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Coordinated Gene Regulation in the Initial Phase of Salt Stress Adaptation*

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*Running title: Dynamics of gene expression upon salt stress in yeast

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Background: Transcriptional responses upon stress involve transient activation and repression of gene expression.
Results: Vacuolar H^+-ATPase and glycerol biosynthesis affect the efficient coordination of transcriptional activation and repression upon osmostress.
Conclusion: Intracellular ion homeostasis, osmolyte production and MAP kinase function modulate the dynamics of a transcriptional stress response.
Significance: Underlining the importance of cell physiology as a modulator of gene regulation.

ABSTRACT

Stress triggers complex transcriptional responses, which include both gene activation and repression. We used time resolved reporter assays in living yeast cells to gain insights into the coordination of positive and negative control of gene expression upon salt stress. We found that the repression of "housekeeping" genes coincides with the transient activation of defense genes and that the timing of this expression pattern depends on the severity of the stress. Moreover we identified mutants which caused an alteration in the kinetics of this transcriptional control. Loss of function of the vacuolar H^+-ATPase (*vma1*) or a defect in the biosynthesis of the osmolyte glycerol (*gpd1*) caused a prolonged repression of housekeeping genes and a delay in gene activation at inducible loci. Both mutants have a defect in the relocation of RNA polymerase II complexes at stress defense genes. Accordingly salt activated transcription is delayed and less efficient upon partially respiratory growth conditions where glycerol production is significantly reduced. Furthermore the loss of Hog1 MAP kinase function aggravates the loss of RNA polymerase II from housekeeping loci, which apparently do not accumulate at inducible genes. Additionally the Def1 RNA polymerase II degradation factor, but not a high pool of nuclear PolII complexes, is needed for efficient stress induced gene activation. The data presented here indicate that the finely tuned transcriptional control upon salt stress is dependent on

physiological functions of the cell, such as the intracellular ion balance, the protective accumulation of osmolyte molecules or the RNA Pol II turnover.

Cells respond to environmental insults by changing their global gene expression pattern. These transcriptional programs are destined to coordinately activate stress defense genes and to suppress the expression of housekeeping functions. In recent years and especially by the use of model systems such as yeast we have accumulated considerable knowledge about the function of signal transduction pathways and how they rapidly activate different classes of "stress defense" genes in the genome (1). Global gene expression experiments have shown that many types of stress lead to the transient upregulation of hundreds of defense functions and to the simultaneous repression of housekeeping genes during adaptation (2,3).

Adaptation to hyperosmotic stress is one of the best understood stress responses in yeast at the molecular level (4). The high osmolarity glycerol (HOG)[4] MAP kinase pathway is the central signal transduction route operating upon osmostress (5,6). Upon activation, its Hog1 MAP kinase triggers an adaptive transcriptional program in the nucleus, which involves several specific transcription factors such as Sko1, Hot1, Rtg1/3, Msn2 or Smp1 (7-12). Although Hog1 remodels the cellular expression profile in a profound manner, the function of Hog1 in gene activation is actually dispensable for yeast growth under osmostress conditions as long as the cell can efficiently accumulate glycerol as an osmoprotectant (13). This shows that the adaptation of gene expression goes hand in hand with other physiological adaptations. Among those, the activated biosynthesis of osmolytes and the extrusion or intracellular distribution of cations are especially relevant for salt and osmostress survival (14-16).

Glycerol is the main osmolyte produced in yeast to counteract the water loss which occurs during acute hyperosmotic stress. It is synthesized by the stress inducible enzymes Gpd1 and Gpp2 which convert the glycolytic intermediate glyceraldehyde-3-phosphate to glycerol (17-19). It is important to note that efficient osmoadaptation by glycerol accumulation seems to be a major physiological determinant which in turn regulates HOG pathway activity by a feedback mechanism (20). Another important physiological aspect to consider is that the elevated intracellular ionic force itself interferes with transcriptional adaptation. Salt stress causes the dissociation of many DNA bound proteins such as transcription factors or RNA polymerase complexes from their chromosomal association sites. The activation of ion transporters at the plasma membrane has been identified as a mechanism to overcome this transcriptional barrier (21). Additionally the intracellular sequestration of toxic ions in the vacuole is yet another determinant of salt tolerance in yeast, since stress activated vacuolar H^+-ATPase function is necessary for the recovery from osmotic shock in a process parallel to the HOG pathway (22).

The physiology of the cell critically conditions the efficiency and timing of the transcriptional response. It has been shown that severe osmotic stress leads to an increasing delay in the induction of stress defense genes. This effect can be explained by a delay in HOG pathway signaling, a later nuclear translocation of Hog1 and the increasing dissociation of DNA associated protein complexes from the genome (21,23-25). Accordingly an important function of Hog1 is to enable rapid transcriptional activation under conditions, which generally inhibit gene expression. This function triggers a general relocation of active transcription complexes from highly expressed genes to stress induced loci (26,27). Perturbations of the transcriptional response by changes in the cells physiology are not restricted to osmotic stress. It has been recently shown that also oxidative stress, at threshold levels, interferes with efficient transcriptional activation and, more importantly, that mutants in ROS detoxification systems show much less dynamic transcriptional responses (28). Thus, the dynamic of a gene expression response to stress is to a great part dependent on cell physiology. Additionally, the tolerance of a yeast cell is determined by its history and for example the previous exposure to stress can modulate the next wave of transcriptional

adaptation. In this process both the accumulation of defense proteins as well as the establishment of transcriptional memory in the experienced cells has been implicated (29,30). It is clear from these results that a transcriptional stress response can only be fully understood in the physiological context of the cell. The processes however, which determine a specific transcriptional profile, remain in most cases unexplored. Here we apply time elapsed reporter studies to identify physiological determinants of the positive and negative gene regulation upon osmotic shock. We find that intracellular glycerol production and vacuolar H^+-ATPase activity modulate the timing and efficiency of the transcriptional response and that the turnover of RNA Pol II rather than its absolute levels might be required for efficient activation of gene expression upon salt shock.

EXPERIMENTAL PROCEDURES

Yeast strains and culture conditions – *S. cerevisiae* strains used in this study were: wild type BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and the mutant alleles *kha1::KanMX4*; *mdm38::KanMX4*; *pho89::KanMX4;* *tok1::KanMX4;* *trk2::KanMX4;* *hog1::KanMX4;* *gpd1::KanMX4;* *nha1::KanMX4;* *nhx1::KanMX4;* *trk1::KanMX4;* *vma1::KanMX4;* *vma2::KanMX4;* *vma8::KanMX4;* *vnx1::KanMX4;* *pmc1::KanMX4;* *vam3::KanMX4;* *vam6::KanMX4;* *def1::KanMX4;* *iwr1::KanMX4 (31);* wild type W303-1A (*MATa*; *ade2-1*; *ura3-1*; *his3-11,-15*; *leu2,3-112*; *trp1*) and its derivatives *RPB3-3HA*; *RPB3-3HA hog1::KanMX4*; *RPB3-3HA gpd1::KanMX4* (this study); *RPB3-3HA vma1::KanMX4* (this study) and *ena1-4::HIS3*. Yeast cultures were grown in yeast extract-peptone containing 2% dextrose (YPD) or 2% galactose (YPGal) with or without the indicated supplementation of NaCl or KCl. Synthetic growth medium contained 0.67% yeast nitrogen base, 50mM succinic acid pH 5.5 and 2% dextrose (SD) or 2% galactose (SGal). According to the auxotrophies of each strain, methionine (10mg/l), histidine (10mg/l), leucine (10mg/l), or uracil (25mg/l) were added.

Plasmids – The plasmid p413-GRE2-lucCP$^+$ for the expression of destabilized luciferase under control of the *GRE2* promoter is described in (32). The p413-TDH3-lucCP$^+$ construct expressing destabilized luciferase under control of the constitutive *TDH3* promoter was obtained by cloning the *lucCP*$^+$ gene into the Gateway destination vector pAG413-GPD-ccdB (*HIS3*; *CEN*) (33).

Reverse transcriptase assays – Total RNA was isolated by acid phenol extraction from yeast cells grown in the indicated condition. Total RNA samples were DNaseI digested and purified with the RNeasy Mini kit (Qiagen). A total of 5μg RNA was converted into DNA using the Superscript III first strand cDNA synthesis kit (Invitrogen). The amount of DNA was quantified with the indicated gene specific primers by quantitative PCR in real time using the EvaGreen qPCR Master Mix (Biotium) on an Applied Biosystems 7500 Sequence Detection System. The *ACT1* gene expression was used as a reference. The expression level was determined in triplicate from three independent cDNA samples.

Chromatin Immunoprecipitation (ChIP) – ChIP was performed essentially as described previously (34). Quantitative PCR analyses were performed in real time using the EvaGreen qPCR Master Mix (Biotium) on an Applied Biosystems 7500 Sequence Detection System. The following genomic regions were assayed: *GRE2* (-301/-121); *STL1* (-485/-292); *TDH3* (-133/+14); *RPL2B* (-159/-14). The *POL1* (+1796/+1996) coding sequence was used as an internal control. All ChIP experiments were performed at three independent chromatin samples.

Continuous growth assays – For sensitivity assays in continuous growth, fresh overnight precultures of the yeast wild type strain (BY4741) in YPD or YPGal medium were diluted in triplicate in the same medium with or without the indicated concentration of NaCl in multi-well plates to the same OD. Growth was then constantly monitored under the indicated salt stress conditions in a Bioscreen C system (Thermo) for the indicated times. The growth curves were processed in Microsoft Excel, and the half maximal cell density was calculated for each growth condition. The time to reach half-

maximal cell density (t_{50}) under each stress condition was compared to the t_{50} under nonstress conditions. This ratio was taken as an indicator of the relative growth efficiency.

Live cell luciferase assays – Yeast cells transformed with the destabilized luciferase reporter genes GRE2-lucCP$^+$ or TDH3-lucCP$^+$ were grown to mid log phase in SD medium. Culture aliquots were then incubated for 90 min with 0.5mM luciferin (Sigma) at 28^{o}C. 120 µl aliquots of cells were then distributed in white 96-well plates (Nunc) and the indicated concentrations of NaCl added from appropriate stock solutions. The light emission was immediately and continuously quantified in a GloMax microplate luminometer (Promega) in three biological replicates. Data were processed in Microsoft Excel software and normalized for the amount of cells in each assay. The relative induction or repression was obtained by calculating the log2-ratio of the induced or repressed light value normalized for the value obtained in the mock treated culture at the same time point. EC_{50} values were calculated as described in Dolz-Edo et al. (28).

Measurement of glycerol accumulation – Yeast wild type cells were grown to mid log phase in YPD or YPGal medium. Triplicate samples were taken before or after the indicated time points after addition of 0.8M NaCl. Cells were resuspended in water and glycerol extracted by boiling the samples for 10 min. Glycerol was quantified in the supernatant using the glycerol assay kit (Sigma) with fluorometric detection at λ_{ex} = 525 nm/λ_{em} = 586-648nm on a GloMax microplate fluorometer (Promega).

RESULTS

Time resolved quantification of positive and negative gene regulation upon salt stress – Salt stress activates a complex transcriptional response in yeast cells, which involves both activation at defense genes and repression at "housekeeping" genes. In order to study this adaptive response in a truly kinetic manner, we used live cell reporter assays based on the expression of destabilized luciferase (lucCP$^+$; (32)). We chose to compare the expression patterns in a time resolved manner for the highly inducible *GRE2* gene and the glycolytic *TDH3* gene (Figure 1A). *GRE2* encodes a stress inducible methylglyoxal reductase, whose expression is repressed upon normal growth conditions. *GRE2* serves here as a prototypical stress induced locus. *TDH3* encodes a glyceraldehyde-3-phosphate dehydrogenase and is one of the most highly expressed genes in yeast upon normal growth. *TDH3* is chosen here as a typical "housekeeping" gene.

The expression profiles of both genes were determined upon different salt (NaCl) doses. As expected, *GRE2* expression was highly and transiently activated upon all stress treatments (Figure 1B). Increasing salt concentrations provoked a successive delay in gene induction with the result that the *GRE2* expression peak continuously shifted to later time points with greater salt doses. The *TDH3* gene showed an opposite behavior. Here the initial high expression levels were transiently repressed and the duration of low expression was continuously prolonged upon higher stress doses. Positive and negative control of gene expression appeared to be coordinated in a way that the highest *GRE2* expression coincided with the lowest levels of *TDH3*. Consequently, the release from repression at the *TDH3* gene occurs exactly at the moment when high *GRE2* levels start to decline. Loss of transcriptional activity at the housekeeping gene, however, occurred immediately upon salt shock and did not seem to correlate with the onset of transcription at the inducible locus. In fact, upon all stress treatments and in a certain time window, which increases upon harsher stress conditions, gene expression is absent at both the inducible and the housekeeping gene (Fig. 1B).

A genetic screen for the identification of physiological determinants which modulate the coordinated activation and repression of gene expression upon salt stress – Having created a quantitative tool for the detection of the transient loss of gene expression upon stress, we next wanted to identify physiological functions that altered the coordinated transcriptional control upon salt stress. We tested whether defects in the intracellular ion homeostasis or glycerol production affected the timing of the transcriptional response at the *TDH3* housekeeping gene. We therefore expressed the TDH3-lucCP$^+$ reporter in mutants

of the major intracellular Na^+, K^+ and H^+ transporters located at the plasma membrane or the vacuolar, mitochondrial, golgi or endosomal membranes and in a *gpd1* mutant deficient in salt induced glycerol biosynthesis. Additionally we used a mutant strain deficient in the activity of the Hog1 MAP kinase responsible for the transcriptional activation of osmostress inducible genes. As shown in Figure 2A, the transient loss of TDH3-lucCP$^+$ expression upon salt shock is very reproducible and unaffected in many of the yeast mutants studied here, including a mutant in the major stress inducible cytoplasmatic Na^+ extrusion system *ena1-4*. However, specific mutant strains were identified with an altered pattern of regulation. The *gpd1* and *vma1* (subunit of the vacuolar H^+-ATPase) mutant strains showed an increased loss of the housekeeping gene expression (Figure 2B). In the case of the *gpd1* strain we additionally observed a delay in the recovery of reporter activity after the salt shock. We also identified mutant strains with an opposite phenotype, which is an apparently less pronounced decrease of TDH3-lucCP$^+$ expression during salt stress. The *hog1* and *trk1* (major K^+ importer at the pasma membrane) mutant strains showed this behavior. Additionally, the *hog1* mutant recovered TDH3-lucCP$^+$ expression much more inefficiently after salt exposure (Figure 2B). The same mutant strains were next analyzed for their effect on the gene induction kinetics upon salt stress.

Defects in the timing of positive gene regulation upon NaCl stress in the gpd1 and vma1 mutants – Loss of Vma1 or Gpd1 function caused a more severe loss of gene expression at a housekeeping gene during salt stress. We addressed the question whether the same mutations also caused a different pattern of gene activation at stress defense genes. Therefore we determined the dose response profiles of the inducible *GRE2* gene by the use of the live cell luciferase reporter assay in comparison to wild type. As shown in Figure 3A, the *vma1* and *gpd1* mutants activated the GRE2-lucCP$^+$ reporter in a less efficient manner. Specifically we detected for the *vma1* mutant a reduction in the induced synthesis rates and induction folds and an increasing delay in gene activation clearly detectable at >0.4 M NaCl. Loss of Gpd1 function caused a general delay of *GRE2* activation at any NaCl concentration tested accompanied by a reduction of synthesis rates and induction folds (Figure 3B). We additionally measured the dose dependent activation of the *GRE2*-luciferase reporter in a *hog1* deletion strain (data not shown) and expectedly found its activation more severely reduced (Figure 3B). Finally we also examined a *trk1* mutant strain for the dynamic pattern of *GRE2* induction, which in this case was indistinguishable from wild type cells (Figure 3B). Thus although the *trk1* mutant showed an apparent alteration in the loss of gene expression at a housekeeping locus, it was not affected in the positive control of gene expression upon salt stress.

We additionally included other vacuolar mutants to test their effect on the dynamic of gene activation upon salt stress using the GRE2-luciferase reporter. Deletion of different subunits of the vacuolar H^+-ATPase such as Vma2 (Figure 3C) or Vma8 (data not shown) again showed a strong decrease of *GRE2* activation. Mutants affected in general vacuolar morphogenesis (*vam3*, *vam6*) showed a significant but weaker reduction of reporter activation, while deletion of specific vacuolar cation transporters Vnx1 (Na^+) or Pmc1 (Ca^{2+}) did not alter the *GRE2* induction profile (shown for *vam6* and *vnx1* in Figure 3C). These results correlated very well with the sensitivity of the different vacuolar mutant strains upon high salt stress (Figure 3D).

We next wanted to confirm by direct transcriptional assays that the lack of Gpd1 or Vma1 function negatively affected the timely induction of salt inducible defense genes. We therefore measured the mRNA production at two highly inducible genes, *GRE2* and *CTT1*, by RT-PCR. As shown in Figure 4, the *gpd1* and *vma1* mutants showed a less efficient and delayed induction of transcription at both stress activated genes. We concluded that both the efficient production of osmolytes such as glycerol and the vacuolar ion homeostasis are determinants of the timing of transcriptional induction upon salt stress.

Environmental conditions which interfere with glycerol accumulation cause

important gene induction defects upon salt stress – According to the previous results, an impairment of glycerol accumulation interfered with the timely and efficient upregulation of defense genes upon salt shock. We next wanted to know to which extent growth conditions, which disfavor an efficient glycerol accumulation, would affect transcriptional activation. Glycerol is the main osmolyte produced by *Saccharomyces cerevisiae* upon hyperosmotic stress and its biosynthesis depends on precursors from glycolysis. Therefore we reasoned that growth on only partially fermentable carbon sources could decrease the glycolytic flux and hence affect glycerol production. We confirmed this by comparing the intracellular glycerol accumulation of yeast cells grown in galactose or glucose upon NaCl shock. As shown in Figure 5A, galactose growth reduced glycerol accumulation of the cells by approximately 60%. Accordingly, yeast cells grown with galactose as the carbon source were less tolerant to salt stress and showed a greatly decreased growth efficiency under NaCl stress conditions (Figure 5B). We then measured the dose regulated transcriptional activation of the *GRE2* gene upon glucose and galactose growth by the real time luciferase assay (Figure 5C). *GRE2* induction occurred with an increasing delay towards higher salt stress conditions in galactose medium. Additionally the quantitative analysis of the *GRE2* activation efficiency (Figure 5D) revealed that galactose grown cells reach half maximal *GRE2* activation at much lower NaCl concentrations (186mM) as compared to glucose grown cells (257mM). Activated gene expression at *GRE2* is dynamically increased within a broad NaCl concentration range (0.1-0.8M) in glucose, but in a much more limited range (0.1-0.3M) in galactose. These data demonstrate that the capacity of the yeast cell to counteract osmostress by glycerol accumulation is critically modulated by the carbon source, which in turn determines the efficiency and kinetics of transcriptional activation.

We then tested how glycerol production and vacuolar ATPase activity contributed to the resistance to NaCl stress. As expected, growth of a *gpd1* mutant strain was progressively delayed with increasing salt concentrations (Figure 5E). A weaker sensitivity phenotype was observed for the *vma1* mutant, where a significant growth delay was only observed upon severe NaCl stress. However, in galactose medium, where glycerol accumulation is impaired, the loss of Vma1 function led to a very strong salt sensitivity (Figure 5E). These data indicated that both glycerol production and vacuolar ion sequestration might independently contribute to salt resistance of yeast cells.

Loss of Gpd1 and Vma1 function causes defects in the distribution of RNA PolII complexes during salt stress – Activated gene expression upon salt shock seemed to be delayed and to occur with reduced efficiency in *gpd1* or *vma1* mutant strains. We wanted to find out whether both mutations affected the redistribution of RNA polymerase II complexes from housekeeping to stress induced genes. Therefore we followed the association of RNA PolII at both types of genes during the first minutes of NaCl stress by in vivo ChIP. As shown in Figure 6A, we confirmed that the delayed gene induction at the salt inducible *GRE2* and *STL1* loci previously observed in a *gpd1* mutant strain was due to a delayed and less efficient recruitment of RNA PolII at the respective promoter regions. Further inspection of RNA PolII occupancy at two highly expressed genes (*TDH3*, *RPL2B*; Figure 6B) revealed that indeed the loss of gene expression upon salt stress observed in yeast wild type cells was accompanied by a transient physical loss of polymerase complexes from these gene promoters. In the first instances of salt stress, loss of PolII from housekeeping promoters is comparable in wild type and *gpd1* mutants. However, in *gpd1* mutant cells PolII reassociation occurs with a clear delay. We then extended the ChIP analysis of PolII redistribution to the *vma1* mutant. In this case we also observed a delayed, but not reduced, accumulation of RNA PolII at the inducible *GRE2* and *STL1* promoters (Figure 6C). However, at housekeeping genes, the *vma1* mutant did not show a more pronounced loss of RNA PolII (Figure 6D). These data demonstrated that cells lacking Gpd1 or Vma1 function have difficulties in rapidly mounting transcriptional initiation at stress defense genes.

Additionally it seems that the delayed RNA PolII association with inducible promoters is not the consequence of a slower dissociation of polymerase from housekeeping genes.

Dissociation of RNA PolII from housekeeping genes occurs independently of its recruitment at inducible genes – We have shown above that activated transcription upon salt stress occurs somehow coordinated with very low expression levels at housekeeping genes. We wanted to know whether the induced recruitment of the transcription machinery at stress genes caused its loss from highly expressed genes. To this end, we took advantage of the *hog1* mutant strain, which is known to be defective in the activated recruitment of RNA PolII at the vast majority of osmostress incucible genes (26,27). This defect was confirmed for the *GRE2* gene in response to NaCl stress (Figure 7A). However, the same initial dissociation of RNA PolII was observed for the *hog1* mutant at the *TDH3* and *RPL2B* genes (Figure 7B, C). Moreover, reassociation of the transcription machinery was severely delayed in the *hog1* strain. These data indicated that the loss of RNA PolII complexes from housekeeping genes was independent of the stimulated transcriptional initiation at stress inducible genes. Loss of transcriptional activity at housekeeping genes might therefore be the direct result of the salt stress, as previously described for NaCl shock (21). We thus used a less severe salt treatment by KCl to assay the association kinetics of RNA PolII by ChIP. KCl caused a similar transient association of the transcription machinery at *GRE2*, which again was completely dependent on Hog1 function. In this case the dissociation of RNA PolII was less pronounced at *RPL2B* and not observed at *TDH3*, while *hog1* mutants showed a substantial and prolonged loss of transcription complexes at both housekeeping genes also upon KCl stress. These data show that the dissociation of RNA PolII complexes occurs dependent on the severity of cationic stress but independently of Hog1 function. The MAP kinase function in turn is essential for the reassociation of transcription complexes at housekeeping genes.

Effects of RNA PolII turnover and nuclear import on the coordinated gene expression upon salt stress – So far we have determined that physiological processes such as the intracellular ion homeostasis and osmolyte biosynthesis affect the kinetics and efficiency of the transcriptional response to salt stress. Given that the luciferase reporter applied here allow to monitor dynamic gene expression changes in a very sensitive manner, we investigated how the recycling and nuclear abundance of RNA PolII complexes affected positive and negative regulation upon salt stress. We considered two mutant strains, *def1* and *iwr1*. Def1 marks arrested RNA PolII elongation complexes for proteasomal degradation (35). As shown in Figure 8A, we observed a slightly decreased decay of the housekeeping luciferase reporter in a *def1* mutant. Additionally we detected an apparently slower recovery of gene expression after salt shock in this mutant. The most prominent defect, however, was manifested in the *def1* mutant for the activated *GRE2* expression (Figure 8A), which was reduced to less than 40%.

Iwr1 is an RNA PolII nuclear import factor and its mutation causes a reduction of active PolII transcription complexes in the nucleus (36,37). As depicted in Figure 8B, the *iwr1* mutant strain showed an increased loss and delayed recovery of housekeeping gene activity upon salt shock, a phenotype, which resembled the *gpd1* mutant lacking proper osmolyte production. However, in this case the efficient induction of the *GRE2* stress gene was completely normal (Figure 8B). These data suggested that a reduced pool of RNA PolII does not affect the transient activation of stress genes but might be limiting for reactivating housekeeping genes after stress adaptation. Furthermore it is possible that removal and turnover of non productive RNA PolII complexes during salt shock is necessary for efficient gene activation. Taken together we report here physiological processes to critically affect the dynamics of gene expression during the adaptation to high salt stress.

DISCUSSION

In this work we identify physiological functions, which determine the dynamics of an adaptive transcriptional response in the case of salt stress. This scenario is chosen here because adaptation to salinity stress requires a considerable modification in gene expression but the stress itself imposes a serious hurdle for efficient transcription. Therefore this type of adverse environmental condition is a good model to improve our understanding of the interplay between the physiological state of the cell and its ability to trigger an adequate activation of defense genes. It is well known that an increasing hyperosmotic challenge causes a delayed transcriptional response of yeast cells (38). Some molecular aspects of how high salinity interferes with the activation of gene expression have been revealed recently. Accordingly, hyperosmolarity slows down signal transduction through the HOG MAP kinase pathway because of molecular crowding, delays the nuclear accumulation of activated Hog1 MAP kinase and generally interferes with the association of specific and general transcription factors to chromosomal DNA (21,23-25). Thus, yeast cells must have mechanisms to overcome the general inhibition of transcription under salt stress conditions, which can be described now in detail with the data presented here (Figure 9).

An immediate consequence of salt stress is the instantaneous loss of gene expression from highly transcribed housekeeping genes. This rapid inhibition is not dependent on the stress dose and occurs similarly at the beginning of different NaCl exposures (Figure 1), however the salt dose determines how long the housekeeping functions remain untranscribed. Loss of housekeeping gene expression is caused by the dissociation of RNA PolII complexes from their promoters (Figures 6 and 7). It is important to note that this loss of transcription complexes is not the cause of their reallocation at stress inducible promoters as has been suggested recently (26), because a *hog1* mutant with largely absent transcriptional initiation at stress induced loci shows an increased and prolonged loss of PolII complexes from housekeeping genes. Thus, the immediate, and most likely passive, loss of transcriptional activity upon salt challenge seems to lead to a lag phase of adaptation where apparently neither constitutive nor stress inducible genes are expressed (Figure 1). This phase becomes continuously longer with increasing salt doses. Previous work has identified the Hog1 dependent activation of plasma membrane transporters such as Nha1 to be important in this pre-transcriptional adaptation (21). In this work we identify other physiological determinants of the transcriptional salt response, which are the biosynthesis of glycerol and the vacuolar ATPase activity.

Our results show that glycerol accumulation in the first minutes upon salt stress exposure determines the onset and efficiency of transcriptional activation. Impairment of glycerol biosynthesis causes a prolonged loss of housekeeping gene expression and a delayed activation at defense genes. Since the protective effect of glycerol is shown here to occur before the first wave of stress defense gene expression we have to evoke mechanisms which do not rely on de novo protein synthesis and therefore different from the well described transcriptional control of glycerol synthesis enzymes Gpd1 or Gpp2. Indeed glycerol accumulation occurs immediately after salt exposure independently of de novo enzyme synthesis (20,39,40). Additionally, non transcriptional mechanisms act on glycolytic enzyme activities such as Pfk2 (41) and on glycerol transporters such as Fps1 (42) to facilitate glycerol biosynthesis and intracellular accumulation upon osmotic shock. Thus, during the lag phase before the activation of gene expression, glycolytic intermediates are temporarily directed towards glycerol production, which in turn facilitates an efficient transcriptional adaptation. In this scenario we expect any decrease in glycolytic flux to cause a poor glycerol accumulation and a delayed transcriptional response. This is shown here by simply switching yeast cells from glucose to galactose metabolism, which is known to reduce the flux through glycolysis and to shift the metabolism partially towards respiration (43). The consequences for osmotic adaptation are shown here (Figure 5) and include a notable reduction in intracellular glycerol accumulation in accordance with previous work (44), a

decrease in growth efficiency and a much less dynamic and efficient activation of defense gene expression upon salt stress. Thus we describe an example were the physiological state of the cell, determined in this case by its mode of carbohydrate metabolism, is an important modulator of its transcriptional stress defense.

Apart from osmolyte production, also the intracellular ion homeostasis plays an important role in determining the transcriptional response to salt stress. According to our results, a lack of vacuolar ATPase activity delays the initiation of transcription at inducible genes upon salt stress (Figures 3, 4, 6). The proton translocating V-ATPase acidifies the vacuolar lumen and thus energizes the import of cations such as Na^+ by other vacuolar transporters (45). Indeed the function of the vacuolar ATPase is important for the survival of yeast cells upon severe salt stress (22,46). Moreover the fragmentation of vacuoles upon ionic stress might be an adaptive mechanism to ensure an enhanced sequestration of toxic Na^+ ions upon salt stress (47). Of note, while the V-ATPase activity is salt inducible, it seems to be important for proper ion homeostasis also under normal growth conditions, as *vma* mutants show elevated HOG pathway activation even in the absence of salt stress (22). These observations fit with a model where in the initial phase of salt stress adaptation both the biosynthesis of glycerol and the sequestration of toxic ions in the vacuoles are important and fast mechanisms to decrease the excess of Na^+ in the cytosol and nucleus. Any interference with this pretranscriptional adaptation will result in a delay of the relatively slower transcriptional response (Figure 9).

Changes in the gene expression pattern upon salt stress are profound and aim to equip the cell with a different protein composition, which is more compatible with the changing environment of the cells. Although transcription initiation complexes have to form at many stress defense loci simultaneously, this process does not seem to depend on an especially high pool of active RNA PolII complexes. We show here that an RNA PolII import deficient cell (*iwr1*) is not affected in the timely induction of osmostress responsive genes. This could indicate that the amount of active PolII complexes in the nucleus is not a limiting factor for a massive gene activation at stress genes. Probably most transcription complexes can be derived from the transiently repressed housekeeping loci. Interestingly we find that a lack of RNA PolII turnover in a *def1* mutant strongly interferes with the efficient activation of a stress gene upon NaCl stress. Def1 plays a crucial role in the targeting, ubiquitilation and degradation of stalled RNA PolII elongation complexes (35,48). This phenomenon has been discovered upon treatment with DNA damaging agents or irradiation, which causes transcriptional arrest. However, ubiquitilation of RNA PolII also occurs upon reduction of its elongation or promoter escape rate in the absence of physical DNA lesions (49). Salt stress could in fact provoke very inefficient transcriptional elongation as it generally interferes with the association of DNA bound proteins in vivo (21). Thus, also salt stress could activate the Def1 mediated RNA PolII removal from unproductive sites in the genome to allow the efficient activation at inducible loci. Our preliminary data shown here might therefore stimulate research on the broader implication of RNA PolII turnover in stress responses. Taken together, our work demonstrates that the dynamics and efficiency of stress induced gene activation is not only conditioned by the directly implicated transcription factors and signal transducers but also by many other physiological adaptations.

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FOOTNOTES

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[3] Both are co-senior authors
[4] The abbreviations used are: HOG, high osmolarity glycerol; ROS, reactive oxygen species; RNA PolII, RNA polymerase II; S.D., standard deviation.

FIGURE LEGENDS

FIGURE 1. **Positive and negative gene expression is coupled upon salt stress.** *A*, schematic overview of the luciferase reporter constructs used in the assay. *B*, coordinated expression of the stress inducible *GRE2* gene versus the repressed housekeeping gene *TDH3*. Yeast strains expressing the GRE2-lucCP$^+$ (green lines) or the TDH3-lucCP$^+$ (red lines) reporter genes were subjected to increasing salt stress by the addition of the indicated concentrations of NaCl. The activity of the reporters was measured continuously in the living cell. Data shown are the mean from three biological replicas with the respective S.D.

FIGURE 2. ***vma1* and *gpd1* mutants show an altered expression profile of the *TDH3* housekeeping gene upon salt stress.** *A*, yeast wild type (BY4741, *upper two panels*; W303-1A, *lower panel*) and the indicated mutant strains expressing the TDH3-lucCP$^+$ reporter gene were subjected to salt stress by the addition of 0.4M NaCl. The expression of the luciferase reporter was measured continuously in the living cell. Data shown are mean values from six independent experiments and represent the relative light emission normalized for the mock treated cells of the same genetic background. *B*, highlighting the mutant strains with an altered TDH3-lucCP$^+$ expression profile from the experiment in (A). Data shown are mean values from six independent experiments; significance values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were obtained with the Student´s t-test for the indicated intervals; error bars represent S.D.

FIGURE 3. ***vma1* and *gpd1* mutants express the stress activated *GRE2* gene less efficiently.** *A*, yeast wild type (BY4741) and the indicated mutant strains harboring the GRE2-lucCP$^+$ reporter gene were subjected to the indicated salt stress conditions (NaCl). The expression of the luciferase reporter was measured continuously in the living cell. Data shown are mean values from three independent experiments; error bars represent S.D. *B*, *C*, fold induction profiles (log2-ratio) of the GRE2-luciferase reporter in the indicated yeast strains upon treatment with 0.4M NaCl. Data shown are the mean values from three independent experiments. Significance values (* $p < 0.01$, ** $p < 0.005$, *** $p < 0.001$) were obtained with the Student´s t-test for the indicated intervals. *D*, salt sensitivity assays with selected vacuolar mutant strains.

FIGURE 4. **mRNA production at salt inducible genes is delayed and less efficient in *vma1* and *gpd1* mutants.** RT-PCR analysis of the *GRE2* (*upper panel*) and *CTT1* (*lower panel*) gene transcription. The indicated yeast strains were treated with 0.4M NaCl and the accumulation of mRNA quantified at the indicated time points. Stress gene expression was normalized with the *ACT1* mRNA as a control. Data shown are mean values from three independent experiments; error bars represent S.D.

FIGURE 5. **Galactose growth reduces glycerol accumulation, growth efficiency and the dynamics of transcriptional activation upon salt stress.** *A*, measurement of intracellular glycerol accumulation in yeast wild type (BY4741). Cells were grown to mid log phase with glucose or galactose as the carbon source and then subjected to osmotic stress by addition of 0.8M NaCl. The glycerol content of the cells was determined in triplicate at the indicated time points. Error bars are S.D. *B*, growth kinetics upon salt stress of yeast wild type cells (BY4741) with glucose or galactose as the carbon source. The growth curves upon increasing concentration of NaCl are depicted in the lower panel for glucose and galactose media. Data shown are the media of three independent yeast cultures for each condition. The error is <15%. The growth efficiency relative to non stress condition was calculated for each stress dose and is depicted in the upper panel. Error bars are S.D. *C*, comparison of the dose response profile for the *GRE2* gene upon NaCl stress for glucose and galactose growth. Yeast wild type cells harboring the GRE2-lucCP$^+$ reporter gene were grown in glucose or galactose containing minimal medium and then treated with the indicated NaCl doses. The expression of the luciferase reporter was measured continuously in the living cell. The time

needed to reach maximal reporter expression is indicated upon both growth conditions. Data shown are mean values from three independent experiments. The error is <15%. *D*, the maximal reporter activity was calculated for the glucose or galactose grown cells and plotted against the NaCl concentration. The maximal luciferase activity was adjusted to 100 for each carbon source. The stressor concentration to reach 50% of the maximal reporter gene activity (EC_{50}) is indicated for both growth conditions. Error bars are S.D. *E*, growth kinetics of *gpd1* and *vma1* mutant strains compared to wild type; (left panel) growth upon NaCl stress is compared in wild type and *gpd1* mutant in glucose media, (middle and right panel) growth upon NaCl stress is compared in wild type and *vma1* mutant in glucose and galactose media. Data shown are the media of three independent yeast cultures for each condition. The error is <15%.

FIGURE 6. **The function of Gpd1 and Vma1 is important for the fast redistribution of RNA PolII from housekeeping to stress inducible genes.** Chromatin immunoprecipitation (ChIP) analysis of Rpb3 in the indicated strain backgrounds. The cells were grown to mid log phase and then subjected to a brief osmotic shock (0.4M NaCl). RNA PolII occupancy was quantified by Rpb3-HA ChIP at the indicated gene promoters and normalized to the *POL1* control region. Occupancy levels before stress were arbitrarily set to 1. Data shown are mean values from three independent chromatin samples. Error bars are S.D. *A*, comparison of Rpb3 association with the inducible *GRE2* and *STL1* promoters in wild type yeast (W303-1A) and the *gpd1* deletion mutant. *B*, comparison of Rpb3 association with the *TDH3* and *RPL2B* housekeeping gene promoters in wild type yeast (W303-1A) and the *gpd1* deletion mutant. *C*, comparison of Rpb3 association with the inducible *GRE2* and *STL1* promoters in wild type yeast (W303-1A) and the *vma1* deletion mutant. *D*, comparison of Rpb3 association with the *TDH3* and *RPL2B* housekeeping gene promoters in wild type yeast (W303-1A) and the *vma1* deletion mutant.

FIGURE 7. **Dissociation of RNA PolII complexes from housekeeping loci upon salt stress is independent of recruitment at inducible genes and aggravated by the loss of Hog1 function.** Chromatin immunoprecipitation (ChIP) analysis of Rpb3 in wild type yeast (W303-1A) and the *hog1* deletion mutant. The cells were grown to mid log phase and then subjected to a brief osmotic shock (0.4M NaCl or KCl as indicated). RNA PolII occupancy was quantified by Rpb3-HA ChIP at the inducible *GRE2* promoter (A) and the *TDH3* and *RPL2B* housekeeping promoters (B, C) and normalized to the *POL1* control region. Occupancy levels before stress were arbitrarily set to 1. Data shown are mean values from three independent chromatin samples. Error bars are S.D.

FIGURE 8. **Def1, but not Iwr1, is important for efficient *GRE2* induction upon salt stress.** Yeast wild type (BY4741) and the *def1* and *iwr1* mutant strains expressing the TDH3-lucCP$^+$ (left panels) or GRE2-lucCP$^+$ (right panels) reporter gene were subjected to salt stress by the addition of 0.4M NaCl. The expression of the luciferase reporters was measured continuously in the living cell. Data shown are mean values from six independent experiments and represent the relative light emission (log2-ratio) normalized for the mock treated cells of the same genetic background. *A*, comparison of the housekeeping (*GPD*) and stress inducible (*GRE2*) luciferase reporter in wild type and the *def1* mutant. *B*, comparison of the housekeeping (*TDH3*) and stress inducible (*GRE2*) luciferase reporter in wild type and the *iwr1* mutant. Significance values (* $p < 0.05$, ** $p < 0.002$) were obtained with the Student´s t-test for the indicated intervals.

FIGURE 9. **Schematic overview of the early events in the adaptation of gene expression to salt stress.** The upper panel illustrates the association of RNA PolII complexes along the salt shock at housekeeping and inducible genes; the lower panel summarizes the gene expression profiles at the same genes. Salt stress causes a rapid loss of transcription complexes and gene expression activity at housekeeping genes, which is followed by a lag phase of low transcriptional activity in general. The length of the lag phase is determined by different processes such as the biosynthesis of

osmolytes (glycerol) or the intracellular ion distribution (vacuolar ATPase). Additionally, Def1 might play a role in the disassembly of RNA PolII at housekeeping genes and the assembly of active transcription complexes at stress induced genes. The lag phase is followed by the transient gene expression burst at activated genes while housekeeping genes remain completely inactive. Finally, the decline in activated expression at defense genes sets the mark for the subsequent recovery of RNA PolII complexes and expression at housekeeping genes.

Figure 1

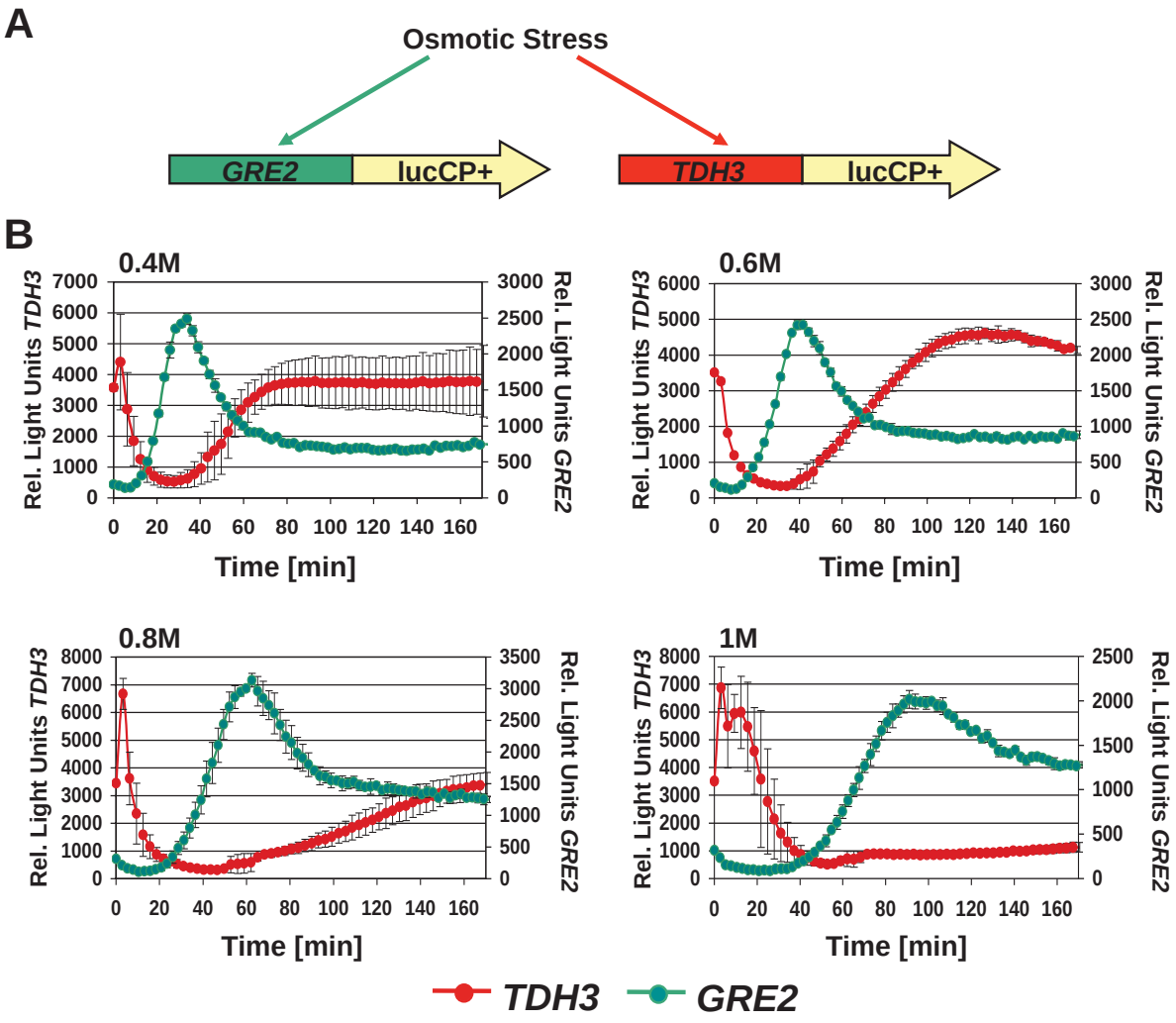


Figure 2

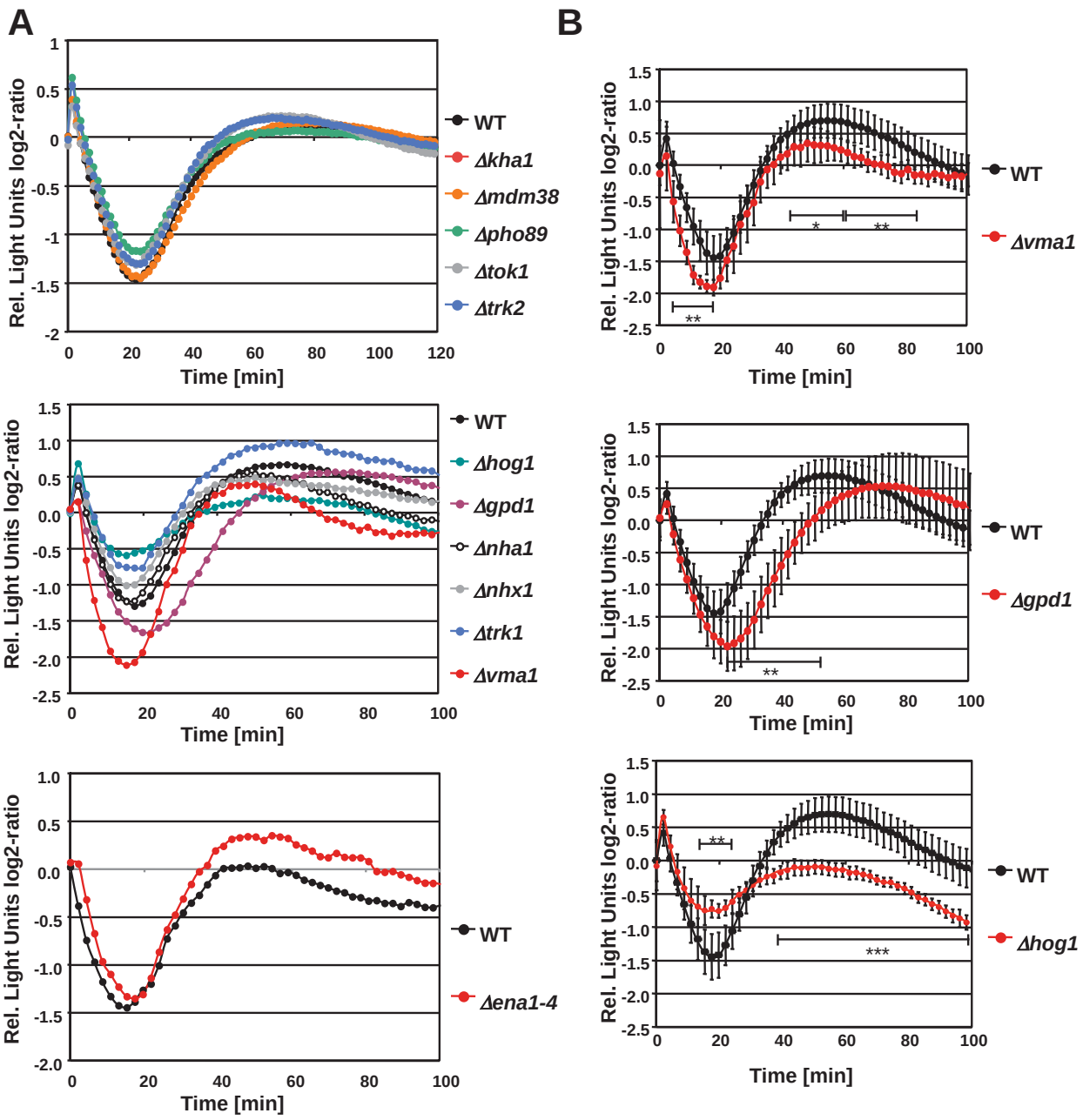


Figure 3

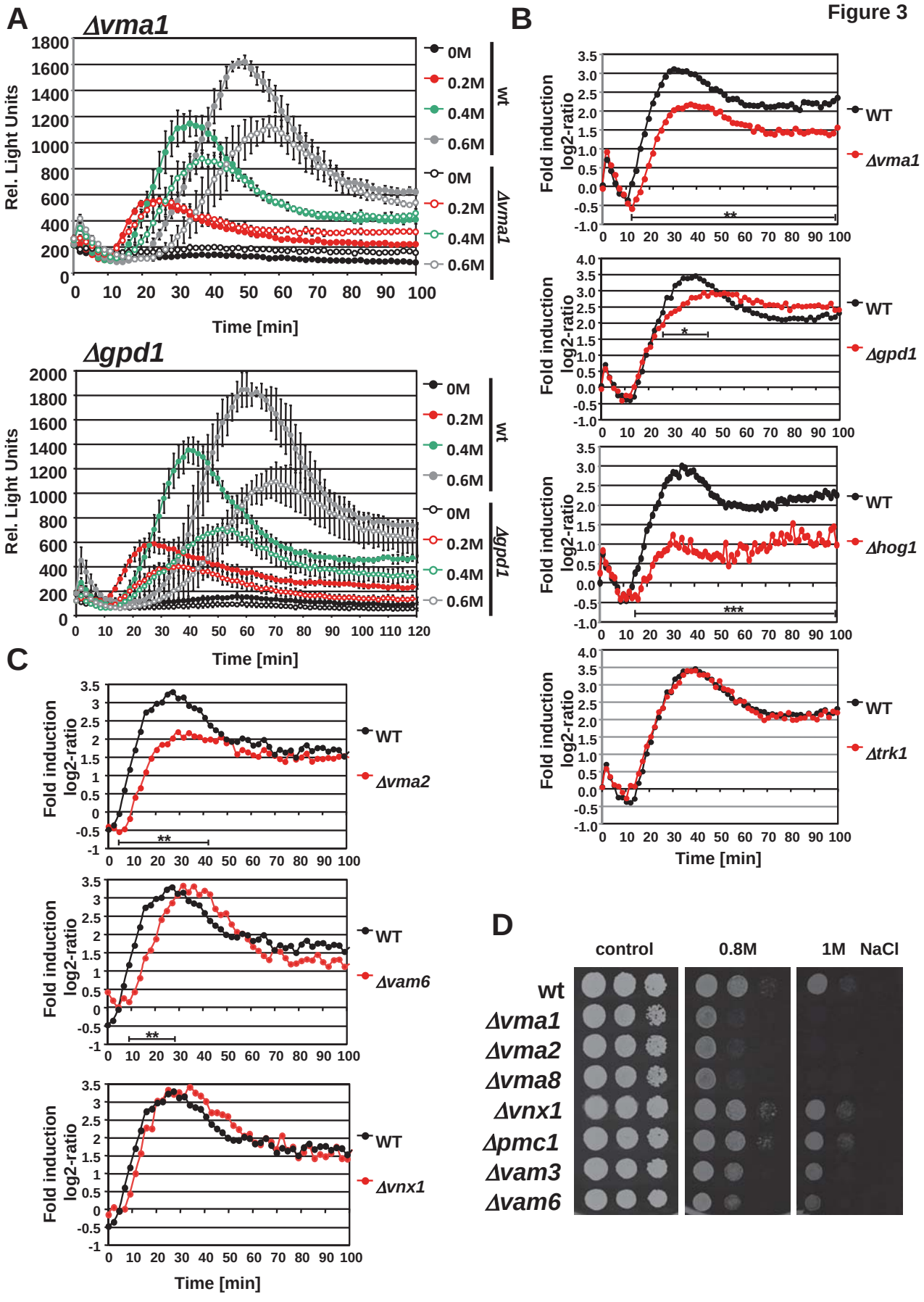


Figure 4

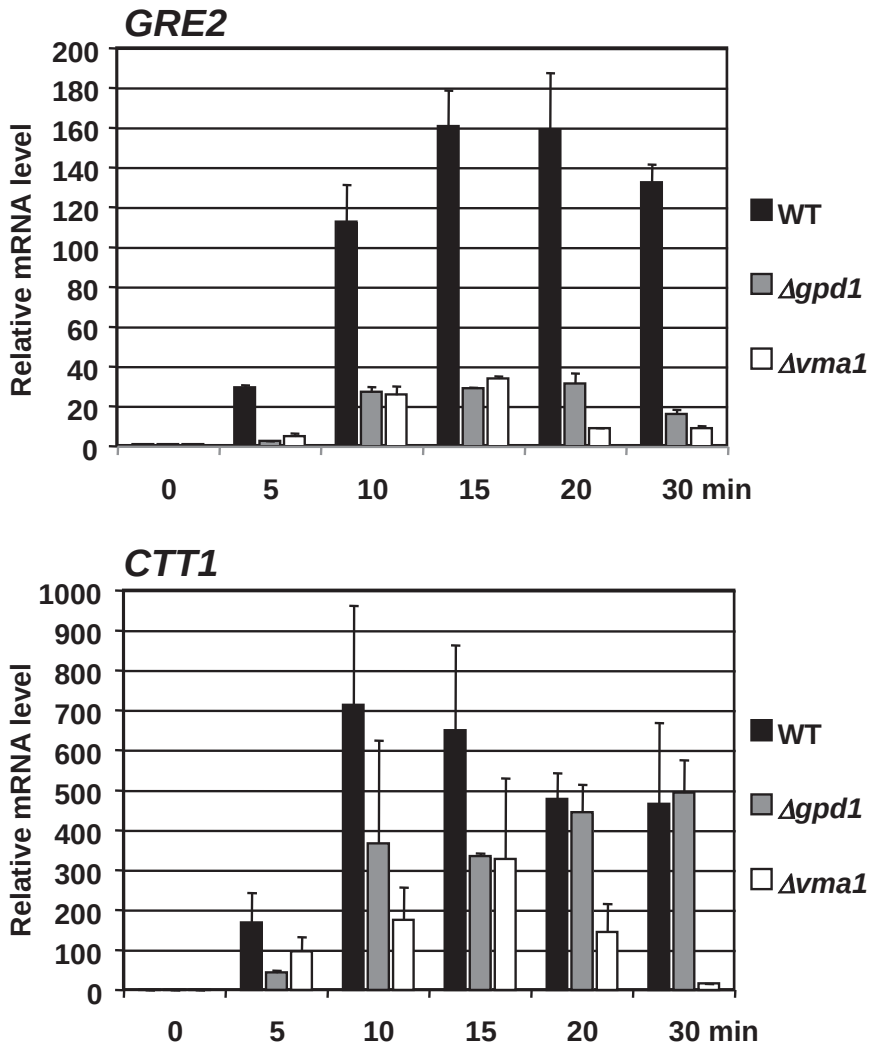


Figure 5

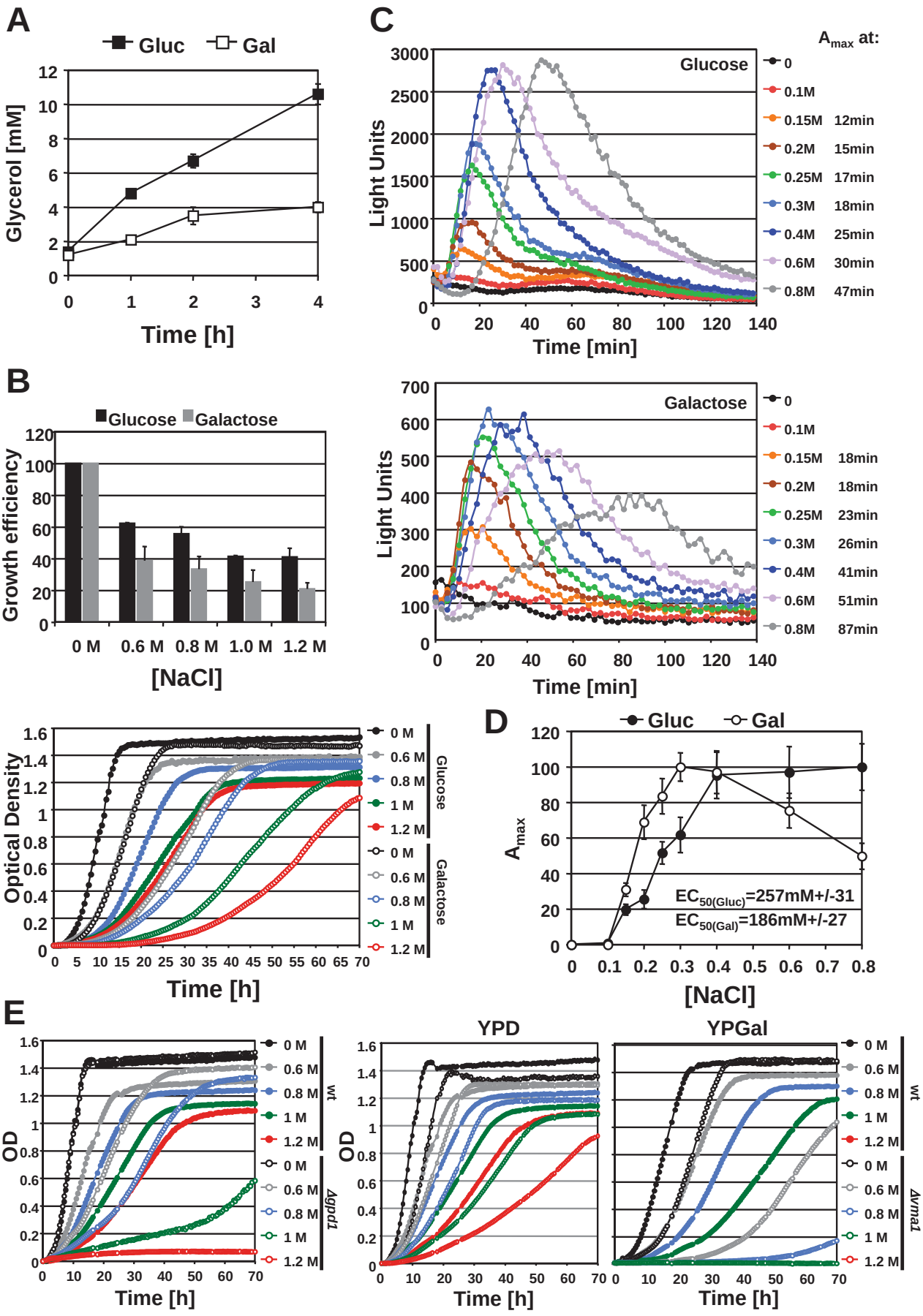


Figure 6

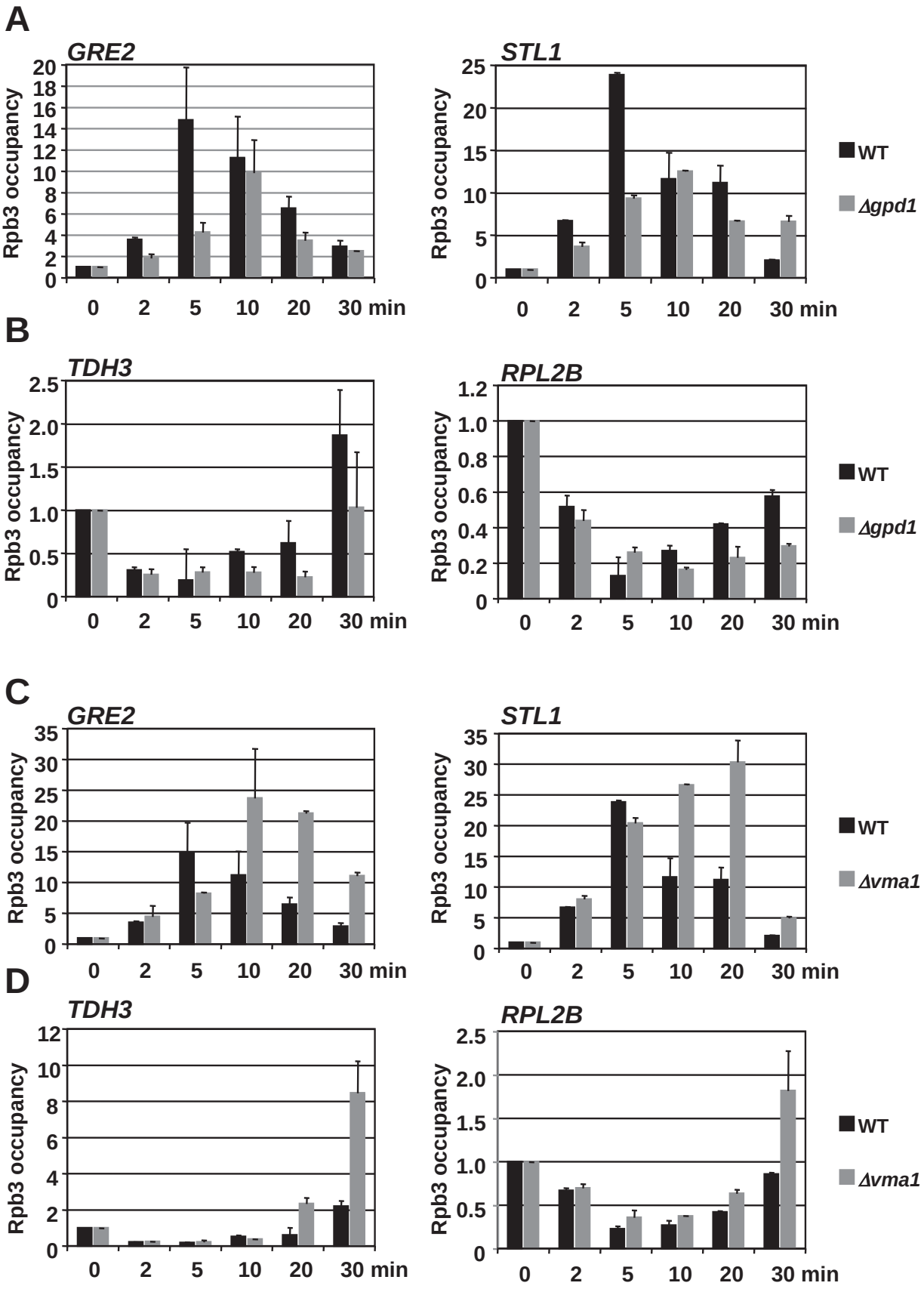


Figure 7

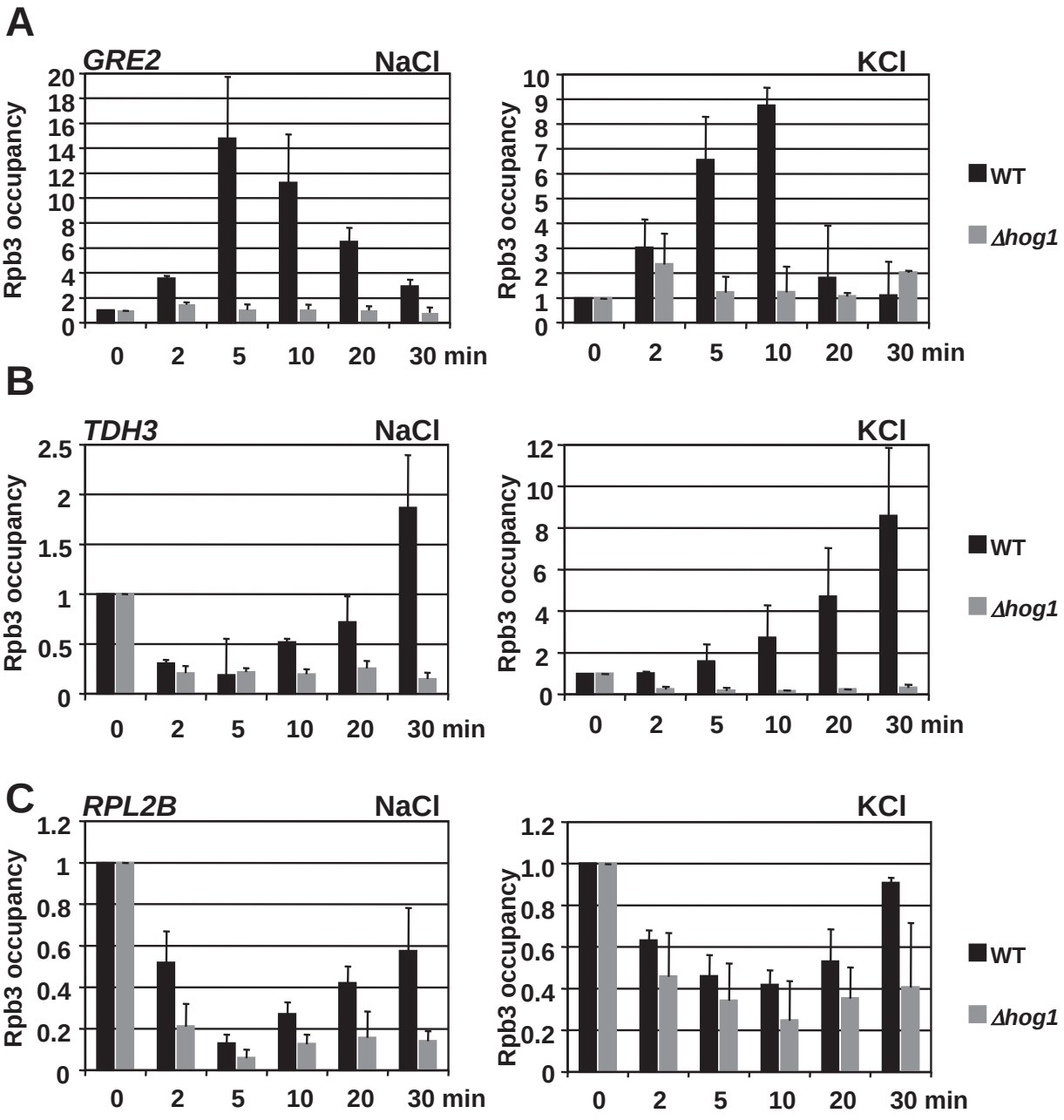


Figure 8

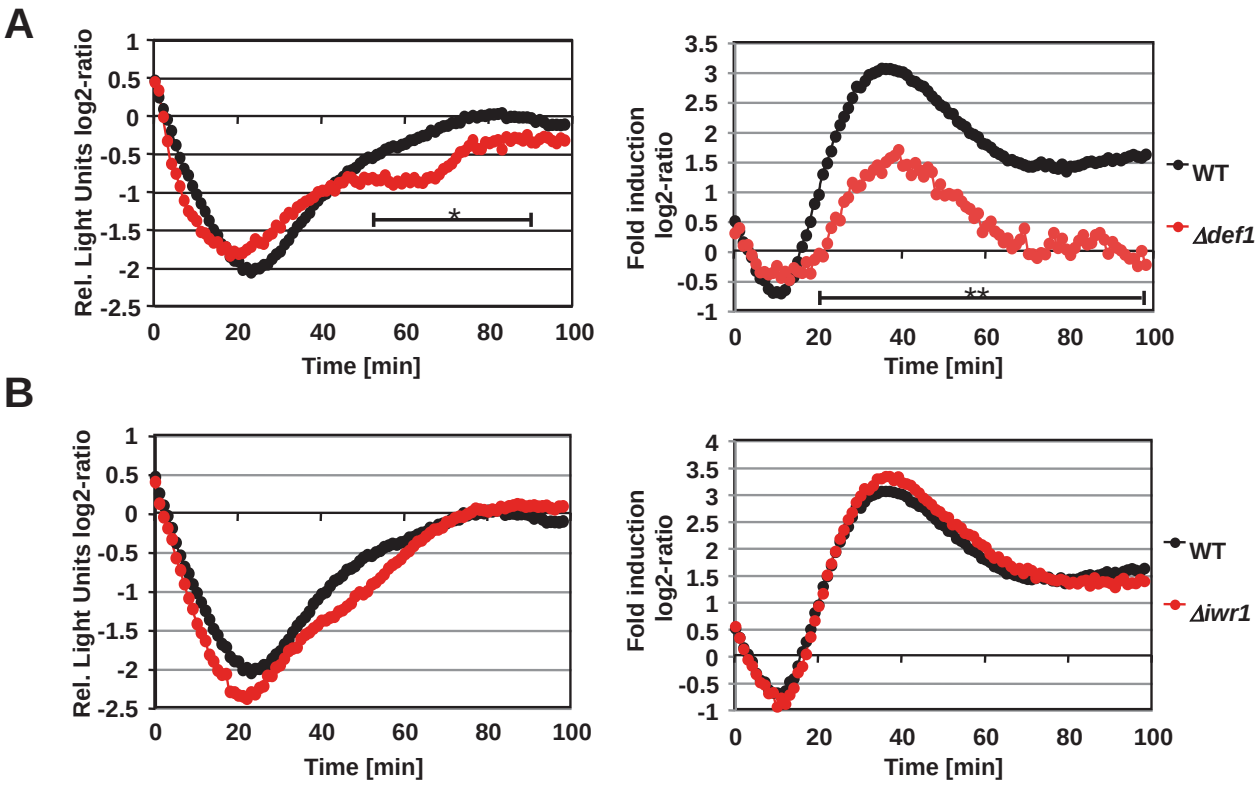
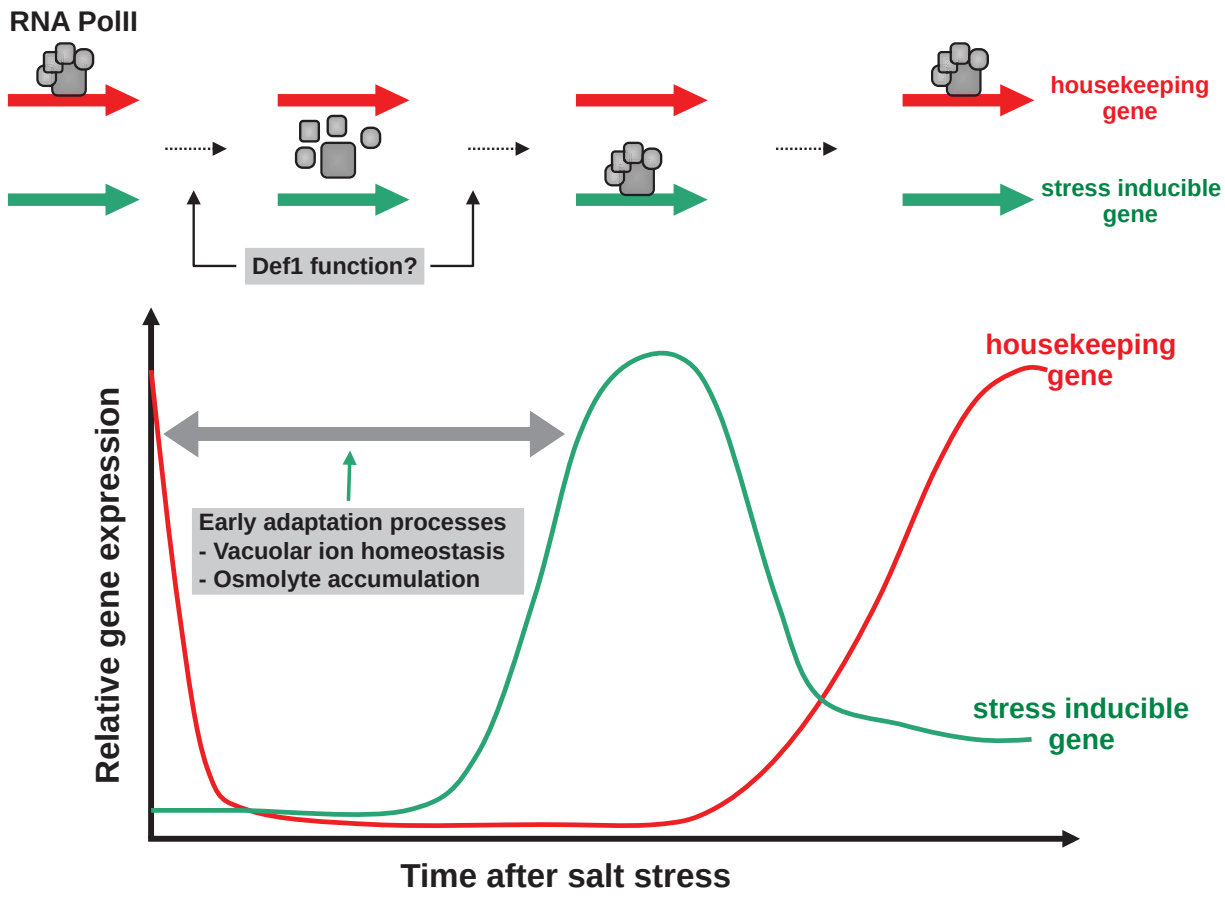


Figure 9



Gene Regulation:

Coordinated Gene Regulation in the Initial Phase of Salt Stress Adaptation

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