Ure2 Is Involved in Nitrogen Catabolite Repression and Salt Tolerance via Ca\(^{2+}\) Homeostasis and Calcineurin Activation in the Yeast Hansenula polymorpha*\(^{1,5}\)

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Disruption of \(H_p\)URE2 resulted in a low expression of genes encoding nitrate-assimilatory proteins; sensitivity to \(Li^+\), \(Na^+\), and \(Cd^{2+}\); no induction of \(ENA1\); low levels of the GATA-type transcription factor \(Gat1\); and low intracellular \(Ca^{2+}\) levels. \(Gat1\) levels were also very low in a \(\Delta cnb1\) mutant lacking the regulatory subunit of calcineurin. The strain \(\Delta ure2\) was very sensitive to the calcineurin inhibitor FK506 and displayed several phenotypes reminiscent of \(\Delta cnb1\). The reporter \(\alpha\)CDRE-lacZ, containing calcineurin-dependent response elements in its promoter, revealed that calcineurin activation was reduced in \(H_p\Delta ure2\). Expression of \(Sc\)URE2 in \(\Delta ure2\) rescued nitrogen catabolite repression and \(Cd^{2+}\) tolerance but not those phenotypes depending on calcineurin activation, such as salt tolerance and nitrate assimilation gene derepression. \(H_p\Delta ure2\) showed an increased expression of the gene \(PMR1\) encoding the Golgi \(Ca^{2+}\)-ATPase, whereas that of \(PMC1\) encoding the vacuolar \(Ca^{2+}\)-ATPase remained unaltered. \(PMR1\) up-regulation was abolished by deletion of the GATA-type transcription factor \(GAT2\) in a \(H_p\Delta ure2\) genetic background, and normal \(Ca^{2+}\) levels were recovered. Moreover, overexpression of \(GAT2\) or \(PMR1\) yielded strains mimicking the phenotype of the \(H_p\Delta ure2\). This suggests that the low \(Ca^{2+}\) levels in the \(H_p\Delta ure2\) mutant are due to high levels of \(Pmr1\) that replenish the Golgi \(Ca^{2+}\) content, thus acting as a negative signal for \(Ca^{2+}\) entry into the cell. We conclude that \(H_p\)Ure2 is involved in salt tolerance and also in nitrate assimilation gene derepression via \(Ca^{2+}\) homeostasis.

In Saccharomyces cerevisiae, Ure2 plays a central role in nitrogen catabolite repression (NCR)\(^*\) (i.e. the genes related to the utilization of poor nitrogen sources are repressed in the presence of preferred nitrogen sources) (1). The first insights into \(L\)URE2 were obtained by Lacroute \textit{et al.} (2, 3), who isolated \(ure2\) mutants incapable of carrying out NCR. They also isolated \(U\)RE3, a non-Mendelian, non-mitochondrial mutation with the same phenotype as \(ure2\) (4, 5). \(U\)RE3 later proved to be an altered form of Ure2 inherited by prion mechanisms (6). NCR involves the localization of GATA-type transcription factors Gln3 and Gat1 outside the nucleus, in the presence of preferred nitrogen sources. This prevents the expression of genes related to the utilization of poor nitrogen sources. In this framework, \(U\)re2 is involved in cytoplasm localization of Gln3 (1, 7–9). Several studies show the relationship between the TOR signaling pathways and NCR. Thus, in the presence of the Tor (target of rapamycin) kinase inhibitor rapamycin, both \(Gat1\) and Gln3 are present in the nucleus, as occurs in poor nitrogen sources; in addition, \(U\)re2 was found to be phosphorylated (7, 10, 11). The \(\Delta ure2\) strain also shows an improvement in \(Na^+\) and \(Li^+\) tolerance, this being due to the induction of the \(Na^+\) extrusion ATPase gene, \(ENA1\), which is positively modulated by \(Gat1\) and Gln3 (12). Likewise, \(U\)re2 deletion suppresses the sensitivity of calcineurin mutants to \(Na^+\), \(Li^+\), and \(Mn^{2+}\), increasing their survival during treatment with mating pheromones; this depends on Gln3 and Ena1 (13). Molecular cloning of \(U\)RE2 by Magasanik’s group, besides confirming its role in NCR, revealed that it encodes a protein with high similarity to glutathione \(S\)-transferases (GST) (14). Despite this, such \textit{in vitro} activity has not yet been detected. However, Cooper and coworkers (15) found that \(\Delta ure2\) mutants are hypersensitive to cadmium and nickel ions and hydrogen peroxide. Nevertheless, using recombinant Ure2, glutathione peroxidase (GPx) activity

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\(^*\) The abbreviations used are: NCR, nitrogen catabolite repression; GPx, glutathione peroxidase; NR, nitrate reductase; qRT-PCR, quantitative RT-PCR; CDRE, calcineurin-dependent response element; ER, endoplasmic reticulum.

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with cumene hydroperoxide, hydrogen peroxide, or tert-butylhydroperoxide as substrates has been reported (16).

_Hansenula polymorpha_ is able to use nitrate as sole nitrogen source. Nitrate enters cells via the high affinity nitrate transporter Ynt1 (17–19). It is reduced to ammonium by the consecutive action of nitrate and nitrite reductase. YNT1, YNR1, and YNI1 genes, respectively, encode the main high affinity nitrate transporter, nitrate reductase (NR) and nitrite reductase. These three genes are subjected to NCR in the presence of preferred nitrogen sources, such as ammonium or glutamine (19, 20). Their expression is also dependent on nitrate induction mediated by two Zn(II)$_2$Cys$_6$ transcriptional factors, Yna1 and Yna2 (20). However, the mechanisms underlying NCR in _H. polymorpha_ are unknown. In _Neurospora crassa_, the protein NMR1 is involved in the negative modulation of nitrate assimilation genes and others involved in the utilization of non-preferred nitrogen sources. NMR1 interacts with the GATA factor NIT2 in the presence of preferred nitrogen sources to prevent NIT2-dependent gene transcription (21, 22). In _Aspergillus nidulans_, NmrA acts similarly to NMR1, on the GATA factor AreA (23). However, neither NMR1 nor NmrA is similar to Ure2. Moreover, gsta, a URE2 ortholog in _A. nidulans_, is not involved in NCR but contributes to heavy metal and xenobiotic compound tolerance (24).

Because the nitrate assimilatory pathway in the yeast _H. polymorpha_ is known to be subject to NCR, we investigated the mechanism involved. One question was whether NCR is framed within Ure2 activity in _H. polymorpha_, as in _S. cerevisiae_, or via mechanisms closer to those reported for filamentous fungi. Once we found that Ure2 was involved in _H. polymorpha_ NCR, we focused on its mechanisms of action because these have only been studied in depth in _S. cerevisiae_.

We found that _HpUre2_ showed sensitivity to Na$^+$ and Li$^+$, in contrast to _S. cerevisiae_, and an unexpected drop in nitrate assimilation gene expression. We concluded that Ure2 is involved in nitrogen catabolite repression and salt tolerance via Ca$^{2+}$ homoeostasis and calcineurin in _H. polymorpha._

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—The _H. polymorpha_ strains used in this work and their genotypes are listed in the supplementary Table I. All strains are derivatives of NCYC495 leu2 ura3 strain. The wild type (WT) was obtained by transforming the _Candida_ strain with integrative vectors bearing _ura3_ marker. The wild type (WT) was obtained by transforming the _Candida_ strain with integrative vectors bearing _ura3_ marker. The wild type (WT) was obtained by transforming the _Candida_ strain with integrative vectors bearing _ura3_ marker. The wild type (WT) was obtained by transforming the _Candida_ strain with integrative vectors bearing _ura3_ marker.

**Nucleic Acid Isolation and Quantitative Real-time PCR**—Yeast DNA and λ DNA were isolated as described elsewhere (25). Total RNA was extracted using the RNeasy Mini Kit™, according to the manufacturer’s instructions (Qiagen). RNA integrity was electrophoretically verified by ethidium bromide staining and $A_{260}/A_{280}$ ratio. Genomic DNA was removed with 10 units of RNase-free DNase (Roche Applied Science) for each 10 μg of RNA. DNase treatment efficiency was checked by the absence of PCR products using _HpACT1_ as a template. Total RNA (1 μg) was reverse transcribed using the commercial TaqMan® kit according to the manufacturer’s instructions (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) was carried out in the iCycler iQ real-time PCR detection system (Bio-Rad) using the Fast Start SYBR Green Master master mix (Roche Applied Science). Four serial dilutions of cDNA, 3 μl of each, were amplified in triplicate for each amplicon in a volume of 20 μl. The relative changes in gene expression from qRT-PCR experiments were analyzed as described (26). _HpACT1_ was used as a reference gene.

**Gene Disruption and Yeast Vectors—**_URE2, CNB1, GAT1, GAT2, and PMC1_ disruption is described in the supplementary material. All of the primer sets for PCR-mediated gene disruption, tagging, or qRT-PCR are described in supplementary Table II. All vectors are described in supplementary Table III.

**lacZ Gene Fusions**—The _pPHI_ 359 vector (27) was used to fuse _ENA1_ and _PMR1_ gene promoters to _lacZ_, yielding _pENA1-lacZ_ and _pPMR1-lacZ_. The regions from −1164 to +39 relative to the ATG of _ENA1_ and −1009 to +39 of the _PMR1_ were amplified by PCR using _Pfu_ from genomic DNA using the primers _ENA1-lacZ-F_ and _PMR1-lacZ-R_ for _ENA1_ and using _PMR1-lacZ-F_ and _PMR1-lacZ-R_ for _PMR1_. The vector _pAMS367_ bearing 4xCDRE-lacZ (kindly provided by M. Cyert, Stanford University) was modified at the Stul site by introducing the _H. polymorpha_ _LEU2_ marker.

**Growth Tests**—The sensitivity of different yeast strains to cations and other compounds was assayed by a drop test. Strains were grown in YPD liquid medium, and then cultures were diluted to obtain 10$^6$ to 10$^7$ cells in 5 μl and then spotted on solid medium.

**Epitope Tagging of Ure2, Gat1, and Gat2**—Ure2, Gat1, and Gat2 were tagged at their C termini with six copies of the peptide epitope from the HA protein of human influenza virus using the vector _pHAI_. This vector derives from _pANL31_ (28), which contains the _eGFP_ (green fluorescence protein) ORF and the zeocin resistance marker (ble). The _eGFP_ sequence was replaced by a 256-bp fragment containing six copies of the HA epitope obtained by PCR using the pair of oligonucleotides tagF and tagR from the _S. cerevisiae_ vector _pYM3_ (29). The +1 to +905 DNA region from _URE2_ was amplified by PCR using the primers _Ure2exp-F_ and _Ure2-HAtag-R_, the +401 to +1523 DNA region from _GAT1_ was amplified using the primers _GAT1GFP-F_ and _GAT1GFP-R_, and the +46 to +1278 DNA region from _GAT2_ was amplified using the primers _GAT2-HA-F_ and _GAT2-HA-R_. All sequences were cloned in frame with the _6HA_ sequence to render the vectors _pHA-URE2, pHA-
GAT1, and pHA-GAT2. These were linearized at the BclI, NarI, and NruI sites, respectively, to facilitate their homologous integration and used to transform yeast. Transformants were selected by growth on YPD plates containing 100 μg/ml Zeocin™ (Invitrogen). HA tagging was confirmed by PCR with primers designed to bind outside the construct (C-URE2-F, C-GAT1-F, and RT-DAL80-F) and at the HA epitope (tagR). Western blot analysis using anti-HA antibody (Roche Applied Science) provided further confirmation.

**SDS-PAGE and Immunoblot**—Crude extracts were obtained from 100 mg of cells in a FastPrep device (Thermosavant LifeSciences) after 20 s at 6.0 m/s with 300 μl of Lysis Buffer I (50 mM Tris-HCl, pH 7.4, 15 mM EDTA, 15 mM EGTA, 10 mM Na3P2O7, 10 mM NaF, 1× Complete Mini protease inhibitor mixture (Roche Applied Science), 2 mM PMSF, 1 mM benzamidine, and 0.5-mm diameter glass beads. The supernatant resulting from the low speed centrifugation of extracts (820 × g for 1 min) was clarified at 10,580 g for 10 min at 4 °C and mixed with 4× Sample Buffer (4× Sample Buffer: 12% (w/v) SDS, 6% (v/v) 2-mercaptoethanol, 30% (w/v) glycerol, 0.05% (w/v) Serva blue G, 150 mM Tris-HCl, pH 7). Samples were heated at 95 °C for 7 min and then subjected to SDS-PAGE and immunoblot. Mouse monoclonal anti-HA antibodies (1:1000; Roche Applied Science) and Immobilon Western (Millipore) were used to detect Ure2, GAT1, and GAT2.

**Measurement of Ca2+ Intracellular Content**—Ca2+ content of the cells was determined as described previously (30). Briefly, samples of cells were filtered, washed twice with ice-cold 100 mM MgCl2, and extracted with 100 mM HCl. Ca2+ was determined by atomic absorption spectrophotometry.

**DNA Sequencing**—DNA sequencing was performed using AmpliTaq polymerase with a BigDye Terminator version 3.1 cycle sequencing kit on an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer).

**Miscellaneous Methods**—Yeast cells were electrotansformed as described previously (31). β-Galactosidase activity was determined as in Ref. 27. Determinations of lithium influx and efflux were previously described (32).

**RESULTS**

**Molecular Cloning and Sequence Analysis of HpURE2**—An HpURE2 partial DNA sequence is present in the H. polymorpha genome data base (Genolévures) (33). Based on this sequence, a 462-bp DNA fragment was obtained by PCR and used to screen an H. polymorpha λEMBL3 genomic library (34). Several phages were isolated, and DNA sequencing of the partial DNA sequence is present in the genome database (Genolevures) (33). Based on this sequence, the ORF of 34.3 kDa. The sequence of HpURE2 is present in GenBank™ under accession number AJ698949.

BLASTp analysis revealed the highest identity of HpUre2, about 70%, with Ure2 of different yeasts, such as Debaryomyces Hansenii, Pichia stipitis, Candida maltosa, and Candida albicans. The identity with ScUre2 decreases to 63%. Identity was also found with different members of the GST superfamily. For more details, see the supplemental material.

**TABLE 1**

| NR activity | NO3− | NO3− + NH4+ |
|-------------|------|-------------|
| WT          | 100  | 100         |
| Δure2       | 85 ± 27.4 | 258 ± 50.4 |
| Δure2 URE2  | 115 ± 14.2 | 116 ± 31.5 |
| nURE2       | 105 ± 36.6 | 37.7 ± 20.1 |

**TAble 2**

Analysis of nitrate assimilation gene expression in WT and Δure2 strains

Ammonium-grown cells of the WT and Δure2 were washed and incubated in synthetic medium containing 5 mM ammonium, 5 mM nitrate, or 5 mM ammonium plus 2.5 mM nitrate. Relative expression was determined by qRT-PCR. The experiments, only one of which is shown, were repeated three times without significant differences. ND, not determined.

**Nitrate assimilation gene expression**

| Nitrate assimilation gene expression | Δure2a | WTb |
|-------------------------------------|--------|-----|
| NO3− | NO3− + NH4+ | NO3− | NO3− + NH4+ |
| YNR1 | 0.7 | 4 | 9700 | 240 |
| YNI1 | 0.5 | 4 | ND | ND |
| YNT1 | 0.6 | 4 | ND | ND |
| GAP1 | 1.2 | 1 | 3 | 1 |
| URE2 | 0 | 0 | 5 | 2 |

a The expression is normalized to the WT in the same medium.

b The expression is normalized to the expression in ammonium. HaACT1 was used as reference gene.

**HpUre2 Is Responsible for Nitrogen Catabolite Repression and Derepression**—To test the role of HpURE2 in NCR, a Δure2 null mutant strain was obtained. NR activity, induced by nitrate and repressed by preferred nitrogen sources, was used as a read-out of NCR. In nitrate plus preferred nitrogen sources, such as ammonium, NR was higher in Δure2 than WT (Table 1), indicating that NCR was almost abolished in Δure2. Indeed, qRT-PCR showed that in nitrate plus ammonium, nitrate assimilation gene expression was higher in Δure2 than WT (Table 2). However, it was very striking that in nitrate, Δure2 presented lower NR activity than WT and also lower nitrate assimilation gene expression, indicating that these genes are not fully derepressed in the absence of URE2.

To gain further insight into HpUre2 regulation, we studied its phosphorylation state in response to rapamycin, to which HpUre2 (Fig. 1), like ScUre2, was very sensitive (35), and to nitrogen sources. As shown in Fig. 1, Ure2 was phosphorylated in response to preferred nitrogen sources, mainly in glutamine, and became dephosphorylated in synthetic medium without any nitrogen source or proline. Rapamycin also triggered Ure2 dephosphorylation.

These results allow us to conclude that URE2 is involved in NCR in H. polymorpha, although the lower expression of nitrate assimilation genes YNT1, YNR1, and YNI1 in Δure2 also indicated a positive role of Ure2. Moreover, the HpUre2 phosphorylation state depends on the nitrogen source, most likely via the TOR signaling pathway.

**Ure2 Is Involved in Na+/Li+ Tolerance**—In S. cerevisiae, Ure2 is involved in salt tolerance (12, 13), which prompted us to
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Aure2 Participates in the Calcineurin-dependent Response—In S. cerevisiae, it has been shown that ENA1 expression is subject to a complex regulatory network, where Gln3, Gat1, and calcineurin are the positive signals (12, 36, 37). However, in HpAure2, the expected increase in ENA1 expression and concomitant cation tolerance was not observed. This suggested that GATA factors and/or calcineurin-dependent ENA1 expression could be negatively affected in Aure2. Therefore, we tested calcineurin involvement using its inhibitor FK506 (38). We found that \( \Delta \)ure2 was very sensitive to this macrolide, even in the absence of Na\(^{+}\) or Li\(^{+}\), which further increased \( \Delta \)ure2 sensitivity to FK506 (Fig. 5). This suggested a positive effect of Ure2 on calcineurin activation. In this framework, we observed a strong parallelism between \( \Delta \)cnb1, lacking the regulatory subunit of calcineurin, and \( \Delta \)ure2, in response to Mn\(^{2+}\), Na\(^{+}\), and SDS (Fig. 6). \( \Delta \)ure2\( \Delta \)cnb1 showed a phenotype very close to \( \Delta \)cnb1, although slightly more sensitive, indicating that Ure2 could even act beyond Cnb1 (Fig. 6). We also found that ENA1 was scarcely expressed in response to Na\(^{+}\) in \( \Delta \)cnb1, as in \( \Delta \)ure2 (data not shown). These results strongly suggested that calcineurin activation in \( \Delta \)ure2 was negatively affected.

URE2 Deletion Reduces Calcineurin-dependent Gene Expression and Total Cell Ca\(^{2+}\) Content—The effect of Ure2 on calcineurin was measured by \( \Delta \)xCDRE-lacZ reporter (39) in WT and \( \Delta \)ure2 strains. \( \Delta \)ure2 showed about a 2.5-fold lower induction of \( \Delta \)xCDRE-lacZ, in response to Na\(^{+}\), indicating poor activation of calcineurin-dependent gene expression in \( \Delta \)ure2 (Fig. 7). Because calcineurin activation is Ca\(^{2+}\)-dependent, we studied the role of Ca\(^{2+}\) in \( \Delta \)ure2 phenotypes. The addition of 50 mM Ca\(^{2+}\) restored \( \Delta \)ure2 tolerance to Li\(^{+}\), whereas 6 mM EGTA increased \( \Delta \)ure2 sensitivity to it (Fig. 8). We also observed that extra Ca\(^{2+}\) produced a higher ENA1-lacZ expression in WT than in \( \Delta \)ure2 (Fig. 9).

study the role of HpUre2 in Na\(^{+}\)/Li\(^{+}\) tolerance. For this, \( \Delta \)ure2 was tested for growth in media containing Na\(^{+}\) or Li\(^{+}\) and was found to be more sensitive to these cations than WT (Fig. 2). This contrasts with S. cerevisiae, where \( \Delta \)ure2 shows greater Na\(^{+}\) and Li\(^{+}\) tolerance than WT (12). Cation influx indicated that \( \Delta \)ure2 accumulated Li\(^{+}\) faster than WT. Likewise, the efflux kinetics showed that \( \Delta \)ure2 was unable to extrude Li\(^{+}\) as WT did (Fig. 3). Because ENA1 encodes the main ATPase involved in Na\(^{+}\)/Li\(^{+}\) extrusion, we determined its expression levels in WT and \( \Delta \)ure2 by qRT-PCR. Na\(^{+}\) and Li\(^{+}\) induced the level of ENA1 in WT between 10- and 13-fold; in contrast, no induction was observed in \( \Delta \)ure2 (Fig. 4). These results reinforced our idea that efflux of these cations was impaired in \( \Delta \)ure2. The same results were found using a strain bearing the ENA1-lacZ construct (data not shown). Our findings allow us to conclude that Ure2 is positively involved in Na\(^{+}\)/Li\(^{+}\) tolerance, up-regulating ENA1 expression.

URE2 Deletion Reduces Calcineurin-dependent Gene Expression and Total Cell Ca\(^{2+}\) Content—The effect of Ure2 on calcineurin was measured by \( \Delta \)xCDRE-lacZ reporter (39) in WT and \( \Delta \)ure2 strains. \( \Delta \)ure2 showed about a 2.5-fold lower induction of \( \Delta \)xCDRE-lacZ, in response to Na\(^{+}\), indicating poor activation of calcineurin-dependent gene expression in \( \Delta \)ure2 (Fig. 7). Because calcineurin activation is Ca\(^{2+}\)-dependent, we studied the role of Ca\(^{2+}\) in \( \Delta \)ure2 phenotypes. The addition of 50 mM Ca\(^{2+}\) restored \( \Delta \)ure2 tolerance to Li\(^{+}\), whereas 6 mM EGTA increased \( \Delta \)ure2 sensitivity to it (Fig. 8). We also observed that extra Ca\(^{2+}\) produced a higher ENA1-lacZ expression in WT than in \( \Delta \)ure2 (Fig. 9).

FIGURE 1. Rapamycin and nitrogen sources regulate Ure2 phosphorylation. A, Ure2–6HA electrophoretic mobility. Ure2–6HA was analyzed by SDS-PAGE from cells grown to early exponential phase in synthetic medium plus 5 mM ammonium (time 0 lane 1), washed, and then deprived of nitrogen for 15, 30, and 120 min (lanes 2–4) or incubated 15, 30, and 120 min in synthetic medium plus 5 mM glutamine (lanes 5–7). Cells incubated for 120 min in synthetic medium plus 5 mM ammonium or 1 mM proline are also shown (lanes 8 and 9). B, the ure2 low mobility shift band is due to phosphorylation. Protein extracts from cells deprived of nitrogen or incubated in glutamine were treated with \( \lambda \)-protein phosphatase. 50 mM EDTA was added to inhibit \( \lambda \)-protein phosphatase. C, rapamycin causes Ure2–6HA dephosphorylation. Protein extracts from Ure2–6HA strain were incubated for 120 min in 5 mM glutamine with (+) or without (−) 0.5 \( \mu \)g/ml rapamycin or deprived of nitrogen. D, rapamycin growth sensitivity. Serial 10-fold dilutions of the WT and \( \Delta \)ure2 were spotted on solid medium containing YPD plus rapamycin at the indicated concentration.

FIGURE 2. \( \Delta \)ure2 is sensitive to Li\(^{+}\) and Na\(^{+}\). WT, \( \Delta \)ure2, \( \Delta \)ure2\( \Delta \)URE2, and nURE2 strains were grown in YPD. Serial 10-fold dilutions were spotted on pH 5.5 buffered synthetic medium plus LiCl and NaCl at the indicated concentrations. Cells were incubated at 37 °C for 2 days.

FIGURE 3. HpURE2 deletion affects cell Li\(^{+}\) content. A, Li\(^{+}\) accumulation. Cells from WT (○) and \( \Delta \)ure2 mutant (□) were grown in pH 5.5 buffered synthetic medium to an OD\(_{600}\) of 0.6–1, washed, and resuspended in fresh medium containing 30 mM LiCl. Samples were taken for intracellular Li\(^{+}\) determination. B, Li\(^{+}\) efflux. Cells were loaded with 30 mM LiCl for 30 min, washed, and resuspended in Li\(^{+}\)-free medium, and intracellular Li\(^{+}\) was determined. The experiments, only one of which is shown, were repeated three times without significant differences.
Furthermore, total cell Ca^{2+} content was lower in ∆ure2 with respect to WT (Fig. 10). Altogether, we conclude that Ure2 is involved in Ca^{2+} homeostasis and consequently in calcineurin activation.
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To gain further insights into calcineurin activation in Δure2, we studied the activation of vacuolar H\(^+\)/Ca\(^{2+}\) exchanger Vcx1. In S. cerevisiae, calcineurin inhibits Vcx1-dependent H\(^+\)/Ca\(^{2+}\) exchange. Calcineurin decreases Ca\(^{2+}\) tolerance of pmc1 mutants by inhibiting the function of Vcx1; this Ca\(^{2+}\) tolerance is restored by inactivation of calcineurin (40). To test the activation of Vcx1 in Δure2, we analyzed Ca\(^{2+}\) sensitivity of Δpmc1, Δcnb1Δpmc1, and Δure2Δpmc1. As shown in Fig. 11, Δpmc1 was very sensitive, whereas Δcnb1Δpmc1 and Δure2Δpmc1 were more resistant. As expected, Δcnb1Δpmc1 was much more tolerant to Ca\(^{2+}\) than Δure2Δpmc1 because the latter conserves some calcineurin activity. These results also show the low activation of calcineurin in Δure2.

Gat1 Levels Are Regulated by the Calcineurin Pathway, Being Lower in Δure2—Because ENA1 and nitrate assimilation genes YNT1, YNR1, and YNI1 are down-regulated in Δure2 and positively regulated by Gat1, we explored the levels of HpGat1 in Δcnb1 and Δure2. HpGat1 is a positive GATA factor also involved in nitrate assimilation gene expression in H. polymorpha (supplemental Figs. S1 and S2). Accordingly with S. cerevisiae Ure2 mechanisms, HpGat1 would enter the nucleus in the Δure2 strain, up-regulating ENA1 expression. The strain Δgat1 showed Li\(^+\) and Na\(^+\) sensitivity, although less than in Δure2, and low levels of ENA1 expression (supplemental Figs. S3 and S4). We found that the Gat1 levels in Δure2 were very low (Fig. 12), in contrast to those found in S. cerevisiae (41–44). Therefore, HpUre2 deletion did not increase ENA1 activation via Gat1 but clearly lowered it. This also explains the lower derepression of the nitrate assimilation genes YNT1, YNR1, and YNI1 in Δure2.

To clarify if calcineurin acts on ENA1 exclusively via Gat1, we measured the levels of ENA1 expression in Δgat1 with or without additional 50 mM Ca\(^{2+}\). An increase of ENA1 expression was observed in response to Ca\(^{2+}\) (data not shown), suggesting that ENA1 induction is under the dual control of calcineurin-Crz1–Gat1 and calcineurin–Crz1. This also explains the higher sensitivity of Δure2 than Δgat1 to Na\(^+\) and Li\(^+\).

Expression of ScURE2 in HpΔure2 Does Not Rescue Calcineurin Activation—Both ScUre2 and HpUre2 present high similarity to GSTs. ScUre2 shows GPx activity, but the latter conserves some calcineurin similarity to GSTs. ScUre2 shows GPx activity, but the former conserved.

Calcineurin Activation

To test the activation of Vcx1 in Δure2, we analyzed Ca\(^{2+}\) sensitivity of Δpmc1, Δcnb1Δpmc1, and Δure2Δpmc1. As shown in Fig. 11, Δpmc1 was very sensitive, whereas Δcnb1Δpmc1 and Δure2Δpmc1 were more resistant. As expected, Δcnb1Δpmc1 was much more tolerant to Ca\(^{2+}\) than Δure2Δpmc1 because the latter conserves some calcineurin activity. These results also show the low activation of calcineurin in Δure2.

Gat1 Levels Are Regulated by the Calcineurin Pathway, Being Lower in Δure2—Because ENA1 and nitrate assimilation genes YNT1, YNR1, and YNI1 are down-regulated in Δure2 and positively regulated by Gat1, we explored the levels of HpGat1 in Δcnb1 and Δure2. HpGat1 is a positive GATA factor also involved in nitrate assimilation gene expression in H. polymorpha (supplemental Figs. S1 and S2). Accordingly with S. cerevisiae Ure2 mechanisms, HpGat1 would enter the nucleus in the Δure2 strain, up-regulating ENA1 expression. The strain Δgat1 showed Li\(^+\) and Na\(^+\) sensitivity, although less than in Δure2, and low levels of ENA1 expression (supplemental Figs. S3 and S4). We found that the Gat1 levels in Δure2 were very low (Fig. 12), in contrast to those found in S. cerevisiae (41–44). Therefore, HpUre2 deletion did not increase ENA1 activation via Gat1 but clearly lowered it. This also explains the lower derepression of the nitrate assimilation genes YNT1, YNR1, and YNI1 in Δure2.

The close correlation between Δure2 and Δcnb1 found throughout this work led us to ask whether Gat1 levels were calcineurin-dependent. We found that Δcnb1 showed even lower Gat1 levels than Δure2 (Fig. 12), allowing us to report for the first time in yeast that levels of a GATA transcription factor were controlled by the calcineurin signaling pathway. In accordance with this, the GAT1 gene presents a putative calcineurin-dependent response element (CDRE) (45) in its 5’-non-coding region. Therefore, the lower levels of Gat1 present in Δure2 are consistent with the low calcineurin activation in this strain compared with WT.

To clarify if calcineurin acts on ENA1 exclusively via Gat1, we measured the levels of ENA1 expression in Δgat1 with or without additional 50 mM Ca\(^{2+}\). An increase of ENA1 expression was observed in response to Ca\(^{2+}\) (data not shown), suggesting that ENA1 induction is under the dual control of calcineurin–Crz1–Gat1 and calcineurin–Crz1. This also explains the higher sensitivity of Δure2 than Δgat1 to Na\(^+\) and Li\(^+\).

Expression of ScURE2 in HpΔure2 Does Not Rescue Calcineurin Activation—Both ScUre2 and HpUre2 present high similarity to GSTs. ScUre2 shows GPx activity, but in vitro GST activity has not been reported. However, deletion of ScURE2 causes increased sensitivity to heavy metal ions, such as Cd\(^{2+}\) and Ni\(^{2+}\) (15). To show whether these potential enzymatic activities of HpUre2 are responsible for Ca\(^{2+}\) homeostasis and calcineurin regulation, we tested whether ScURE2 expression was able to complement HpΔure2 phenotypes. HpΔure2ScURE2 transformants almost fully recovered Cd\(^{2+}\) tolerance, indicating that GST and GPx activities associated with ScUre2 are active in H. polymorpha. NR activity in HpΔure2ScURE2 was lower than HpUre2 in nitrate plus ammonium (supplemental Table IV). Consistent with this, HpUre2ScURE2 fully recovered tolerance to chlorate (Fig. 13), the chlorine analog of nitrate, which is reduced by NR to the toxic chlorite (46), indicating that ScUre2 was able to
exert NCR in *H. polymorpha*. However, ScURE2 expression did not rescue either Li\(^+\) tolerance (Fig. 13) or NR activity levels (supplemental Table IV). These levels were lower in *Hp\(\Delta ure2*ScURE2* than in WT, in both nitrate and nitrate plus ammonium (supplemental Table IV). In nitrate, NR activity is consistently lower in ScURE2*Hp\(\Delta ure2* than in *Hp\(\Delta ure2*; this could be due to the low levels of Gat1 in this strain and also to the capacity of ScUre2 to bind Gat1 even in the presence of a non-preferred nitrogen source like nitrate (supplemental Table IV). Therefore, these results indicate that ScUre2 was unable to activate calcineurin as Ure2 does. The sensitivity of *Hp\(\Delta ure2*ScURE2*, like that of *\(\Delta ure2* to FK506, is consistent with this (Fig. 14).

**PMR1 Expression Is Regulated by Ure2 through Gat2—**To maintain cytosolic Ca\(^{2+}\) homeostasis in *S. cerevisiae*, calcineurin regulates the expression of *PMC1* and *PMR1*, encoding P-type Ca\(^{2+}\)-ATPases involved in Ca\(^{2+}\) transport into the vacuole and ER-Golgi, respectively (47, 48). Analysis of *PMR1* and *PMCI* gene expression in *H. polymorpha* revealed that *PMR1-lacZ* levels were higher in *\(\Delta ure2* than WT, whereas *PMCI* expression remains unaltered in *\(\Delta ure2* (Fig. 15). Moreover, adding extra Ca\(^{2+}\) produced higher induction in WT than in *\(\Delta ure2* (Fig. 15). This result suggests that the lower levels of intracellular Ca\(^{2+}\) observed in *\(\Delta ure2* are due to the constitutively high *PMR1* expression because this would increase ER-Golgi Ca\(^{2+}\) replenishment, and as a result, Ca\(^{2+}\) entry into cells would decrease. In *S. cerevisiae*, the *\(\Delta pmr1* mutant considerably increases Ca\(^{2+}\) entry into cells via a capacitative Ca\(^{2+}\) entry, similar to that found in mammals (49–51). In light of the current *S. cerevisiae* Ure2 mode of action, in *Hp\(\Delta ure2* a GATA factor would enter the nucleus, activating *PMR1*. Analysis of PMR1 expression in strains lacking the positive GATA factors *HpGAT1*, *HpGLN3*, and *HpGAT2* revealed low *PMR1* expression in *\(\Delta gat2*, even in the presence of Ca\(^{2+}\) (Fig. 15). *HpGat2* presents a close sequence similarity with ScGat1 and Hpgat1 (supplemental Figs. S1 and S2). Nevertheless, its deletion has no effect on Li\(^+\) and Na\(^+\) sensitivity, unlike Gat1 (supplemental Fig. S3). In accordance with the observed role of Gat2 in regulating *PMR1*, a strain bearing multicopy *GAT2* (*nGAT2*) showed high *PMR1* expression levels, equal to those seen in *\(\Delta ure2*; on the other hand, *\(\Delta gat2* deletion to obtain *\(\Delta ure2*Gat2* restored *PMR1* expression to those levels observed in WT (Fig. 15). We also determined calcineurin-dependent gene expression by 4xCDRE-*lacZ* in *\(\Delta gat2*, *\(\Delta ure2*Gat2*, and *nGAT2*; in the latter, this expression was the same as in *\(\Delta ure2*, whereas deletion of *Gat2* led to higher levels of expression (Fig. 7). Furthermore, overexpression of *PMR1* by increasing gene dosage reproduces *\(\Delta ure2* phenotypes (Fig. 16). We concluded that Ure2 is involved in Ca\(^{2+}\) homeostasis via Gat2, which is responsible for *PMR1* regulation.

**Gat2 Levels Are Regulated by Calcineurin and Gat1—**Because Gat2 plays a key role downstream from Ure2, we determined Gat2 levels in *\(\Delta cnb1* and *\(\Delta ure2*. *\(\Delta cnb1* showed very low levels of Gat2, almost undetectable by Western blot, whereas in
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FIGURE 17. **CNB1** and **GAT1** deletion decreases Gat2 levels, whereas **URE2** deletion increases them. Gat2–6HA levels in WT, **ure2**, **Δcnb1**, and **Δgat1** were analyzed by SDS-PAGE. Cells grown to early exponential phase in synthetic medium plus 5 mM ammonium were washed and incubated for 120 min in synthetic medium plus 5 mM glutamine (**Gln**), 5 mM ammonium (**NH₄⁺**), or 5 mM nitrate (**NO₃⁻**). 50 μg of protein were analyzed.

**FIGURE 18.** Working model for role of Ure2 in Ca²⁺ homeostasis, nitrate assimilation, and **ENA1** gene expression. Ure2 regulates Ca²⁺ cell content via Gat2, which acts transcriptionally on **PMR1**. **PMR1** regulates Ca²⁺ levels in ER-Golgi, which in turn act on total Ca²⁺ content in the cell, modulating calcineurin activation. This induces **ENA1** and nitrate assimilation gene up-regulation via *Crz1* → *Gat1*. **ENA1** is also induced via *Crz1* directly. Gat2 is transcriptionally activated by Gat1. Lines with arrowheads indicate positive events, whereas lines with bars are inhibitory. Although not shown here, the model assumes that Ure2 also retains Gat1 in the cytosol in the presence of preferred nitrogen sources.

**Δure2** they were higher than WT (Fig. 17). Because Gat1 levels were lower in **Δcnb1** than WT, we studied whether Gat2 was under the control of Gat1. Indeed, in **Δgat1**, Gat2 levels were about 50% of those in WT (Fig. 17). The high levels of Gat2 in **Δure2** are consistent with our results because we report that **PMR1** expression, which is regulated by **GAT2**, is higher in this strain. These high GATA factor levels are apparently contradictory with the down-regulation of calcineurin in **Δure2**. However, because Ure2 is absent, Gat1 can enter the nucleus freely to activate **GAT2** transcription. The results here reported are summarized in the HpUre2 working model (Fig. 18).

**DISCUSSION**

ScUre2 plays a central role in the regulation of nitrogen metabolism (1, 11, 35, 52) and to a lesser extent in salt tolerance (12, 13, 37). **HpURE2** has been shown to be a highly pleiotropic gene because its deletion resulted in an assortment of different phenotypes. Some of the observable characteristics of **Δure2** are (i) rapamycin sensitivity; (ii) decreased tolerance to Na⁺, Li⁺, Mn²⁺, and SDS; (iii) pronounced FK506 sensitivity; and (iv) reduction of nitrate assimilation gene expression (Figs. 1, 2, 5, and 6 and Table 2). These traits reveal similarities but also important differences between ScUre2 and HpUre2. The main structural difference between them is the absence of the Q/N-rich N-terminal region involved in prion-like behavior (54, 55).

We have not yet investigated whether HpUre2 presents such behavior, either in *H. polymorpha* itself or using *S. cerevisiae* as the host. However, analysis of **Δure2** revealed a clear involvement of HpUre2 in NCR (Tables 1 and 2), which suggests that the above N-terminal region seems uninvolved in NCR. This is consistent with what was found in *S. cerevisiae* (55), although new insights into NCR suggest this prion domain contributes to Ure2 stability and functioning in this process. Actually, this domain is required for interaction with the GATA factor Gzf3 (56).

Besides HpUre2, we have also identified two positively acting GATA factors (HpGat1 and HpGat2 (see supplemental material)) and one with a negative role (HpGzf3), all showing significant identity with the *S. cerevisiae* GATA factors. Altogether, these findings indicate that the overall nitrogen regulatory system in the nitrate-assimilating yeast *H. polymorpha* is closer to *S. cerevisiae* than to the filamentous fungi *A. nidulans* and *N. crassa*, which are also able to use nitrate as the sole nitrogen source (57, 58). We also found that HpUre2 undergoes phosphorylation in response to preferred nitrogen sources and is dephosphorylated under nitrogen limitation conditions and by rapamycin (Fig. 1). This confirms our previous observation that in *H. polymorpha*, the TOR signaling pathway responds to nitrogen sources and regulates nitrogen assimilation gene expression (59). The phosphorylation state of Ure2 could play an important role in modulating its interaction with the GATA factors.

Another remarkable difference between HpUre2 and ScUre2 is their role in the response to Na⁺ and Li⁺ stress; whereas **HpΔure2** presents sensitivity to these cations (Fig. 2), **ScΔure2** is resistant. Greater Li⁺ accumulation and low **ENA1** expression account for the Na⁺/Li⁺ sensitivity of **HpΔure2** (Figs. 3 and 4). As occurs in *S. cerevisiae*, both calcineurin and Gat1 regulate **ENA1** positively because strains lacking Gat1 (supplemental Fig. S4) and Cnb1 (data not shown) showed low levels of **ENA1** expression. Two new findings open novel perspectives on this interesting protein. First, Ure2 clearly regulates Ca²⁺ cell content via Gat2, which acts transcriptionally on **PMR1** (Fig. 15). Second, this process is involved in activating the calcineurin pathway. Even more important, Gat1 levels were clearly regulated by calcineurin (Fig. 12). Indeed, because it is widely assumed that Ca²⁺ activates calcineurin in response to different stimuli (60, 61), a poor activation of calcineurin was to be expected in **Δure2**. Accordingly, a strong parallelism in the behavior of **Δcnb1** and **Δure2** was seen: (i) the two strains were

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sensitive to Mn$^{2+}$, Na$^+$, and SDS; (ii) $\Delta ure2\Delta cnb1$ showed a phenotype very close to $\Delta cnb1$ (Fig. 6); (iii) ENA1 expression levels were identically low in both strains (data not shown); (iv) Gat1 levels were very low in both (Fig. 12); (v) $\Delta ure2$ was remarkably sensitive to FK506 (Fig. 5); and (vi) the lower calcineurin activation in $\Delta ure2$ was shown using 4xCDRE-lacZ (Fig. 7). The slight increase in $\Delta cnb1\Delta ure2$ sensitivity with respect to $\Delta cnb1$ could be due to the role of Ure2 in detoxification processes (15). These findings allow us to understand the scarce induction of ENA1 in $\Delta ure2$ in response to Na$^+$/Li$^+$. We are also aware that regulation of Gat1 by calcineurin has a special significance for nitrate assimilation gene derepression in H. polymorpha. We emphasize that our results allow us to conclude that NCR is almost abolished in $\Delta ure2$. In this regard, HpUre2 seems to operate as in S. cerevisiae, retaining GATA factors outside the nucleus when the medium contains preferred nitrogen sources (1, 7–9). However, in $\Delta ure2$, nitrate assimilation gene derepression in nitrate is negatively affected. Now we know that this is due to the low levels of Gat1 present in $\Delta ure2$.

Expression of ScURE2 in Hp$\Delta ure2$ also revealed that HpUre2 displays a more complex regulatory network than ScUre2. Thus, ScUre2 rescued Cd$^{2+}$ tolerance (Fig. 13) and NCR in Hp$\Delta ure2$ (Fig. 13 and supplemental Table IV). This rescue is associated with the GST and GPx activities of ScUre2, respectively, and its capacity to retain HpGat1 outside the nucleus in the presence of preferred nitrogen sources, such as ammonium. In contrast, Li$^+$ tolerance and NR activity were not complemented, indicating the incapacity of ScURE2 to activate the calcineurin in Hp$\Delta ure2$ (Fig. 13 and supplemental Table IV). These results are consistent with our model of the action mechanisms of HpUre2 (i.e. its capacity to interact directly with Gat1 but also indirectly acting on its levels via Ca$^{2+}$ homeostasis and calcineurin).

Calcineurin has been widely reported to be associated with GATA factors in mammalian cells, playing an important role in muscle regeneration and hypertrophy in association with NFATc1 and GATA-2 (62, 63). Blockage of calcineurin down-regulates GATA-6-DNA binding in differentiated vascular smooth muscle cells (64). The hypertrophic effects of calcineurin in cardiomyocytes have been linked with its interaction with GATA-4 transcrip- tion factor (65).

Regarding lower levels of Ca$^{2+}$ in $\Delta ure2$ than in WT, we observed that a lack of Ure2 led to Ca$^{2+}$-ATPase PMR1 gene up-regulation (Fig. 15), presumably as a consequence of a GATA factor being freed from Ure2 and entering the nucleus. Once the GATA factor is in the nucleus, it can act directly on PMR1 or on a second GATA factor affecting PMR1. We therefore propose that PMR1 is regulated by Ure2 through Gat2 in H. polymorpha, because (i) $\Delta GAT2$ deletion led to PMR1 down-regulation (Fig. 15), (ii) its overexpression led to PMR1 up-regulation (Fig. 15), and (iii) $\Delta ure2$ presents high levels of Gat2 (Fig. 17). Because $\Delta GAT2$ is down-regulated in $\Delta gat1$ and $\Delta cnb1$ (Fig. 17), activation of $\Delta GAT2$ expression by Gat1 cannot be ruled out. In S. cerevisiae, the ER-Golgi Ca$^{2+}$ store has been shown to induce a signal that modulates extracellular Ca$^{2+}$ entry (49–51), depending in turn on Ca$^{2+}$-ATPase Pmr1 levels. We consider that the up-regulation of PMR1 via Gat2 in $\Delta ure2$ leads to a decrease in Ca$^{2+}$ entry, resulting in a reduced cytosolic Ca$^{2+}$ store. Deletion of GAT2 from the $\Delta ure2$ genetic background abolished PMR1 up-regulation (Fig. 15), and in accordance with our hypothesis, Ca$^{2+}$ content was restored to WT levels (Fig. 10). Furthermore, the activation of Vcx1 in $\Delta ure2$ could also act concertedly or sequentially along with PMR1 to reduce the cytoplasmic Ca$^{2+}$ available for calcineurin activation.

The Pmr1 Ca$^{2+}$/Mn$^{2+}$-ATPase negatively regulates the rapamycin-sensitive TOR complex (TORC1) in S. cerevisiae, Mn$^{2+}$ in the Golgi being involved in TORC1 signaling inhibition (66). However, our results are consistent with the role of Pmr1 in Ca$^{2+}$ transport. In agreement with this, Ca$^{2+}$ addition produced a lower ENA1-lacZ induction in $\Delta ure2$ than in WT (Fig. 9), as would be expected for a strain where Ca$^{2+}$ homeostasis is jeopardized.

We have elucidated the mechanisms underlying Ca$^{2+}$ homeostasis, Na$^+$/Li$^+$ tolerance, and nitrate assimilation gene derepression involving Ure2 in H. polymorpha, which provide new insights into the role of this protein. Whether or not the same mechanisms exist in other yeasts is an intriguing question. So far, how Ure2 acts in nitrogen regulation and facilitates Na$^+$/Li$^+$ tolerance has only been characterized in S. cerevisiae. Indeed, the function of ScUre2 in NCR is fully complemented by the Ure2p of different Candida and Saccharomyces yeast species (53). In contrast, we report here that ScURE2 expressed in Hp$\Delta ure2$ complements NCR but not those phenotypes where calcineurin is involved, suggesting that the mechanisms so far elucidated for ScUre2 are not universal.

In summary, we uncover the central role of Ure2 in Ca$^{2+}$ homeostasis and its implication in calcineurin pathway activation. The GATA factor Gat1 is also shown to be regulated by calcineurin.

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