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Functional expression of the maize mitochondrial URF13 down-regulates galactose-induced *GAL1* gene expression in *Saccharomyces cerevisiae* ☆

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Abstract

Genes for the enzymes that metabolize galactose in *Saccharomyces cerevisiae* are strongly induced by galactose and tightly repressed by glucose. Because glucose also represses mitochondrial activity, we examined if derepression of the *GAL1* galactokinase gene requires physiologically active mitochondria. The effect of mitochondria on the expression of *GAL1* was analyzed by a novel approach in which the activity of the organelles was altered by functional expression of URF13, a mitochondrial protein unique to the Texas-type cytoplasmic male sterility phenotype in maize. Mitochondrial targeting and functional expression of the URF13 protein in yeast result in a decrease of the mitochondrial membrane potential similar to those observed in cells treated with mitochondrial inhibitors such as antimycin A or sodium azide. Activation of URF13 in galactose-induced cells results in the inhibition of *GAL1* expression in the absence of repressing concentrations of glucose. Our data reveal the existence of a regulatory pathway that connects the derepression of the *GAL1* gene with mitochondrial activity.

Keywords: Yeast; Gene expression; Mitochondria; Membrane potential; Flow cytometry

Mitochondrial dysfunction and alterations in mitochondrial activity have been shown to cause a wide range of changes in physiological and metabolic responses in a variety of different organisms. In human, mitochondrial defects have been implicated in degenerative diseases, aging, diabetes, cancer, and apoptosis [1,2].

A growing body of evidence indicates that, in mammalian and yeast cells, mitochondria are involved in oxygen sensing and the induction of some hypoxia-responsive genes. The adaptive response of mammalian cells to hypoxia is mediated by the ubiquitous hypoxia-inducible factor 1 (HIF-1) whose targets include the genes for erythropoietin, vascular endothelial growth factor, and glycolytic enzymes [3]. HIF-1 DNA-binding activity and activation of hypoxia-responsive genes have been shown to occur via a mitochondrion-dependent signaling process involving the generation of reactive oxygen species [4]. In *Saccharomyces cerevisiae*, the genes *OLE1* (Δ-9 fatty acid desaturase), and *CYC7* (iso-2-cytochrome *c*) are two hypoxic genes that have been found to require a functional respiratory chain and cytochrome *c* oxidase for their induction by anoxia [5].

Genome-wide transcriptional analysis of petite yeast cells indicates that many nuclear genes (~4%) are affected by the absence of mitochondrial DNA [6,7]. The best

☆ *Abbreviations: GAL1*, the gene encoding the yeast galactokinase; MTP, mitochondrial-targeting N-terminal peptide; T-*urf13*, the gene encoding the maize URF13 protein.

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system of interorganellar communication between nucleus and mitochondria described in budding yeast is the retrograde signaling (RTS) in which the expression of some nuclear genes is altered in cells with dysfunctional mitochondria [8]. One example of such genes is the gene encoding the peroxisomal isoform of citrate synthase, *CIT2*. The transcript of the *CIT2* gene is up-regulated as a result of alterations in mitochondrial function due to the addition of respiratory inhibitors, in mutants lacking mtDNA (ρ^0 petites) and in cells in which the mitochondrial form of the citrate synthase, *CIT1*, is deleted [9,10]. The RTS allows cells to adjust their glutamate supplies in response to the functional state of mitochondria [8].

Mitochondrial control of nuclear gene expression has also been described in filamentous fungi. In *Trichoderma reesei*, the transcriptional expression of cellulase genes was found to be sensitive to the functional state of the mitochondria. The transcripts of these genes are down-regulated by chemical agents known to inhibit mitochondrial activity and by oxygen limitation in the culture medium [11].

An excellent example in plants of the involvement of mitochondria in cytoplasmically inherited sterility and sensitivity to environmental pathogens has been described in maize [12]. A mitochondrial gene, T-*urf13*, is unique to *cms-T* maize, which displays cytoplasmic male sterility, and was found to produce a 13 kDa polypeptide, URF13, which is a component of the inner mitochondrial membrane. Sequence analysis indicates that T-*urf13* originated by recombination between a chloroplast gene encoding a tRNA for arginine and two mitochondrial genes: 26S ribosomal RNA and ATPase subunit 6 [13]. This novel gene was found to be associated with cytoplasmic male sterility, and sensitivity to a toxin (T-toxin), produced by the fungal pathogen *Bipolaris maydis*, and to the carbamate insecticide methomyl [14].

Glucose is the primary substrate for the production of energy in the form of ATP in eukaryotic microorganisms. In *S. cerevisiae*, glucose represses the expression of genes that are required for the metabolism of alternative carbon sources, such as galactose [15]. Glucose also represses mitochondrial activity, including proteins required for the flow of electrons and protons through the respiratory pathway [16]. Galactose utilization requires both induction by galactose and the absence of repressing concentrations of glucose. Because mitochondria are physiologically and metabolically functional under these conditions, we asked if expression of the genes involved in galactose utilization in *S. cerevisiae* actually requires active respiration by the organelles.

To address this question, we analyzed the expression of the galactokinase gene, *GAL1*, of *S. cerevisiae*. This gene is coordinately regulated by a complex interplay between a DNA-binding transcriptional activator (Gal4p), a repressor (Gal80p), and a sensor/inducer (Gal3p) that activates transcription of the *GAL* genes in the presence of galactose and ATP [17,18]. Here, we report that expression of *GAL1* is affected by alteration in mitochondrial activity. Functional expression of the maize protein URF13 leads to a reduction of the mitochondrial membrane potential and concomitantly to inhibition of *GAL1* gene expression, in the presence of galactose.

Materials and methods

Yeast strains, plasmids, and growth conditions. The *S. cerevisiae* strains used in this study were W303-1A (*MATa*, ρ^+, *ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1*) [19] and a *gal2Δ* isogenic derivative strain. *GAL2* gene disruption was achieved by integrating the KanMX4 cassette into the *GAL2* locus. Cells were routinely grown at 30 °C in YP medium (1% yeast extract, 2% bacto peptone) with 2% glucose, 2% galactose or 5% glycerol, or in YNB medium (0.67% yeast nitrogen base) supplemented with the required nutritional markers and carbon sources. To test the effect of methomyl on *GAL1* gene expression, a solution of 1 M methomyl (Du Pont) in ethanol was prepared and diluted to a final concentration of 6 mM in the culture media.

The two plasmids containing the T-*urf13* gene, with or without a mitochondria-targeting peptide [20], pEMBLYe30/2-21urf13-TW and pEMBLYe30/2urf13-TW, respectively, were generously provided by Dr. N. Glab (Centre de Génétique Moléculaire, C.N.R.S. UPR2420, associé à l'Université Pierre et Marie Curie, France). Yeast transformation was done according to a previously described procedure [39].

Total RNA isolation and Northern blot analysis. Total RNA was isolated from exponentially growing yeasts as described before [21]. For Northern blot analysis, aliquots containing 10 μg total RNA were fractionated electrophoretically in a 1.2% agarose gel, transferred to a Zeta Probe membrane (Bio-Rad), and hybridized with either *GAL1* or *ACT1* probes following membrane manufacturer's instructions.

Flow cytometry analysis. Exponentially growing cells were stained with rhodamine 123 (final concentration 10 μg/ml) for 30 min at 30 °C, under strong agitation (300 rpm), washed twice with YP, and diluted 1:10 in the same medium without rhodamine prior to analysis by flow cytometry. Rhodamine 123 is an indicator of the mitochondrial membrane potential, and thus serves as a probe for the functional state of the mitochondria. Experiments were performed in a FACStar Plus (Becton–Dickinson), equipped with an argon laser. The fluorescence intensity of rhodamine 123 was measured at 535 nm. Data acquisition was done by LYSYS II version 1.1, HP Pascal 3.22 software. The number of cells analyzed per experiment was fixed at 10,000. Flow cytometry results were analyzed by using the software WinMDI (http://facs.scripps.edu).

Analysis of galactose uptake. Sugar uptake by whole cells was measured as previously described [22]. Briefly, cells were grown to logarithmic phase on either 2% glucose or 2% galactose and then harvested. Cultured cell suspensions (15 mg of cell dry weight/ml) were incubated with radioactively labeled D-[^{14}C]galactose (purchased from Du Pont, NEN Research Products, Boston, MA) at 30 °C for 30 s. Triplicate 100 μl samples were removed, filtered through tared 25 mm diameter 0.45 μm pore-size Millipore nylon filters, soaked in 26% galactose for preventing unspecific binding [23], and washed with 5 ml ice-cold deionized water. The filters were then placed in a 20 ml borosilicate liquid scintillation vial containing 5 ml of Packard Optifluor scintillation cocktail, and the radioactivity was measured in a Packerd C 1600 TR (USA) liquid scintillation counter.

Results

The W303-1A working strain does not contain an imp1 allele

It has been previously shown that in some genetic backgrounds, such as S288C-derived yeast strains, a defect in the galactose transporter gene *GAL2* (*imp1* mutants) leads

to impairment in galactose uptake, and consequently the cells require functional mitochondria for growth on galactose [24–26]. These mutants, however, can still induce *GAL* genes [25,27–30] because galactose enters the cells through other hexose transporters (encoded by *HXTs* genes) with lower affinity and capacity for galactose [22,31,32]. As a consequence *imp1* mutants strains grow slower on galactose and growth becomes dependent on mitochondrial activity and is thus severely impaired in the presence of mitochondrial inhibitors, such as antimycin A.

The W3031-A strain used in this work is considered to have a wild-type *GAL2* gene [28]. Nevertheless, we decided to characterize this strain to exclude the possibility of working with an *imp1* mutant. To that end, we constructed an isogenic strain of W3031-A in which the *GAL2* gene was deleted and compared both strains for their galactose uptake activity and also their ability to grow on galactose in the presence of antimycin A.

A clear galactose-inducible galactose uptake activity was observed in wild-type cells, while this activity was significantly reduced when the *GAL2* gene was deleted from the genome (Fig. 1A). In addition, growth of the *gal2Δ* strain on galactose and in the presence of antimycin A was severely reduced (Fig. 1B). These results show that our working strain has indeed a wild type *GAL2* gene.

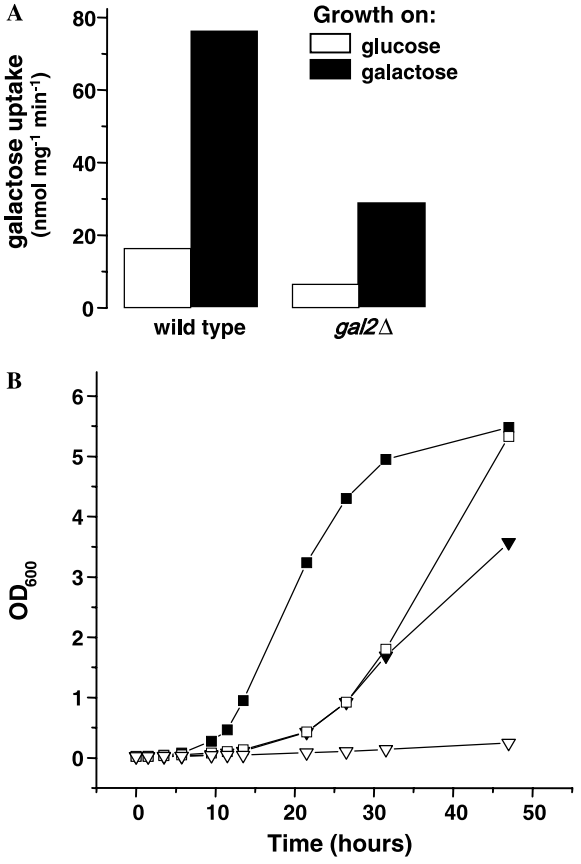

Fig. 1. Yeast strain W303-1A has a wild type *GAL2* gene. (A) Cells from W303-1A and a *gal2Δ* isogenic derivative strain were pre-grown on YP in the presence of 2% of the indicated carbon sources. Galactose uptake was then measured as described in Materials and methods. The data represented are the average of three independent experiments. (B) Growth curves of the W303-1A strain in the absence (■–■) and the presence (□–□) of antimycin A; *gal2Δ* strain in the absence (▼–▼) and the presence (▽–▽) of the drug. Antimycin A was added to the medium to a final concentration of 10 μg/ml.

Glucose lowers the mitochondrial membrane potential and represses expression of the GAL1 gene

Glucose is a major effector of the metabolic and physiological state of *S. cerevisiae* cells. Glucose represses mitochondrial respiration [33] and mitochondrial gene expression [16]. Because mitochondrial activity is repressed in cells growing on culture media containing glucose, while in those growing on a non-fermentable carbon source, such as glycerol, the organelles are fully active, we analyzed the kinetics of induction of the *GAL1* mRNA in cells cultured under both conditions. The *GAL1* transcript was rapidly induced, within 20 min of the addition of galactose, in cells grown on glycerol; in contrast, cells grown on glucose, washed and then induced with galactose, expressed the *GAL1* transcript only after 2–3 h (Figs. 2A and B). It is important to mention that the concentration of glucose was below the detection limit in both cultures before the addition of galactose (data not shown). Fig. 1C shows the effect of glucose, glycerol, and sodium azide on mitochondrial activity, as judged by measuring the membrane potential with rhodamine 123 using flow cytometry analysis. The mitochondrial membrane potential is strongly repressed by 2% glucose compared to that measured in glycerol-grown cells, and is equivalent to that measured in the presence of sodium azide, an inhibitor of complex IV of the electron transport chain [34]. The result indicates that triggering of the *GAL1* gene expression may depend on the metabolic state of the mitochondria; cells with repressed mitochondrial activity induce *GAL1* gene much more slowly than those with active mitochondria.

Functional expression of the maize URF13 down-regulates expression of the GAL1 gene

The T-*urf13* gene, in *cms-T* maize, encodes a 13 kDa polypeptide that is translocated into the mitochondrial inner membrane. An interaction between the carbamate insecticide methomyl and URF13 results in permeabilization of the inner mitochondrial membrane [12]. Expression of the T-*urf13* gene in *S. cerevisiae* has also been shown to confer methomyl sensitivity on mitochondria, resulting in the inhibition of respiratory growth and the inability of yeast cells to grow on glycerol as the sole carbon source [20,35]. Because URF13 is a naturally occurring protein, and unlike the oxidative phosphorylation inhibitors is not toxic to the cell [20], we analyzed the expression of the *GAL1* gene under controlled conditions in which URF13 is functionally active. Two plasmids coding for URF13, with or without a mitochondrial-targeting peptide, were used in this work [20]. The first plasmid, pEMBLYe30/

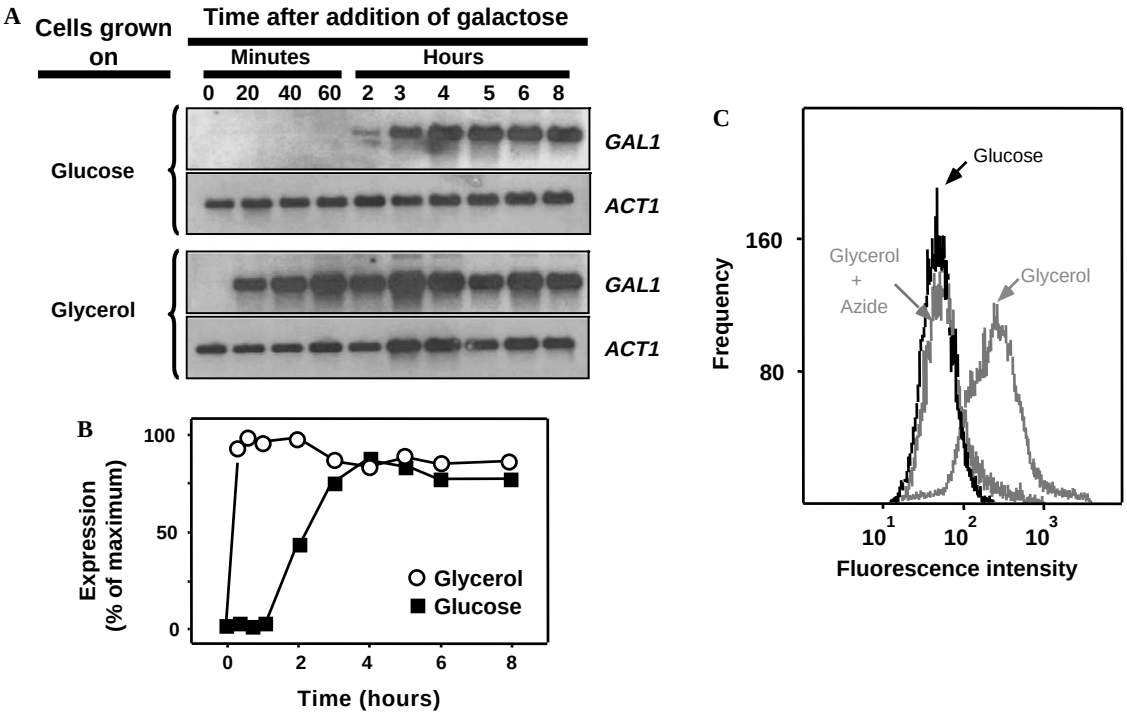

Fig. 2. Effect of glucose on the expression of the *GAL1* transcript and on mitochondrial membrane potential. (A) Cells were grown on 2% glucose or 5% glycerol, as indicated, washed and then induced with 2% galactose. Aliquots of the culture were withdrawn at different times after the addition of galactose and total RNA (10 μg) was isolated, fractionated electrophoretically on a 1.2% agarose gel, transferred to a Zeta Probe membrane, and hybridized with labeled probes as indicated. The actin (*ACT1*) transcript is included as a control. (B) The autoradiograms shown in (A) were scanned with a densitometer and data were normalized relative to the corresponding values obtained with actin; the data are plotted as the percentage of maximum values versus time after the addition of galactose. (C) Yeast cells were grown in YP medium in the presence of 5% glycerol for 12 h, washed twice in the same medium, and then incubated for 2 h in YP media containing 5% glycerol, 2% glucose or 5% glycerol in the presence of 65 μg/ml NaN_3. Flow cytometry measurements of rhodamine-stained cells were performed as described in Materials and methods.

2urf13-TW (referred to hereafter as pURF13), contains the coding sequence of T-*urf13* flanked by the promoter and terminator of the yeast phosphoglycerate kinase gene (*PGK1*). This plasmid should encode a polypeptide, URF13, which will not be targeted to the mitochondria and therefore serves as a control. The second plasmid, pEMBLYe30/2-21urf13-TW (referred to as pMTP-URF13), is identical to the first plasmid, except that the T-*urf13* coding sequence is preceded by a mitochondrial-targeting N-terminal peptide (MTP) of the nuclear gene ATPase subunit 9 of *Neurospora crassa* (see Fig. 3B) [20]. To determine whether URF13 affects mitochondrial activity in the presence of methomyl, yeast transformants containing pMTP-URF13 were grown on glycerol medium, and the mitochondrial membrane potential was monitored by flow cytometry after the addition of sodium azide or methomyl. The addition of sodium azide to respiring cells grown on glycerol, in which 85% of the population show a high mitochondrial membrane potential (Fig. 3A), results in a drastic drop in the potential: 91% of the cells now show a low mitochondrial membrane potential. The effect of activating URF13 on the mitochondrial membrane potential was then analyzed by the addition of methomyl to a final concentration of 6 mM. As shown in Fig. 3A, the addition of methomyl resulted in 37% of cells in a low mitochondrial membrane potential state. This result indicates that activating URF13 reduces the mitochondrial membrane potential. It is important to mention that we observed no significant difference in growth rate between cells transformed with pMTP-URF13 treated with methomyl and control cells (no added methomyl) after switching to a medium containing no methomyl (data not shown).

To analyze the effect of activation of URF13 on the expression of the *GAL1* transcript, cells transformed with pURF13 or pMTP-URF13 were grown on glucose, shifted to a medium with no carbon source (YP), treated with methomyl for 1 h, and then induced with galactose, and aliquots were withdrawn at different times for *GAL1* mRNA analysis. Figs. 3B and C show the kinetics of induction of the *GAL1* transcript in both transformants. Derepression of the *GAL1* transcription was strongly inhibited in cells in which URF13 was targeted to the mitochondria. This result indicates that alteration of mitochondrial activity by activation of URF13 results in impairment of the induction of the *GAL1* gene.

Discussion

The mitochondrion is an important organelle in which a variety of cellular functions are carried out, including the oxidation of pyruvate and other fuels by the tricarboxylic

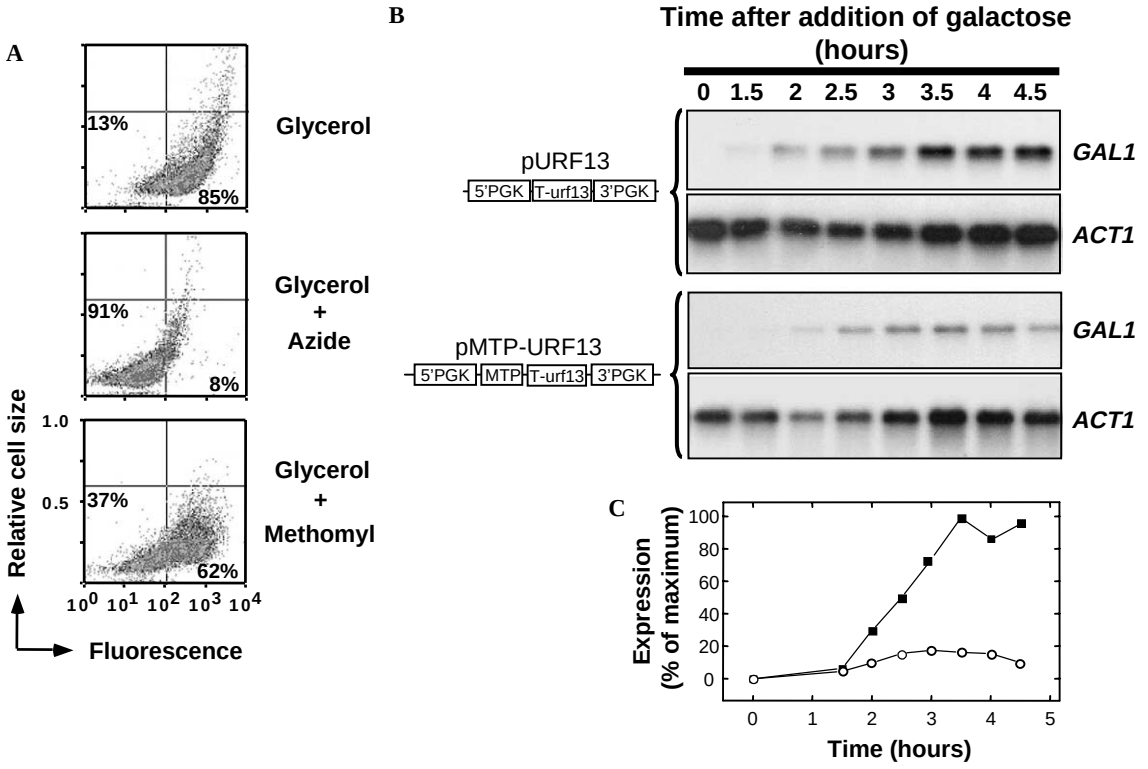

Fig. 3. Effect of the maize URF13 protein and methomyl on the derepression of *GAL1*. (A) Mitochondrial membrane potential was measured in cells in which the URF13 was directed to the inner mitochondrial membrane. Cells were transformed with pMTP-URF13 containing the T-*urf13* coding sequence preceded by the mitochondrial-targeting N-terminal peptide (MTP) of the nuclear gene ATPase subunit 9 of *N. crassa*. Cells were grown in YNB in the presence of glycerol and then incubated for 30 min in the presence 65 μg/ml sodium azide or 6 mM methomyl, as indicated. Membrane potential was measured by flow cytometry analysis of rhodamine-stained cells (see Materials and methods for details). (B) Cells transformed with the vector pURF13 or pMTP-URF13 were pre-grown on YNB in the presence of 2% glucose, washed, resuspended in YNB and incubated for 1 h in the presence of 6 mM methomyl, and then induced with 2% galactose. RNAs were isolated from cells collected at the indicated times, and 10 μg of RNA samples was fractionated and hybridized with labeled probes as indicated. (C) The autoradiograms shown in (B) were measured by densitometry and normalized with reference to the corresponding values obtained with actin (■–■, pURF13; ○–○, pMTP-URF13). The data are plotted as the percentage of maximum values versus time after the addition of galactose.

acid (TCA) cycle, electron transfer and respiration, the synthesis of ATP, and the biosynthesis of numerous amino acids, lipids, and heme [36]. Although mitochondria were considered as specialized organelles performing these processes many years ago, it has been recognized that mitochondria also play a central role in the regulation of a variety of physiological and metabolic events in the cell. To extend our knowledge of genes controlled by mitochondrial activities, we asked if interorganellar communication plays a role in the expression of genes involved in the metabolism of alternative carbon sources. In this study, we analyzed the effect of perturbation or reduction of mitochondrial activity on the expression of one of the *GAL* genes, *GAL1*.

The kinetics of induction of the *GAL1* transcript by galactose differ significantly in cells grown on fermentable and non-fermentable carbon sources, such as glucose or glycerol, respectively. When cells were grown on 2% glucose and then induced with galactose, the *GAL1* transcript was detected after 2 h. In contrast, in cells grown on 5% glycerol, the transcript was readily detected after 20 min of induction by galactose. To analyze the effect of glucose on mitochondrial activities, we measured the mitochondrial membrane potential using flow cytometry analysis. The results show that glucose lowers the mitochondrial membrane potential to a value comparable to that obtained in the presence of sodium azide, an inhibitor of the electron transport chain. In contrast, in the presence of glycerol the yeast cells have a high mitochondrial membrane potential. If expression of the *GAL1* gene requires active mitochondria, then the observed delay in the derepression of cells grown on glucose could be the time required for the organelle to attain its full physiological capacity. It is important to note that this delay in the derepression of the *GAL1* transcript, in cells grown on glucose, is not due to a lack of the activator of the *GAL* system, Gal4p, whose expression is also repressed by glucose. This is based on the fact that the kinetics of induction of *GAL1* expression in a mutant with constitutive *GAL4* expression is as slow as in wild-type strain [37]. To analyze if derepression of *GAL1* requires active organelles, we used a novel approach, in which the maize URF13 protein was functionally expressed in the inner mitochondrial membrane of yeast cells.

Methomyl interacts with URF13 and results in permeabilization of the inner mitochondrial membrane [12]. Addition of methomyl to cells carrying URF13 targeted

to mitochondria results in a decrease of the mitochondrial membrane potential and partially inhibits the induction of the *GAL1* transcript by galactose. Therefore, perturbation of mitochondrial activities blocks galactose-induced *GAL1* gene expression.

The gene *imp1* is a partially defective allele of *GAL2*. This allele is found in many but not all yeast laboratory strains. It has been shown that the *imp1* allele causes significantly impaired galactose uptake and a phenotype which depends on mitochondrial function for growth on galactose [24].

Our results show that the W303-1A strain used in the present work harbors a normal *GAL2* gene, as previously shown by other groups [26,28]. Thus, the lack of induction of the *GAL1* gene observed when mitochondrial activity was impaired is a defect in galactose metabolism due to impaired galactose uptake. Our results are in accordance with a recent report that indicates that galactose metabolism, including *GAL1* induction by galactose, is impaired by iron deficiency in yeast cells, which also leads to respiratory deficiency and defective mitochondrial function [38].

Our results demonstrate that repression of mitochondrial activity inhibits expression of the *GAL1* transcription; conversely, expression of *GAL1* requires active mitochondria. Because glucose represses mitochondrial activity, it is tempting to speculate that glucose repression of the genes required for the utilization of alternative carbon sources, like galactose, is partially mediated by mitochondrial activity.

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