Arsenic stress elicits cytosolic Ca\(^{2+}\) bursts and Crz1 activation in *Saccharomyces cerevisiae*

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Although arsenic is notoriously poisonous to life, its utilization in therapeutics brings many benefits to human health, so it is therefore essential to discover the molecular mechanisms underlying arsenic stress responses in eukaryotic cells. Aiming to determine the contribution of Ca\(^{2+}\) signalling pathways to arsenic stress responses, we took advantage of the use of *Saccharomyces cerevisiae* as a model organism. Here we show that Ca\(^{2+}\) enhances the tolerance of the wild-type and arsenic-sensitive *yap1* strains to arsenic stress in a Crz1-dependent manner, thus providing the first evidence that Ca\(^{2+}\) signalling cascades are involved in arsenic stress responses. Moreover, our results indicate that arsenic shock elicits a cytosolic Ca\(^{2+}\) burst in these strains, without the addition of exogenous Ca\(^{2+}\) sources, strongly supporting the notion that Ca\(^{2+}\) homeostasis is disrupted by arsenic stress. In response to an arsenite-induced increase of Ca\(^{2+}\) in the cytosol, Crz1 is dephosphorylated and translocated to the nucleus, and stimulates CDRE-driven expression of the *lacZ* reporter gene in a Cnb1-dependent manner. The activation of Crz1 by arsenite culminates in the induction of the endogenous genes *PMR1*, *PMC1* and *GSC2*. Taken together, these data establish that activation of Ca\(^{2+}\) signalling pathways and the downstream activation of the Crz1 transcription factor contribute to arsenic tolerance in the eukaryotic model organism *S. cerevisiae*.

INTRODUCTION

Arsenic (As) is a metalloid naturally occurring in the Earth’s crust. Highly toxic arsenic ion species are released into the environment primarily through leaching from geological formations into aquifers, although anthropogenic sources also account for environmental contamination. Although it is used in therapeutics, chronic exposure to arsenic compounds constitutes a global health problem and has been associated with many diseases, including cancer (Tseng, 2007). It is therefore important to clarify the cellular and molecular mechanisms involved in the response of eukaryotic cells to arsenic stress. In this context, the remarkably high degree of conservation between *Saccharomyces cerevisiae* and higher eukaryotes makes yeast a valuable model organism to unravel the complex and fundamental mechanisms underlying arsenic stress responses.

Eukaryotic cells, from yeast to humans, respond promptly and precisely to adverse stimuli by a complete reprogramming of gene expression. This process is tightly orchestrated by specific transcription factors, which mediate the induction of genes conferring protective activity. Indeed, microarray transcriptional profiling of yeast cells exposed to arsenic compounds performed by us and others has revealed that many cellular pathways, including those involved in oxidative stress defence, redox maintenance, glutathione biosynthesis and arsenic detoxification, are enriched in arsenic-treated cells (Haugen et al., 2004; Thorsen et al., 2007; Menezes et al., 2008). The activity of Yap1, a member of the YAP (yeast AP1-like) family of transcription factors, has been shown to be essential to regulate the induction of reactive oxygen species (ROS) homeostasis machinery in cells exposed to arsenic stress (Menezes et al., 2004, 2008). Although multiple arsenic adaptation pathways, involving transcription factors such as Yap8, Met4, Hog1 and Abf1, have been described (Rodrigues-Pousada et al., 2010), the complex mechanism of arsenic stress responses is still far from being completely understood.

Calcium ion (Ca\(^{2+}\)) is an essential element playing a central role as intracellular messenger in eukaryotic cells.

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Abbreviations: CaM, calmodulin; CaN, calcineurin; CDRE, CaN-dependent response element; c.p.s., counts per second; CsA, cyclosporin A; ER, endoplasmic reticulum; qPCR, quantitative PCR; RLU, relative luminescence units.

The microarray data discussed in this paper are available from the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE33427.

Two supplementary figures and two supplementary tables are available with the online version of this paper.
The utilization of Ca\(^{2+}\) to regulate a wide range of cellular processes, in response to a variety of environmental insults, is a strategy exploited by virtually all eukaryotic organisms. In yeasts, it has been reported that exposure of cells to many stress conditions, including iron overload and ethanol shock, disrupts Ca\(^{2+}\) homeostasis (Batiza et al., 1996; Kanzaki et al., 1999; Matsumoto et al., 2002; Peiter et al., 2005; Araki et al., 2009; Popa et al., 2010; Li et al., 2011). However, in some conditions such as H\(_2\)O\(_2\), it has been shown that the zinc-finger transcription factor Crz1. Like the mammalian orthologue NFAT (Yoshimoto et al., 2001), CaN is a highly conserved serine/threonine phosphatase involved in the biosynthesis of the cell wall, Gsc2. The stress-induced increase of cytosolic free Ca\(^{2+}\) is triggered by either the mobilization of extracellular sources, mainly via the high-affinity Ch1/Mid1 channel (Matsumoto et al., 2002; Peiter et al., 2005; Popa et al., 2010), or the release of vacuolar sources through the Yvc1 ionic channel (Denis & Cyert, 2002). Homeostasis is restored by the sequestration of Ca\(^{2+}\) into the vacuole, through the action of the Ca\(^{2+}\)-ATPase Pmc1 and the Ca\(^{2+}\)/H\(^{+}\) exchanger Vcx1 (Palmer et al., 2001). Alternatively, Pmr1 and Cdr1 can direct Ca\(^{2+}\) to the secretory pathway in the endoplasmic reticulum (ER) and Golgi. Under conditions of high cytosolic Ca\(^{2+}\) concentrations, the essential Ca\(^{2+}\) receptor protein calmodulin (CaM) binds to Ca\(^{2+}\), undergoing a conformational change that allows the activation of target proteins such as the CaM-regulated kinases Cmk1/Cmk2 and calcineurin (CaN) (Cyert, 2001). CaN is a highly conserved serine/threonine phosphatase composed of a catalytic and a regulatory subunit, encoded by CNA1 and CNB1, respectively, the latter being essential for enzyme activity and induction by CaM (Cyert et al., 1991; Cyert & Thorner, 1992; Cyert, 2003). CaN carries out multiple functions in yeast, in particular the regulation of the zinc-finger transcription factor Crz1. Like the mammalian orthologue NFAT (Yoshimoto et al., 2002), upon dephosphorylation by CaN, Crz1 rapidly relocates to the nucleus (Stathopoulos-Gerontides et al., 1999) and mediates transcriptional activation through the CDRE (CaN-dependent response element). PMC1 and PMR1, encoding Ca\(^{2+}\) transporters, and GSC2, encoding a cell wall biosynthetic enzyme, are among the Crz1 target genes (Matheos et al., 1997; Stathopoulos & Cyert, 1997; Denis & Cyert, 2002).

Interestingly, our recent microarray data suggest that arsenic stress affects the expression of genes involved in Ca\(^{2+}\) signalling pathways, and these findings prompted us to investigate the role of the Ca\(^{2+}\) ion in the yeast stress response to arsenite. Here we report that arsenite disrupts Ca\(^{2+}\) homeostasis and triggers the activation of Crz1, which in turns regulates the induction of genes encoding the Ca\(^{2+}\) transporters Pmr1 and Pmc1, and encoding a protein involved in the biosynthesis of the cell wall, Gsc2.

**METHODS**

**Bacterial and yeast strains, plasmids and growth conditions.**

The *Escherichia coli* strain XL1-Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZAM15 Tn10 (Tetr)]] (Stratagene, Agilent Technologies) was used as the host for routine plasmid amplification purposes. Outgrowth was performed in super optimal broth with catabolite repression (SOC) medium at 37 °C. Plasmids were selected on LB plates supplemented with 100 µg ampicillin ml\(^{-1}\).

A list of *Saccharomyces cerevisiae* strains and oligonucleotide primers used in this work is presented in Tables 1 and S1, available with the online version of this paper, respectively. The microhomology PCR method (Güldener et al., 1996) was used to generate the double mutant strains as follows: (1) *crz1Δcrz1*, deletion of the *CRZ1* coding region in the *cni1* mutant; (2) *yap1Δcrz1*, deletion of the *CRZ1* coding region in the *yap1* mutant; and (3) *YAA3-Δyap1*, deletion of the complete *YAP1* coding region in the *YAA3* strain. The double mutant *yap1Δcrz1* was generated through the transformation of linearized plasmid pFL1 (encoding a *YAP1* version in which the HIS3 gene was inserted in the internal *RamH* of *YAPI*) in the *cni1* strain. Gene disruptions were confirmed by PCR analysis of genomic DNA using upstream (A1) and downstream (A4) specific primers (Table S1). pEVPI1-Apopeaquorin (Batiza et al., 1996) was used to measure cytosolic free Ca\(^{2+}\) levels. Strains were grown in synthetic medium (SM; 0.67 % (w/v) ammonium sulfate/yeast nitrogen base without amino acids, 2 % (w/v) glucose, supplemented with 20 µg L-histidine-HCl monohydrate ml\(^{-1}\), 60 µg leucine ml\(^{-1}\), 20 µg uracil ml\(^{-1}\) and 20 µg L-methionine ml\(^{-1}\)). Selective synthetic media were prepared omitting the amino acid corresponding to the auxotrophic marker. For solid media, agar was added to a final concentration of 2 % (w/v). All spectrophotometric measurements of yeast biomass were performed using a polystyrene 45 mm path-length cuvette and the Bio-Rad spectrophotometer. Exponential growth phase cells were attained through the dilution of overnight cultures to an OD\(_{600}\) 0.1 ± 0.01 in fresh media followed by incubation to OD\(_{600}\) 0.5 ± 0.05. For each experiment cells were treated with NaAsO\(_2\) (Sigma-Aldrich) and/or CaCl\(_2\) (Merck) under the conditions indicated in the respective figures. For RNA extraction, samples were washed with appropriate buffers and stored at −80 °C. Standard methods were used for genetic analysis, transformation and gene disruption procedures (Ausubel et al., 1995).

**Growth assays.** Phenotypic growth assays were carried out by spotting 5 µl of sequentially diluted cultures (approx. 5 x 10\(^{-1}\), 5 x 10\(^{-2}\), 5 x 10\(^{-3}\), 5 x 10\(^{-4}\) and 1 x 10\(^{-5}\) cells) in SM containing up to 1.5 mM NaAsO\(_2\) and/or 50 mM CaCl\(_2\). Growth was recorded after 2 days of incubation at 30 °C. To monitor cell growth in liquid media early exponential phase cultures (OD\(_{600}\) 0.5 ± 0.05) were diluted to 0.1 ± 0.01 and treated with 1.5 mM NaAsO\(_2\). The cultures were incubated for 28 h at 30 °C with orbital agitation (200 r.p.m.) and the OD\(_{600}\) was monitored at intervals of 2 h.

**Microarray analysis.** Total RNA was isolated as described elsewhere (Puig et al., 2005) from exponentially growing BY4741 yeast cells that were either exposed or not to 2 mM sodium arsenite for 60 min; 50 µg total RNA was sent for labelling and hybridization to Affymetrix Yeast Genome S98 arrays. For further information about sample preparation, please see the Duke Microarray Core Facility at http://www.genome.duke.edu/cores/microarray/. The data were analysed by using both Partek Genomics Suite and dChip softwares.

**In vivo monitoring of the Ca\(^{2+}\) pulse induced by arsenic stress.** Monitoring of cytosolic Ca\(^{2+}\) was performed using the apopeaquorin reporter system (Batiza et al., 1996). For this purpose, the yeast strains BY4742 and *yap1* were transformed with plasmid pEVPI1-Apopeaquorin, kindly provided by Dr Patrick H. Masson. For the luminescence assays, stationary phase pre-cultures were diluted to OD\(_{600}\) 0.1 ± 0.01 in fresh SM and grown to OD\(_{600}\) 1 ± 0.1. To reconstitute functional aequorin, 50 µM native coelenterazine (Sigma; dissolved in methanol) was added to the cell suspension and the cells were incubated for 2 h at 30 °C in the dark. Cells were harvested by...
centrifugation and washed three times with PBS (10 mM phosphate, pH 7.4, 138 mM NaCl, 2.7 mM KCl) to remove excess coelenterazine. The pellet was resuspended in SM and the cell suspension was transferred to a 96-well microplate. Before the induction of arsenic stress, the baseline luminescence was determined by 1 min of recording at 10 s intervals using a Perkin Elmer Victor3 luminometer. The pellet was resuspended in SM and the cell suspension was centrifuged and washed three times with PBS (10 mM phosphate, 1.5 mM KCl). Immediately after centrifugation, permeabilized with chloroform and assayed for enzyme activity using a BioTek Epoch microplate spectrophotometer. Miller units were calculated as described elsewhere (Miller, 1972). Experiments were performed using biological triplicates and the SD of the mean is shown.

**RESULTS AND DISCUSSION**

**Table 1. S. cerevisiae strains used in this work**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4742</td>
<td>MATa his3 leu2 lys2 ura3</td>
<td>EUROSCARF*</td>
</tr>
<tr>
<td>crz1</td>
<td>MATa his3 leu2 lys2 ura3 YNL027W::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>cnb1</td>
<td>MATa his3 leu2 lys2 ura3 YKL190W::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>crz1cnb1</td>
<td>MATa his3 leu2 lys2 ura3 YKL190W::kanMX4 YNL027W::HIS3MX4</td>
<td>This study</td>
</tr>
<tr>
<td>yap1</td>
<td>MATa his3 leu2 lys2 ura3 YML007W::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>yap1crz1</td>
<td>MATa his3 leu2 lys2 ura3 YML007W::kanMX4 YNL027W::HIS3MX4</td>
<td>This study</td>
</tr>
<tr>
<td>yap1cnb1</td>
<td>MATa his3 leu2 lys2 ura3 YKL190W::kanMX4 YML007W::HIS3MX4</td>
<td>This study</td>
</tr>
<tr>
<td>BY4741</td>
<td>MATa his3 leu2 met15 ura3</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>mid1</td>
<td>MATa his3 leu2 met15 ura3 YNL291C::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>cch1</td>
<td>MATa his3 leu2 met15 ura3 YGR217W::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>YAA3</td>
<td>MATa his3::CRZ1-GFP-HIS3 leu2 ura3 met15</td>
<td>Araki et al. (2009)</td>
</tr>
<tr>
<td>YAA3-yap1</td>
<td>MATa his3::CRZ1-GFP-HIS3 leu2 ura3 met15 YML007W::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>YAA4</td>
<td>MATa his3::CRZ1-GFP-HIS3 leu2 ura3 met15 YKL190W::kanMX4</td>
<td>Araki et al. (2009)</td>
</tr>
<tr>
<td>YAA5</td>
<td>MATa his3 leu2 lys2 ura3 aur1::AUR1-C-4xCDRE-lacZ</td>
<td>Araki et al. (2009)</td>
</tr>
<tr>
<td>YAA6</td>
<td>MATa his3 leu2 lys2 ura3 YNL027W::HIS3MX4 aur1::AUR1-C-4xCDRE-lacZ</td>
<td>Araki et al. (2009)</td>
</tr>
<tr>
<td>YAA7</td>
<td>MATa his3 leu2 lys2 ura3 YKL190W::kanMX4 aur1::AUR1-C-4xCDRE-lacZ</td>
<td>Araki et al. (2009)</td>
</tr>
</tbody>
</table>

*EUROPean Saccharomyces Cerevisiae ARchive for Functional analysis.

β-Galactosidase assays. β-Galactosidase activity measurements in cells harbouring the CDRE–lacZ reporter construct were performed as previously described (Menezes et al., 2004). For this purpose, BY4742, cnb1 and crz1 exponential phase cells (OD600 0.5 ± 0.05), encoding the CDRE–lacZ reporter construction, were treated with either 1 mM NaAsO2 or 20 mM CaCl2 at 30 °C for 90 min. Cells were harvested by centrifugation, permeabilized with chloroform and assayed for enzyme activity using a BioTek Epoch microplate spectrophotometer. Miller units were calculated as described elsewhere (Miller, 1972). Experiments were performed using biological triplicates and the SD of the mean is shown.

Real-time PCR. RNA was extracted from early exponential phase cultures that were either untreated or exposed for 30 min to 1.5 mM NaAsO2. DNA was removed by on-column DNase I digestion (RNase-Free DNase Set, Qiagen). Total RNA (1 μg) was reverse-transcribed with Transcriptor Reverse Transcriptase (Roche Diagnostics). Quantitative PCRs (qPCRs) were performed in a LightCycler 480 Instrument (Roche), using LightCycler 480 SYBR Green 1 Master (Roche) and the oligonucleotides listed in Table S1. Relative standard curves were constructed for each gene, using triplicate serial dilutions of cDNA. The relative expression of the genes was calculated by the relative quantification method with efficiency correction, using LightCycler 480 Software 1. Actin was used as a reference gene. All assays were done in triplicate.

**RESULTS AND DISCUSSION**

Genome-wide transcriptional analysis reveals that arsenic stress affects the expression of Ca2+-related genes

Aiming to investigate the response of S. cerevisiae cells to mid-term arsenic exposure, we determined the mRNA expression profile of wild-type cells upshifted to 2 mM Na3HAsO4-enriched medium for 60 min. Besides the upregulation of genes included in functional categories...
previously shown to be targeted by arsenic compounds, such as sulphur/methionine metabolism and redox homeostasis (Haugen et al., 2004; Thorsen et al., 2007; Menezes et al., 2008), our transcriptomic analysis revealed a significant alteration of the expression profile of genes related to Ca\(^{2+}\) signalling pathways (Table S2). Despite several reports of genome-wide experiments, this is believed to be the first time that such a response has been detected, probably because none of the earlier studies addressed the mid-term response (60 min) induced by arsenate. Among these genes were CRZ1, encoding the master transcriptional regulator of Ca\(^{2+}\)-mediated signalling; RCN1 and CMP2, encoding proteins related to CaN function; CMD1, CMK2 and RCK2, encoding CaM and proteins directly regulated by CaM binding, respectively; and the calcium transporters encoded by MID1 and CCH1 (Table 2). Based on these results we hypothesized that Ca\(^{2+}\) signalling pathways could be involved in the response of yeast cells to arsenic stress.

### Exogenous Ca\(^{2+}\) increases arsenic tolerance in a Crz1-dependent manner

To address the contribution of Ca\(^{2+}\) signalling to arsenic stress responses we first examined the growth phenotypes of the wild-type, the arsenic-sensitive mutant yap1 (Menezes et al., 2004), and the respective isogenic crz1 and cnb1 knockout strains in synthetic media supplemented with CaCl\(_2\). All the strains revealed similar growth patterns both in the absence and in the presence of CaCl\(_2\) concentrations up to 100 mM (Fig. 1a, upper panels). The crz1 and yap1crz1 strains did not exhibit any growth defect under the Ca\(^{2+}\)-replete condition compared with the wild-type strain, indicating that the cells were not under Ca\(^{2+}\) stress. Indeed, all the strains showed normal growth in media containing up to 200 mM CaCl\(_2\) (Fig. S1). As described previously (Menezes et al., 2004), the yap1 mutant strain displayed high growth sensitivity to 1 mM As(III), whereas the wild-type strain was resistant to 1 mM arsenite and moderately sensitive to higher concentrations. Remarkably, arsenic-stressed wild-type and yap1 cells, simultaneously treated with 50 mM CaCl\(_2\), were clearly more tolerant to arsenite stress (Fig. 1a, middle and lower panels). Moreover, acquisition of tolerance mediated by Ca\(^{2+}\) was shown to be dose-dependent, as media supplementation with 100 mM CaCl\(_2\) further enhanced the growth of these strains in the presence of arsenite. Our results therefore show that an increase in the availability of exogenous Ca\(^{2+}\) partially relieves arsenic toxicity and that Ca\(^{2+}\) may be implicated in the adaptive response to arsenic stress.

The zinc-finger transcription factor Crz1 is a central player in the Ca\(^{2+}\) signalling cascade. To determine whether Ca\(^{2+}\) contributes to arsenic tolerance through the activation of Ca\(^{2+}\) signalling pathways, we monitored, simultaneously, the growth of the respective crz1 isogenic strains. First, we found that the crz1 mutant was moderately sensitive to 1 and 1.5 mM As(III) (see Fig. 1a, lower and middle panels, and Fig. 1b, c), revealing therefore a link between the Crz1 pathway and the arsenic stress responses in yeast. Second, when CRZ1 was disrupted in the yap1 strain, the Ca\(^{2+}\)-mediated phenotype recovery of the double mutant was compromised in the presence of 1 mM arsenite plus 100 mM CaCl\(_2\), when compared with the yap1 single mutant, being completely abrogated in medium supplemented with only 50 mM CaCl\(_2\). Finally, the growth recovery of crz1 in the presence of Ca\(^{2+}\) was not as efficient as for the wild-type strain under all the stress conditions tested. Taken together, these results provide strong evidence of the importance of Crz1 and Ca\(^{2+}\) signalling pathways to full adaptive S. cerevisiae arsenic stress responses.

Under moderate arsenic stress conditions (1 mM NaAsO\(_2\)), Ca\(^{2+}\) induced some degree of tolerance even in the absence of Crz1 (Fig. 1a, middle panel), implying that Ca\(^{2+}\) may also mediate arsenic tolerance through the activation of Crz1-independent targets of the Ca\(^{2+}\) signalling cascade. The CaM kinase Cmk2, the related protein kinase Rck2, the glutamate decarboxylase Gad1 and the ER-residing proteins Hph1/Hph2 are potential candidates, due to their protective functions under oxidative and cell wall stress (Cyert, 2001; Sánchez-Piris et al., 2002; Heath et al., 2004), which are known forms of cellular damage caused by arsenic stress (Menezes et al., 2008; Thorsen et al., 2009).

### Table 2. Ca\(^{2+}\)-related genes whose expression is affected by arsenic

The mean fold induction of triplicate samples is shown. A complete list of all Ca\(^{2+}\)-related genes whose expression is altered by arsenic compounds is shown in Table S2.

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
<th>Function</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) signalling</td>
<td>CRZ1</td>
<td>Transcription factor that activates transcription of genes involved in stress response</td>
<td>2.09</td>
</tr>
<tr>
<td>CaN complex</td>
<td>RCN1</td>
<td>Protein involved in CaN regulation during calcium signalling</td>
<td>2.78</td>
</tr>
<tr>
<td>Ca(^{2+}) binding</td>
<td>CMP2</td>
<td>CaN A; one isoform (the other is CNA1) of the catalytic subunit of CaN</td>
<td>1.90</td>
</tr>
<tr>
<td>CaM binding</td>
<td>CMD1</td>
<td>CaM; Ca(^{2+})-binding protein that regulates Ca(^{2+})-independent and -dependent processes</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>CMK2</td>
<td>CaM-dependent protein kinase</td>
<td>–1.99</td>
</tr>
<tr>
<td>Ca(^{2+}) transport</td>
<td>RCK2</td>
<td>Protein kinase involved in the response to oxidative and osmotic stress</td>
<td>–1.40</td>
</tr>
<tr>
<td></td>
<td>MID1</td>
<td>N-Glycosylated integral membrane protein of the ER membrane and plasma membrane</td>
<td>–1.64</td>
</tr>
<tr>
<td></td>
<td>CCH1</td>
<td>Voltage-gated high-affinity calcium channel</td>
<td>–1.77</td>
</tr>
</tbody>
</table>
Indeed, we found that CMD1, CMK2 and RCK2 were upregulated by arsenic in our transcriptomic analysis (Table 2).

Since the activation of Crz1 by Ca\(^{2+}\) is dependent on CaN function, we included in the phenotypic analysis the cnb1 mutant, which is defective in the regulatory subunit of the CaN complex. As reported before, inactivation of Cnb1 renders the phosphatase complex non-functional (Cyert, 2003). Curiously, the cnb1 mutant exhibited increased tolerance to high-dose arsenite stress compared with the wild-type strain, which was further increased when CaCl\(_2\) was added to the medium (Fig. 1a, lower panel). The double mutant yap1cnb1 displayed similar growth patterns under low-dose arsenite stress (Fig. S2). Although yap1cnb1 was very sensitive to 1 mM arsenite, its growth was rescued through supplementation of the medium with CaCl\(_2\). Under a high arsenite dose (1.5 mM), yap1cnb1 growth recovery occurred only when media were supplemented with 100 mM CaCl\(_2\) (Fig. 1a, middle and upper panels).

In order to understand the distinct behaviour of crz1 and cnb1 strains, we performed epistasis analysis. For this purpose, we constructed the double mutant crz1cnb1 and compared the growth phenotypes of the three strains exposed to arsenic stress in the presence and absence of CaCl\(_2\). The results in Fig. 1(d) clearly show that cnb1 mutation is dominant over crz1 mutation. This response is not specific to arsenic stress conditions, since cnb1 is also dominant over crz1 in the presence of high CaCl\(_2\) concentrations (see Fig. S1). These results were not surprising, since CaN is upstream of Crz1 in the Ca\(^{2+}\)/CaM/CaN signalling pathway, regulating its translocation into the nucleus under conditions that trigger the mobilization of Ca\(^{2+}\) in the cytosol. A plausible explanation for this phenotypic paradox may lie in the fact that CaN regulates many substrates and therefore carries out multiple functions in yeast (Cyert, 2001). Indeed, it has already been reported that CaN mutants display defects that are not mimicked by the crz1 mutant, which reinforces the participation of Crz1 in the regulation of additional yeast proteins (Cyert, 2003). Our results suggest that CaN is possibly exerting dual effects: (a) a protective function through the regulation of Crz1, and (b) the mediation of arsenic toxicity by either activating proteins conferring toxicity or repressing those contributing to tolerance.
Arsenite elicits a transient Ca\textsuperscript{2+} release into the cytosol

A transient increase in free cytosolic calcium is a mechanism used by eukaryotic cells to activate the Ca\textsuperscript{2+} signalling pathways and thus to regulate many cellular processes in response to specific environmental cues (Viladevall et al., 2004; Araki et al., 2009). Given that an excess of extracellular Ca\textsuperscript{2+} favours cell adaptation to arsenic stress and that Crz1 activity is required for arsenic tolerance, we next evaluated whether exposure to arsenite was accompanied by an increase of free cytosolic Ca\textsuperscript{2+} in media containing standard CaCl\textsubscript{2} concentrations (approx. 0.8 mM) (Abelovska et al., 2007). For this purpose we used the apoaequorin-based methodology, which relies on the induction of aequorin activity by Ca\textsuperscript{2+} in a dose-dependent fashion (Batiza et al., 1996). To spontaneously reconstitute functional aequorin, wild-type and yap1 cells transformed with the pEVP11-Apoaequorin plasmid were incubated in the presence of the cofactor coelenterazine as described in Methods. The baseline luminescence of both cultures, recorded for 10 min at 30 °C, did not increase over time (Fig. 2a, b, broken lines). The wild-type and yap1 cells responded promptly to an arsenite shock, displaying a sharp rise followed by a rapid fall in luminescence, thus reflecting arsenite-induced fluctuations in free cytosolic Ca\textsuperscript{2+} (Fig. 2a, b, solid lines). The Ca\textsuperscript{2+} pulse amplitude exhibited by the arsenic-sensitive mutant yap1 was slightly, but reproducibly, higher than that of the wild-type strain (compare Fig. 2a and b).

These results indicate that arsenic compounds disturb Ca\textsuperscript{2+} homeostasis and further support the hypothesis that the activation of the Ca\textsuperscript{2+} signalling cascade may represent a mechanism employed by yeast cells to deal with arsenic stress.

Under stress conditions, an increase of cytosolic free Ca\textsuperscript{2+} can be generated by the mobilization of extracellular or vacuolar sources. The results of the phenotypic analysis reported here indicate that the enhancement of extracellular Ca\textsuperscript{2+} availability promotes tolerance, suggesting that the arsenic-induced cytosolic Ca\textsuperscript{2+} burst might result from the mobilization of external sources. The facts that the mid1 mutant, which is defective in the plasma membrane high-affinity Ca\textsuperscript{2+} channel, is sensitive to arsenic compounds and that Ca\textsuperscript{2+} media supplementation did not efficiently improve the tolerance of this mutant to 1.5 mM As(III) (Fig. 2c), as happens with the wild-type strain, further support this hypothesis. Curiously, the cch1 mutant exhibited growth patterns similar to those of the wild-type strain (Fig. 2c), suggesting that the role of Chc1 is dispensable for the arsenic-mediated activation of Ca\textsuperscript{2+} signalling. The discrepancy between the two mutants, whose genes encode proteins that cooperate to form a high-affinity Ca\textsuperscript{2+} influx system, has already been described and is attributed to the fact that Mid1 can also operate independently of Chc1 (Popa et al., 2010; Li et al., 2011).

![Fig. 2. Arsenite stress elicits a transient cytosolic Ca\textsuperscript{2+} pulse. Wild-type BY4742 (a) and yap1 (b) cells expressing coelenterazine-reconstituted aequorin were injected or not injected with 1.5 mM As(III) directly in the luminometer plates and luminescence was recorded for 10 min. The arrows indicate the time of arsenite addition. Each determination was repeated at least three times with no significant variations. The dashed lines correspond to the baseline luminescence. RLU are expressed as c.p.s. per OD\textsubscript{600} unit. (c) Mid1 is required for Ca\textsuperscript{2+}-mediated enhancement of arsenic tolerance. Wild-type BY4741 and the isogenic mid1 and cch1 strains were grown to early exponential phase and spotted onto SM supplemented with As(III) and/or CaCl\textsubscript{2}. Growth was recorded after 48 h incubation at 30 °C. A representative experiment is shown.](image-url)
**Crz1 is induced by arsenite stress**

The stress-induced increase of cytosolic free Ca\(^{2+}\) leads to the formation of a Ca\(^{2+}\)/CaM complex that binds to and activates the serine-threonine protein phosphatase CaN. One of the main functions of CaN is to dephosphorylate Crz1, thus triggering its nuclear accumulation when cytosolic Ca\(^{2+}\) levels rise. To provide additional evidence corroborating the importance of Ca\(^{2+}\) signalling and Crz1 activity to arsenic stress responses, we monitored Crz1–GFP phosphorylation status in the wild-type and yap1 strains, and under conditions where CaN activity was specifically inhibited by CsA. As shown in Fig. 3(a), Crz1 was mainly phosphorylated under the control condition in the wild-type and yap1 mutant strain. Treatment of cells with As(III) induced a partial shift of the protein to a faster-migrating form in the SDS-PAGE gel. This form corresponded to the dephosphorylated GFP–Crz1, since it completely disappeared when cells were treated with the CaN inhibitor CsA. To provide further support of CaN-dependent Crz1 activation by arsenic, in vivo fluorescence microscopy was used to monitor Crz1–GFP dynamics in wild-type, yap1 and cnb1 cells subjected to arsenite stress. Crz1–GFP was found dispersed throughout the cytoplasm in the absence of arsenite (Fig. 3b). Exposure to 2.5 mM As(III) induced its rapid translocation into the nucleus of almost all wild-type and yap1 cells within the first 15 min of incubation. Arsenite-induced Crz1–GFP nuclear accumulation was completely abrogated in the cnb1 strain, which is devoid of the regulatory subunit of CaN. Altogether, the results here reported are consistent with the notion that arsenite mediates cytosolic Ca\(^{2+}\) release, leading to the induction of CaN, which in turn leads to Crz1 dephosphorylation and its consequent nuclear accumulation.

**Arsenite stimulates CDRE-dependent gene expression**

The Crz1 transcriptional activator regulates the expression of many genes whose products are involved in diverse cellular functions, and the DNA motif known as CDRE mediates the activation of the large majority of these genes (Denis & Cyert, 2002). To determine whether arsenite stimulates CDRE-driven gene expression we used a reporter construction in which four in-tandem CDRE cis-elements were positioned in the promoter controlling the expression of the lacZ gene (Araki et al., 2009). The β-galactosidase activity values observed for the wild-type strain treated with 20 mM CaCl\(_2\) for 90 min revealed a 10-fold increase compared with the control condition (Fig. 4). The absence of both Crz1 and Cnb1 abrogated the induction of the reporter gene, demonstrating the Ca\(^{2+}\) -responsiveness of the system and its dependence on the intact function of Crz1 and Cnb1. Exposure of wild-type cells to 1 mM arsenite led to an eightfold increase of β-galactosidase activity compared with the untreated condition (Fig. 4), which was a pattern of induction similar to that observed in the yap1 strain (data not shown). Remarkably, some degree of lacZ activation was noted in the crz1 mutant, whereas in cells devoid of cnb1, no β-galactosidase activity was detected (Fig. 4). These observations suggest that in response to arsenite an additional activator protein, whose activity seems to be influenced by the absence of CaN activity, may partially contribute to the induction of lacZ expression. Taken together, these results indicate that CaN-dependent Crz1 activation by arsenite has the potential to induce the expression of endogenous CDRE-regulated genes.

**PMR1, PMC1 and GSC2 are induced by arsenite stress**

To verify whether CaN/Crz1 activation by arsenite stimulates the activation of endogenous target genes under
the Ca$^{2+}$ signalling cascade, we monitored by qPCR the expression levels of the Crz1-regulated genes PMR1 and PMC1, involved in ion homeostasis, and GSC2, encoding a protein implicated in cell wall synthesis (Denis & Cyert, 2002). Fully supporting our hypothesis, the treatment of cells with arsenite induced expression of the three genes in the wild-type as well as in the yap1 strain (Fig. 5). Full activation of PMR1, PMC1 and GSC2 by arsenite was clearly dependent on Crz1 activity, although significant levels of PMR1 expression were still observed in the crz1 strain (Fig. 5a). These data are consistent with the b-galactosidase assay results that show that CDRE-regulated expression is partially dependent on Crz1 activity, although significant levels of PMR1 expression were still observed in the crz1 strain (Fig. 5a). These data are consistent with the β-galactosidase assay results that show that CDRE-regulated expression is partially dependent on Crz1 (Fig. 4), and are similar to those described in an earlier work (Araki et al., 2009). The comparison of PMR1 and PMC1 levels in the wild-type and yap1 strains revealed that expression of these genes was enhanced in the latter (Fig. 5a, b), suggesting either that the maintenance of Ca$^{2+}$ homeostasis in yap1 cells requires increased amounts of Pmr1 and Pmc1, or that Yap1 exerts a negative effect on the expression of these genes. The evidence showing that yap1 cells exhibited increased Ca$^{2+}$ pulse amplitude (Fig. 2) supports the former suggestion. Collectively, these results reinforce the hypothesis that arsenic stress induces a cytosolic Ca$^{2+}$ pulse that activates the Ca$^{2+}$ signalling pathways, which in turn culminates with the induction of at least PMR1, PMC1 and GSC2.

Concluding remarks

The deleterious effects and widespread distribution of the metalloid arsenic in the Earth’s crust have led organisms to develop complex adaptation mechanisms and protection systems that are highly conserved between mammals and yeasts. However, the mechanisms underlying arsenic tolerance/toxicity are still far from being deciphered. Yet, despite the toxic effects of arsenic exposure there has been a resurgence of interest in the utilization of arsenic trioxide (ATO) in the treatment of acute promyelocytic leukaemia (APL) (Kanzaki et al., 1999; Lallemand-Breitenbach et al., 2012). The mechanisms by which ATO selectively induces apoptosis of cancer cells are not yet fully understood;
however, it has been shown that ATO triggers ER stress responses and disturbs Ca\(^{2+}\) homeostasis (Binet et al., 2010). Thus, to improve the effectiveness of arsenic-derived drugs in cancer therapy it is essential to recapitulate the cellular and molecular processes affected by the treatment.

In this work we took advantage of the eukaryotic model yeast *S. cerevisiae* to investigate how Ca\(^{2+}\) signalling pathways contribute to arsenic stress responses in eukaryotic cells. We report for what we believe is the first time that an excess of extracellular Ca\(^{2+}\) relieves arsenic toxicity in two different genetic backgrounds of *S. cerevisiae*. Furthermore, we show that tolerance acquisition mediated by Ca\(^{2+}\) is dependent on the function of Crz1, directly implicating the Ca\(^{2+}\) signalling cascade in this process. Upon arsenic stress, the burst of Ca\(^{2+}\) in the cytosol triggers CaN-dependent Crz1 dephosphorylation and nuclear accumulation, thereby leading to the induction of CDRE-driven expression of the endogenous *PMR1*, *PMC1* and *GSC2* genes.

Although required for Crz1 activation, Cnb1 also seems to mediate arsenic toxicity, either by activating proteins that confer toxicity and/or by repressing those that contribute to tolerance. Further work is in progress in order to clarify this.

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