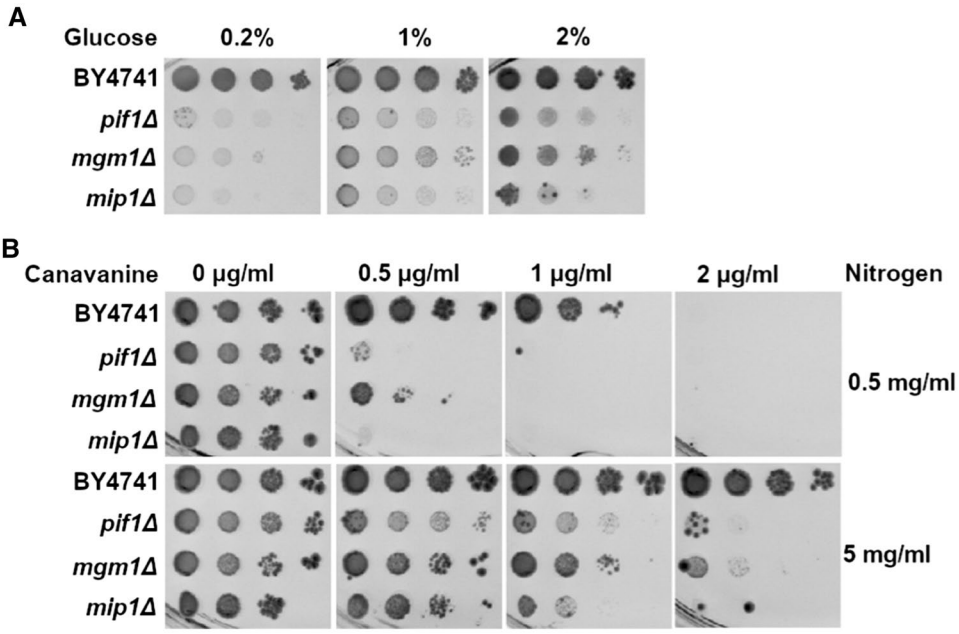


Fig. 9 Nitrogen starvation increases canavanine sensitivity more than glucose starvation. **a** Strains were grown on media containing 2% glucose and either 1/10th the normal amount of ammonium sulfate (top; 0.5 mg/ml) or normal nitrogen (bottom; 5 mg/ml), without arginine, and in the presence of either no canavanine, 0.5 µg/ml, 1 µg/ml, or 2 µg/ml. **b** Strains were grown on (−) arginine media with 0.2%, 1%, or 2% glucose containing 1 µg/ml canavanine. Decreased glucose concentration had little effect on canavanine-induced growth inhibition. Growth after 1 week

Martinez-Force and Benitez measured amino acid pools in fermentative versus respiratory and mixed fermentative–respiratory media. Their data show increases in arginine, lysine, and glutamate as metabolism shifts towards respiratory only. Our findings were somewhat different; while we did see a significant increase in arginine pools in WT, there was no difference in glutamate concentrations between WT and petite, and free lysine was almost 20% greater under fermentative conditions (Fig. 4a, b). Importantly, our results were obtained under conditions of arginine starvation, not like the data obtained by Martinez-Force and Benitez. Therefore, from an arginine biosynthesis standpoint, our results are more relevant because there is no influence of arginine uptake from the media. In addition, we describe here for the first time the differences in the free amino acid profile between respiration-proficient and respiration-deficient yeast grown on solid media where diffusion is limited. Our results show that compared to liquid phase, solid phase growth has a major effect on the status of relative free amino acids in both WT and petite, with petites undergoing the most drastic changes (Figs. 4, 13). *pif1Δ* grown in solid media produced less glutamine, glutamate and ornithine, all of which are amino acids that are part of the arginine biosynthesis pathway (Jauniaux et al. 1978). We show that addition of arginine and glutamate somewhat relieves the growth inhibition imposed by canavanine on solid media (Figs. 3, 5, Fig. S3). The simplest explanation is that under canavanine stress there is a need to produce more arginine to compete with canavanine for uptake into proteins; since petite cells have less glutamate and glutamine to start with (Fig. 4d, f), the reservoir for arginine production is lower than WT cells. Alternatively, the low pools of glutamate and glutamine may itself cause stress on the proteome that is then exacerbated by the stress of canavanine. Another level of complexity is the observation that canavanine reduces the efficiency of targeting of arginine biosynthetic enzymes to the mitochondria, especially in petite cells (Fig. 6). We found that canavanine reduces the ability to generate mitochondrial membrane potential in both WT and petites. This, combined with petites' inherently reduced mitochondrial membrane potential, could explain why canavanine exposure causes such a drastic reduction in the ability of some proteins to be targeted to the mitochondria in respiration-deficient cells (Figs. 6, 7, 8).

We also show that addition of α-ketoglutarate has a small but reliable effect on canavanine tolerance in petite cells, though the effect is not equal between all petite mutants; a stronger effect was observed in *mgm1Δ* cells. The difference between the mutants could be because Mgm1 unlike Mip1 and Pif1 also functions in mitochondria morphology and fusion that may affect permeability of α-ketoglutarate or retention of the relevant enzymes in the mitochondria (Sesaki et al. 2003). Ammonium and α-ketoglutarate are the simplest compounds required for glutamate biosynthesis (Roon et al. 1974). Enzymes of the citric acid cycle are functional in petite cells, but those downstream of α-ketoglutarate (*KGD1*, *SDH1*, *FUM1*, and *MDH1*) are all downregulated in rho^0 petites, and petite cells lacking any of the *RTG* genes show severe reduction in citric acid cycle enzymes upstream of α-ketoglutarate (*CIT1*, *ACO1*, *IDH1*, *IDH2*) (Liu and Butow 1999). It is unknown how respiration capacity affects the cellular concentrations of α-ketoglutarate in yeast. One of the roles of the RTG pathway is to increase the amounts of α-ketoglutarate in mitochondrially stressed

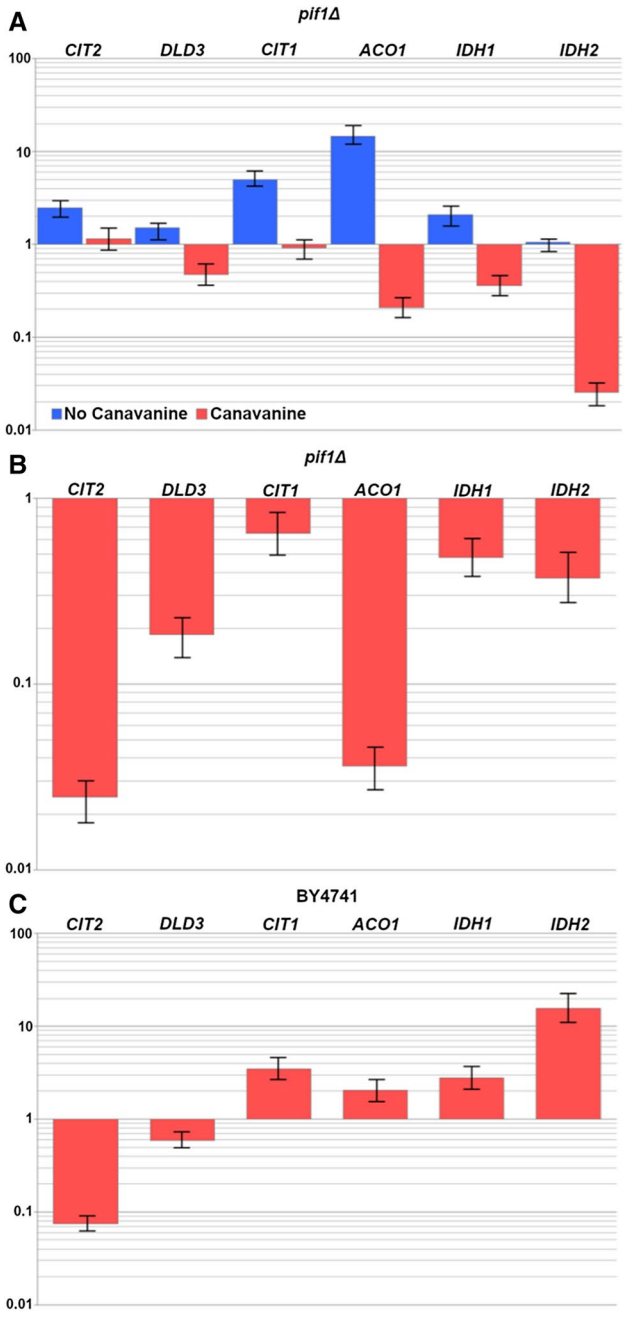


Fig. 10 Canavanine affects the RTG pathway. Fold change was calculated using the Δ/ΔCt method with *ACT1* as a reference gene. **a** Blue columns represent mRNA expression of *pif1Δ* compared to WT when grown on (−) arginine media. Red columns represent mRNA expression of *pif1Δ* compared to WT when grown on (−) arginine media 1 µg/ml canavanine. **b** Bar chart represents mRNA expression of *pif1Δ* grown on (−) arginine media with 1 µg/ml canavanine versus *pif1Δ* grown on (−) arginine media. **c** Bar chart represents mRNA expression of WT grown on (−) arginine media with 1 µg/ml canavanine versus WT grown on (−) arginine media

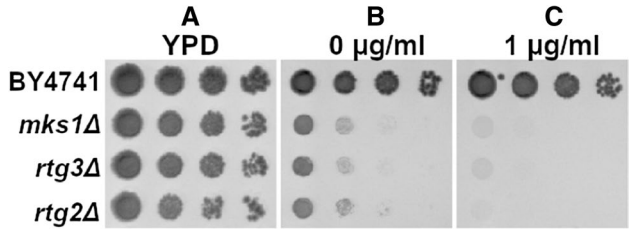


Fig. 11 Genes of the RTG pathway are required for canavanine tolerance. A single colony was placed in 200 µl DDW, serial dilutions were performed, and pronged onto **a** YPD, **b** glucose (−) arginine media without canavanine, and **c** glucose (−) arginine media with canavanine. Growth after 5 days

cells; this is largely modeled after phenotypes of respiratory-deficient cells (Epstein et al. 2001; Liu and Butow 1999; Traven et al. 2001), but it is unknown if the RTG pathway can elevate α-ketoglutarate levels in petites to a comparable α-ketoglutarate level in WT. Our data indicate that there is not enough α-ketoglutarate in petite cells to produce glutamate and glutamine at a WT level (Figs. 4, 5, 13). Furthermore, our finding that canavanine hampers expression of *CIT1*, *ACO1*, *IDH1*, and *IDH2* in petites yet increases expression of these four genes in WT may explain why petites are so much more sensitive to canavanine that WT: inhibition of the RTG response, whether by canavanine or otherwise, inherently decreases petites' ability to generate α-ketoglutarate; however, *CIT1*, *ACO1*, *IDH1*, and *IDH2* are under *HAP* control in respiration-competent cells, thus they are still able to produce α-ketoglutarate. The modest effect of α-ketoglutarate addition on canavanine tolerance could be due to poor uptake or rapid metabolism that is different from glutamate biosynthesis (Contreras-Shannon et al. 2005; Quezada et al. 2013; Smith et al. 2007).

Reduction in the amount of the nitrogen source sensitizes petite cells more than WT to canavanine (Fig. 9). This finding may suggest that the poor production of glutamate is not only a result of poor flow of citric acid cycle metabolites but due to either poor uptake of nitrogen or inefficient activity of the enzymes required for glutamine or glutamate biosynthesis. Gdh1 is a key enzyme in glutamate production that is expressed in fermentative growth and uses α-ketoglutarate as a substrate (DeLuna et al. 2001); we hypothesize that it is either downregulated or inhibited in petite cells. Alternatively, Gdh1 could be directly or indirectly inactivated by canavanine.

The RTG pathway has been extensively studied in petites (Epstein et al. 2001; Liu and Butow 1999, Traven et al. 2001); however, its relation to canavanine has not been reported. This pathway regulates genes involved in glutamate and α-ketoglutarate homeostasis, which leads to the conclusion that the RTG pathway plays a role in nitrogen homeostasis (Magasanik and Kaiser 2002). Our results show that nitrogen starvation increases canavanine sensitivity, and loss of *RTG2*, *RTG3* or *MKS1* eliminates a colony's ability to tolerate canavanine under arginine-limiting conditions

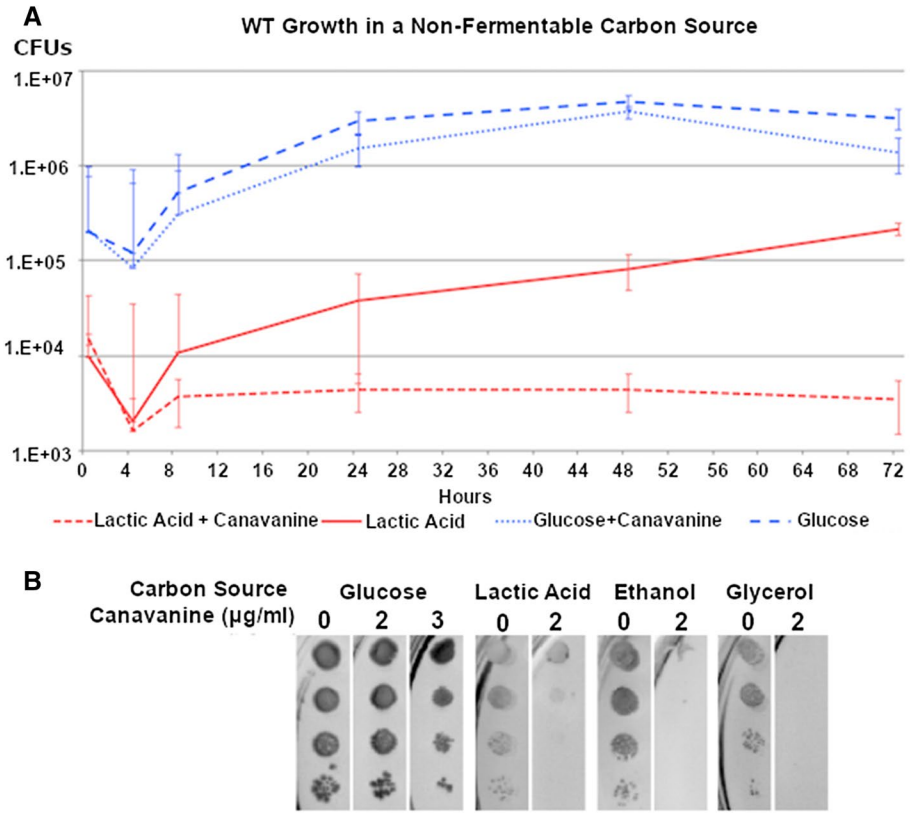


Fig. 12 Canavanine is fungistatic with a non-fermentable carbon source. **a** Growth of BY4741 in glucose (blue) versus lactic acid (red). A single colony of WT BY4741 was grown overnight in 1 ml lactic acid (−) arginine with 1 µg/ml canavanine in 30 °C, 250 RPM shaking. The following day, cultures were diluted 50-fold and continued to grow for a total of 96 h. At seven time points (marked with error bars), 100 microliter of culture was diluted and spread onto YPD plates. **b** BY4741 growth on non-fermentable carbon sources versus growth on glucose with or without canavanine. WT was grown on a non-fermentable carbon source (−) arginine with or without canavanine. Growth after 1 week

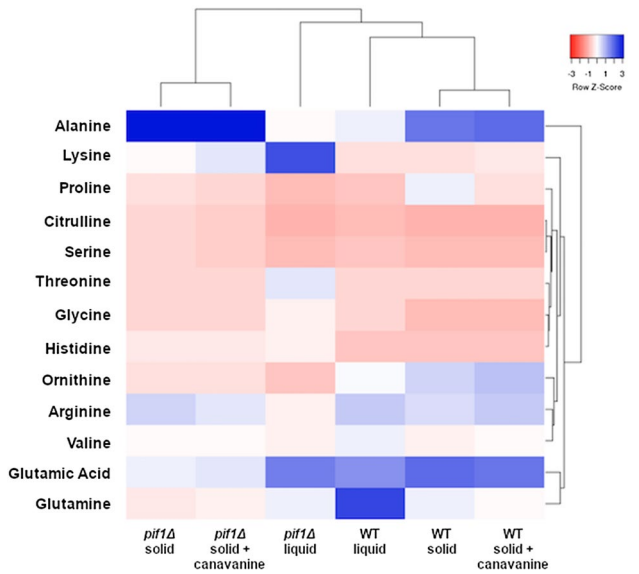


Fig. 13 Heat map showing free amino acid abundance. Only amino acids comprising 2% or greater in at least one condition have been included. Clustering has been applied to both axes (condition and amino acid)

(Figs. 9b, 11). It has been reported that nitrogen starvation induces mitophagy (Camougrand et al. 2008; Kanki et al. 2009), yet *rtg*Δ mutants undergo less mitophagy (Torelli et al. 2015). All *rtg* mutants (*rtg1*Δ, *rtg2*Δ, and *rtg3*Δ) have a great reduction in *CIT2* expression, both in respiration-proficient and -deficient strains (Guaragnella and Butow 2003; Liao and Butow 1993). In fact, petite cells with mutations in any of the *rtg* genes show a reduction in most citric acid enzymes (the exceptions being *SDH1* and *FUM1*) (Liu and Butow 1999). We suggest that the inhibition of the RTG pathway by canavanine is the one explanation for the various petite-related phenotypes we observe here. This is because petites rely more than WT on the RTG pathway for nitrogen homeostasis and amino acid production, two processes closely tied to the mitochondria (Jauniaux et al. 1978; Komeili et al. 2000; Magasanik and Kaiser 2002). At this point we do not know how canavanine interferes with the RTG pathway. The response to canavanine is mediated by Msn2/4 transcription factors that govern cytosolic stress (Shor et al. 2013). Could it be that the cytosolic stress response overrides the mitochondrial one?

Our observations may have an applied side; several fungicides target succinate dehydrogenase and, therefore, respiration (Scalliet et al. 2012). In light of our results, synthetic lethality of the succinate dehydrogenase inhibitor boscalid and canavanine (or similar compounds) is possible.

Finally, we observe that growth in a non-fermentable carbon source greatly sensitizes yeast cells to canavanine. At this point, we do not understand the mechanism of this synthetic interaction. It is possible that canavanine directly interferes with the assembly of respiratory complexes even

in very low concentrations—much lower than needed to inhibit the rest of the proteome. However, other more complicated scenarios are possible. Interestingly, a connection between the Snf1 kinase, which is activated in cells growing on a non-fermentable carbon source, and the response to misfolded proteins were previously reported (Simpson-Lavy et al. 2017). It was previously reported that growth on a carbon source that is not glucose changes the morphology of mitochondria and activates the RTG pathway (Epstein et al. 2001; Guaragnella and Butow 2003; Prevost et al. 2018; Soontorngun 2017). It is, therefore, possible that the toxic effect of canavanine in the presence of a non-fermentable carbon source occurs through inhibition of the RTG pathway or disruption of mitochondria morphology. Zhang and Cao provide a more general view on respiration capacity and response to stress. They suggest that regulation of mitochondrial respiration can modulate metabolic reprogramming and stress response, thus restriction of the ability to regulate respiration either by growth on a non-fermentable carbon source or using respiration-deficient cells may restrict the ability of the cell to deal with canavanine stress (Zhang and Cao 2017).

We think that the mode of inhibition under forced respiratory conditions is different than the mode of inhibition of canavanine imposed on petite cells grown on glucose. This is due to the fungistatic effect of canavanine and a non-fermentable carbon source in liquid culture (compare Fig. 2 with Fig. 12). If so, from an evolutionary standpoint, what is the meaning of such confined respiratory capacity, i.e., the Goldilocks effect?

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