JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2011.02249.x

# Adaptation of *Saccharomyces cerevisiae* to saline stress through laboratory evolution

R. DHAR*†, R. SÄGESSER*†, C. WEIKERT*†, J. YUAN*† & A. WAGNER*†‡

*Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland
†The Swiss Institute of Bioinformatics, Quartier Sorge – Batiment Genopode, 1015 Lausanne, Switzerland
‡The Santa Fe Institute, 1399 Hyde Park Road, Santa Fe, New Mexico 87501, USA

*Keywords:*

adaptation;
experimental evolution;
gene expression;
genomics;
ploidy;
single-nucleotide polymorphism.

## Abstract

Most laboratory evolution studies that characterize evolutionary adaptation genomically focus on genetically simple traits that can be altered by one or few mutations. Such traits are important, but they are few compared with complex, polygenic traits influenced by many genes. We know much less about complex traits, and about the changes that occur in the genome and in gene expression during their evolutionary adaptation. Salt stress tolerance is such a trait. It is especially attractive for evolutionary studies, because the physiological response to salt stress is well-characterized on the molecular and transcriptome level. This provides a unique opportunity to compare evolutionary adaptation and physiological adaptation to salt stress. The yeast *Saccharomyces cerevisiae* is a good model system to study salt stress tolerance, because it contains several highly conserved pathways that mediate the salt stress response. We evolved three replicate lines of yeast under continuous salt (NaCl) stress for 300 generations. All three lines evolved faster growth rate in high salt conditions than their ancestor. In these lines, we studied gene expression changes through microarray analysis and genetic changes through next generation population sequencing. We found two principal kinds of gene expression changes, changes in basal expression (82 genes) and changes in regulation (62 genes). The genes that change their expression involve several well-known physiological stress-response genes, including *CTT1*, *MSN4* and *HLR1*. Next generation sequencing revealed only one high-frequency single-nucleotide change, in the gene *MOT2*, that caused increased fitness when introduced into the ancestral strain. Analysis of DNA content per cell revealed ploidy increases in all the three lines. Our observations suggest that evolutionary adaptation of yeast to salt stress is associated with genome size increase and modest expression changes in several genes.

## Introduction

The ability to characterize the changes that occur during evolutionary adaptation on a genome-wide scale has been a boon for the field of laboratory evolution. Most published studies focus on traits with a simple basis, where changes of major effects in one or few genes can alter a trait during laboratory evolution experiments (Ferea *et al.*, 1999; Blanc & Adams, 2003; Velicer *et al.*, 2006; Stanek *et al.*, 2009). Such traits are important, but they are in the minority. The vast majority of traits have a complex, polygenic basis (Benfey & Protopapas, 2005). We know much less about how genomic change and change in gene expression occurs in such polygenic traits. Our study is a step towards answering this question. We here focus on a prototypical polygenic trait, an organism's response to high concentrations of salt in its environment. The physiological response of an organism to such salt stress is well studied on the molecular and on

*Correspondence:* Andreas Wagner, Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Bldg. Y27, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland.
Tel.: +41 44 635 6141; fax: +41 44 635 6144;
e-mail: andreas.wagner@ieu.uzh.ch

the transcriptome levels (Posas *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001). This fact provides another important motivation to study the salt stress response in an evolutionary context. It allows us to ask whether evolutionary adaptation to salt stress is similar to physiological adaptation, using a genome-scale approach that relies on transcriptome changes in response to salt stress.

Environmental fluctuations and stressors constantly challenge organisms in the wild. Organisms thus use cellular mechanisms to adapt to and to survive environmental fluctuations. Hyperosmotic stress is one prominent environmental stressor, where a cell experiences higher solute concentration outside the cell than inside. This causes water loss from the cell, resulting in a higher intracellular concentration of ions and metabolites, and eventual arrest of cellular activity. Hyperosmotic stress is caused by high concentrations of sugar or salt, e.g. sodium chloride (NaCl). High salt stress is a special case of hyperosmotic stress and has similar effects on a cell as high concentration of sugars (Gasch *et al.*, 2000; Causton *et al.*, 2001). In addition, it causes hyperionic stress because of high extracellular concentrations of $Na^+$ and $Cl^-$ ions, which are imported into the cell and can disrupt cellular ionic equilibrium. Tolerance to $Na^+$ stress thus needs additional ion transport and detoxification mechanisms along with those required for the hyperosmotic stress response (Serrano, 1996; Apse *et al.*, 1999; Gaxiola *et al.*, 1999; Maathuis & Amtmann, 1999; Serrano *et al.*, 1999; Zhu, 2001). The hyperosmotic stress response in yeast is mediated by the high osmolarity glycerol (HOG) pathway, which is a MAPK pathway (Brewster *et al.*, 1993; Dihazi *et al.*, 2004; Saito & Tatebayashi, 2004; reviewed in Hohmann, 2002).Two cell membrane–bound sensors, Sho1p and Sln1p, detect osmotic change, which results in activation of Hog pathway genes, which, in turn, leads to the activation of the downstream genes associated with salt tolerance and adaptation (Martinez-Pastor *et al.*, 1996; Schmitt & McEntee, 1996; Gorner *et al.*, 1998; Ostrander & Gorman, 1999; Rep *et al.*, 1999, 2000; Reiser *et al.*, 2000). Whole transcriptome studies by Gasch *et al.* (2000), Posas *et al.* (2000), Rep *et al.* (2000), Causton *et al.* (2001) and Yale & Bohnert (2001) have identified hundreds of genes whose expression levels are affected by hyperosmotic stress. The genes induced during osmotic stress response include the genes involved in synthesis and regulation of the cellular osmolytes glycerol and trehalose. Another group of genes, specifically activated under saline stress, are associated with ion homeostasis. Some of the genes affected by osmotic stress show transient expression changes, whereas others show expression changes that are stable in time. In addition to genome-wide approaches, many studies have characterized the roles of individual genes in the osmotic stress response of yeast (Haro *et al.*, 1991; Garciadeblas *et al.*, 1993; Mai & Breeden, 1997; Ganster *et al.*, 1998; Mendizabal *et al.*, 1998; Tsujimoto *et al.*, 2000; Betz *et al.*, 2002; Goossens *et al.*, 2002; Hirata *et al.*, 2003; Heath *et al.*, 2004).

Yeast is a good model system for studying osmoadaptation in eukaryotes, as other fungi and plants share many of the stress response pathways and proteins involved in osmoadaptation in yeast. First, the mitogen-activated protein kinase (MAP kinase) cascade, central to the stress response in yeast, is a conserved eukaryotic signal transduction pathway present from fungi to plants (Kosako *et al.*, 1993; Neiman, 1993; Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994; Waskiewicz & Cooper, 1995; Jonak *et al.*, 1999; Kyriakis & Avruch, 2001). Stress signalling in plants is also carried out by MAPK pathways, which are activated by cold, drought, salt, heat and oxidative stress (Jonak *et al.*, 1996; Kovtun *et al.*, 2000; Teige *et al.*, 2004). Second, yeast and plants have highly similar genes required for stress tolerance (Mendoza *et al.*, 1994; Bressan *et al.*, 1998; Pardo *et al.*, 1998; Lee *et al.*, 1999; Hasegawa & Bressan, 2000; Sanders, 2000; Quintero *et al.*, 2002; Zhu, 2002). Third, yeast and plants have similar membrane ion transport and detoxification systems (Gaxiola *et al.*, 1999; Quinteroa *et al.*, 2000). For example, HAL family genes are important for ion homeostasis in yeast as well as in plants (Haro *et al.*, 1991; Gaxiola *et al.*, 1992; Ferrando *et al.*, 1995; Murguía *et al.*, 1996; Rios *et al.*, 1997; Espinosa-Ruiz *et al.*, 1999; Mulet *et al.*, 1999; Gisbert *et al.*, 2000; Yang *et al.*, 2001).

With one exception (Samani & Bell, 2010), all previous studies of salt stress adaptation in yeast focused on *physiological* adaptation. Such adaptation occurs on time scales up to a few hours. The mechanisms of longer-term *evolutionary* adaptation to salt stress are not known. Such adaptations occur on time scales of hundred generations or more. One aim of this study is to compare a population's evolutionary response to salt stress with its physiological response on the transcriptome level. Does evolutionary adaptation mirror physiological adaptation? Does it affect largely the same genes as the physiological response?

A second aim is to investigate the genetic basis of *evolutionary* adaptation of yeast to high saline stress, as far as this is possible for a complex trait. Does the adaptation come about through accumulation of identifiable beneficial point mutations? Does it involve chromosomal rearrangements, as observed in evolution of yeast in glucose-limited or phosphate-limited media? Or does it take place through genetic and epigenetic changes altering expression of genes that help cells adapt to high salt? These are some of the questions we ask.

Understanding of *evolutionary* principles of salt tolerance could also be important for biotechnological applications. First, yeast cells experience high salt concentration in many industrial fermentation processes, and improvement in performance of yeast in such conditions would benefit the industry immensely (Attfield, 1997; Trainotti & Stambuk, 2001; Zheng *et al.*,

2011). Second, the principles of salt tolerance in yeast might be useful for engineering fungi and crop plants for salt tolerance, as both classes of organisms share many components of their stress response systems.

In this contribution, we exposed yeast to high salt concentrations for 300 generations in the laboratory in medium containing sodium chloride (NaCl). We compared the fitness and viability of the evolved lines with the starting yeast strain (ancestral strain), followed by characterization of gene expression changes and genetic changes such as mutations, copy number variations and chromosomal alterations.

## Results

To test for evolutionary adaptation of yeast cells to osmotic stress, we carried out laboratory evolution experiments through 30 serial transfers in batch cultures, comprising approximately 300 cell generations (see Methods). This number of generations is sufficient to show evolutionary adaptations in yeast (Adams & Oeller, 1986; Adams *et al.*, 1992; Dunham *et al.*, 2002; Gresham *et al.*, 2008). For our experiments, we used haploid populations to avoid any potential masking of adaptively significant alleles in the diploid stage (Zeyl *et al.*, 2003). We carried out three parallel replicate evolution lines in which yeast cells grew and divided in yeast peptone medium supplemented with galactose as the sole carbon source and with 0.5 M NaCl (YPGN), which is a high salt medium that exposes cells to high osmolarity stress. We refer to these lines as S lines (S1, S2 and S3). As a control, we also carried out three replicates where the growth medium did not contain NaCl (lines C1, C2 and C3).

### Evolutionary adaptation to NaCl

Before embarking on our experiments, we asked whether NaCl affects the fitness of our ancestral strain. Only in this case would we expect that NaCl exerts a selection pressure to which the strain can adapt. Fitness has two main components in our experiments. These are viability on the one hand and growth or cell division rate on the other hand. We found that osmotic stress does not affect viability of the ancestral strain significantly

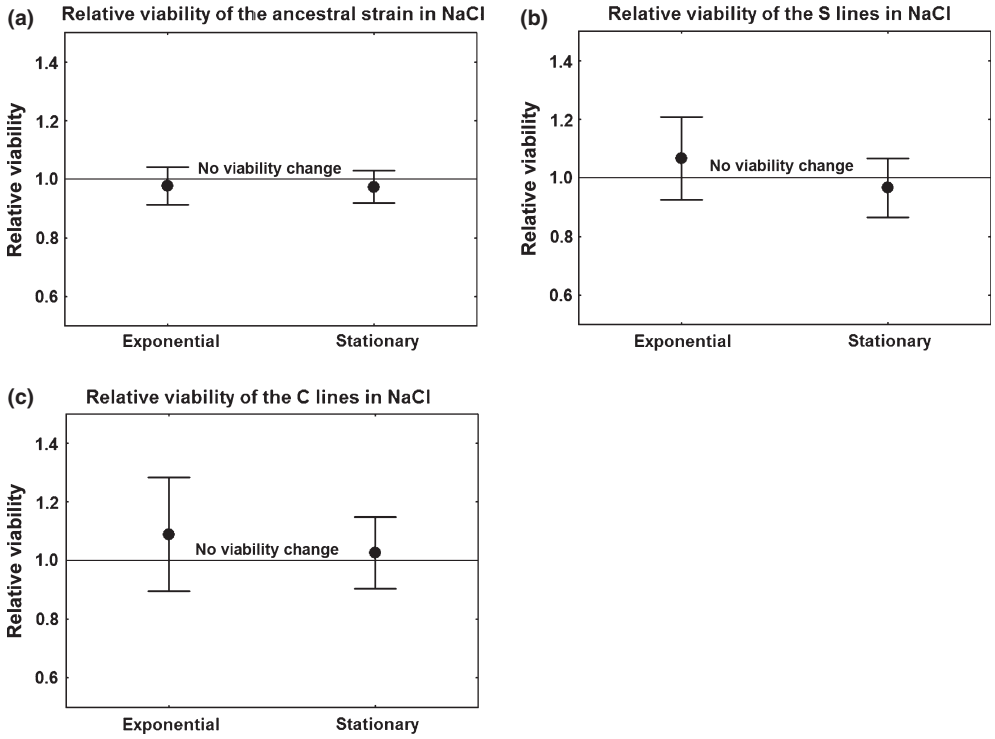


**Fig. 1** (a–c) Viability of the ancestral strain and the evolved lines in medium containing 0.5 M NaCl. Cells from the ancestral strain and the evolved lines from overnight cultures were transferred into YPG medium. Cell samples from these cultures were withdrawn after 16 h (during exponential growth phase), as well as after 24 h (stationary phase), and then diluted and plated. The plates were incubated at 30 °C for 5 days, and the number of colonies was counted. The relative viability of both the ancestral strain and the evolved lines was estimated from the ratio of colonies formed on agar plates with YPGN medium to that of plates with YPG medium. Relative viabilities were measured for (a) the ancestral strain, (b) lines evolved in YPGN medium (S lines) and (c) lines evolved in YPG medium (C lines). From the figures, it is clear that NaCl does not affect the viability of the ancestral strain. The viabilities of the evolved lines S1, S2 and S3 do not increase during the course of adaptation.

(Fig. 1a). Not surprisingly then, viability does not increase in the course of our experiment for lines evolved on NaCl (Fig. 1b) and for lines evolved without NaCl (Fig. 1c).

In contrast to viability, population growth rate is affected by salt stress, as shown in Fig. S1 (Appendix S3). The figure indicates that the ancestral strain grows significantly more slowly in medium containing NaCl. Thus, the fitness of the ancestral strain is lower in salt compared with that of the ancestral strain in medium without any salt. Having established that NaCl does affect the fitness of the ancestral strain through its growth rate, we asked if the growth rate and thus, relative fitness $w$ (see Methods) of three salt evolved lines increase after 300 generations of evolution (Fig. 2a). Fitness increased significantly relative to the ancestral strain, such that the final evolved lines had a relative population mean fitness between $w = 1.11$ and $w = 1.17$, which corresponds to a decrease in the average cell doubling time between 8.2 and 12.3% relative to the ancestral strain (see Methods).

As our high salt medium is a complex medium, it is likely that part of the evolutionary response we observe also reflects adaptation to medium components different from NaCl. To ask whether this is the case, we also measured the relative fitness of the three S lines in the control medium (without NaCl). Not surprisingly, the fitness in this medium had also increased (Fig. 2b) relative to the ancestral strain, which suggests that at least part of the evolutionary adaptation we see is not specific to NaCl as a stressor. However, two lines of evidence show that a substantial fraction of the fitness increase is specific to NaCl. The first is that the fitness increase, when measured in the absence of NaCl, appears substantially and significantly lower than when measured in the presence of NaCl (Fig. 2a,b; Mann–Whitney $U$-test, $P < 0.0001$). The second line of evidence is provided by our three control lines that had evolved without the addition of NaCl to the medium. Figure 2a shows that in every single line, the fitness increase, when measured in medium with NaCl, is consistently and significantly lower than for the lines that had evolved in NaCl (Mann–Whitney $U$-test, $P < 0.0001$). Specifically, the increase in relative fitness $w$ was at least 54 per cent higher in the lines evolved in NaCl compared with the lines evolved without NaCl. In sum, a significant and substantial part of the evolutionary adaptation we observe is because of the selection pressure provided by NaCl.

## Gene expression analysis of the evolved lines

To compare the expression levels of genes in the evolved lines and in the ancestral strain, we performed whole gene transcriptome analysis using yeast microarrays (Affymetrix, High Wycombe, UK). Because previous experiments had shown that many yeast genes change expression in response to salt and to other stressors (Gasch *et al.*, 2000; Posas *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001), we expected that this would also hold for our lines. Thus, we first asked which genes respond to salt in a similar manner in the ancestral strain and in *all* evolved lines. We will refer to such genes for brevity as *shared* genes. For all the analyses presented below, we used all the replicate lines, as well as replicate microarray experiments (see Methods).

In total, we observed 581 shared genes that were induced in salt and 580 shared genes that were repressed in salt with two-fold expression change ($|\log_2(\text{fold change})| \geq 1$, $t$-test, $P < 0.05$, false discovery rate

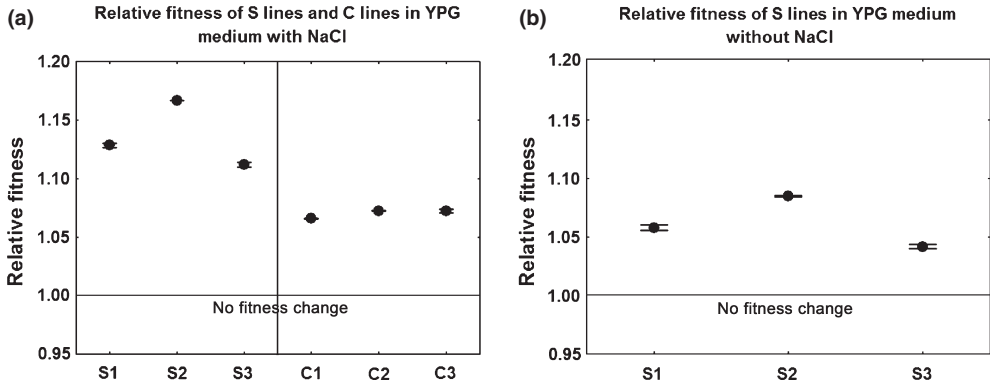


**Fig. 2** Fitness assay of the evolved yeast lines. Fitness assays were performed by inoculating equal numbers of cells from an evolved line and from the GFP-tagged reference yeast strain in the same vessel. The cells were grown for 24 h, and the percentage of non-GFP-tagged cells was counted using FACS. The ratio of the cell numbers of the evolved lines to that of the ancestral strain gives the relative fitness of the evolved lines. (a) Relative fitness of the three replicate yeast lines evolved in medium with salt (S1, S2 and S3) in YPGN medium, and the three replicate yeast lines evolved without NaCl (C1, C2 and C3) measured in YPGN medium. (b) Relative fitness of the lines S1, S2 and S3 in medium without NaCl. The lines evolved in salt show an increase in growth rate by 8–12% in NaCl medium compared with the ancestral strain. These lines also show a growth rate increase in medium without NaCl. However, this increase in growth rate is consistently lower than the increase in NaCl medium. In addition, the lines evolved in medium without NaCl show lower fitness in NaCl medium. Taken together, it can be concluded that part of the adaptation in the salt evolved lines is specific to NaCl.

< 10%). Figure 3a shows a 'volcano plot' ($-\log_{10}$ (*P*-value) vs. $\log_2$(fold change)) for the expression of all yeast genes in response to NaCl in the S lines and the ancestral strain. Figure 3b shows the extent of expression change in the evolved lines (vertical axis) and in the ancestral strain (horizontal axis) for all genes with similar response to NaCl in the evolved lines and the ancestral strain ($|\log_2(\text{fold change})| \geq 1$, *t*-test, $P < 0.05$, false discovery rate < 10%). The genes we labelled by name in the figure include some known stress response genes (e.g. *GPD1, SIP18*), some genes whose expression changed substantially (e.g. *FMP48, NOG1*), and genes in both categories (e.g. *GRE1*). Many of these shared genes are directly associated with saline stress, hyperosmotic stress or the general stress response, and were shown to be affected by hyperosmolarity and salt stress in previous studies of the *physiological* and osmotic stress response (Gasch *et al.*, 2000; Posas *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001). Among the 581 shared induced genes, 192 genes were shown to be affected by stress in previous studies; among the 580 shared repressed genes, 56 were affected by stress in previous studies.

Next, we classified shared genes using the Comprehensive Yeast Genome Database (CYGD) classification from the Munich Information Center for Protein Sequences (MIPS) (Güldener *et al.*, 2005). A detailed analysis is given in the Supporting Information (Appendices S1 and S3, Fig. S2). Here, we only discuss two gene classes in more detail. First, genes associated with 'cell rescue, defence and virulence' contain many general stress response genes, as well as genes that respond specifically to saline stress. One would expect that such genes are induced in response to salt stress, and our data show that induced genes in this category are over-represented, and repressed genes in this category are underrepresented (Appendices S1 and S3, Fig. S2). This group of genes was also found to be significantly

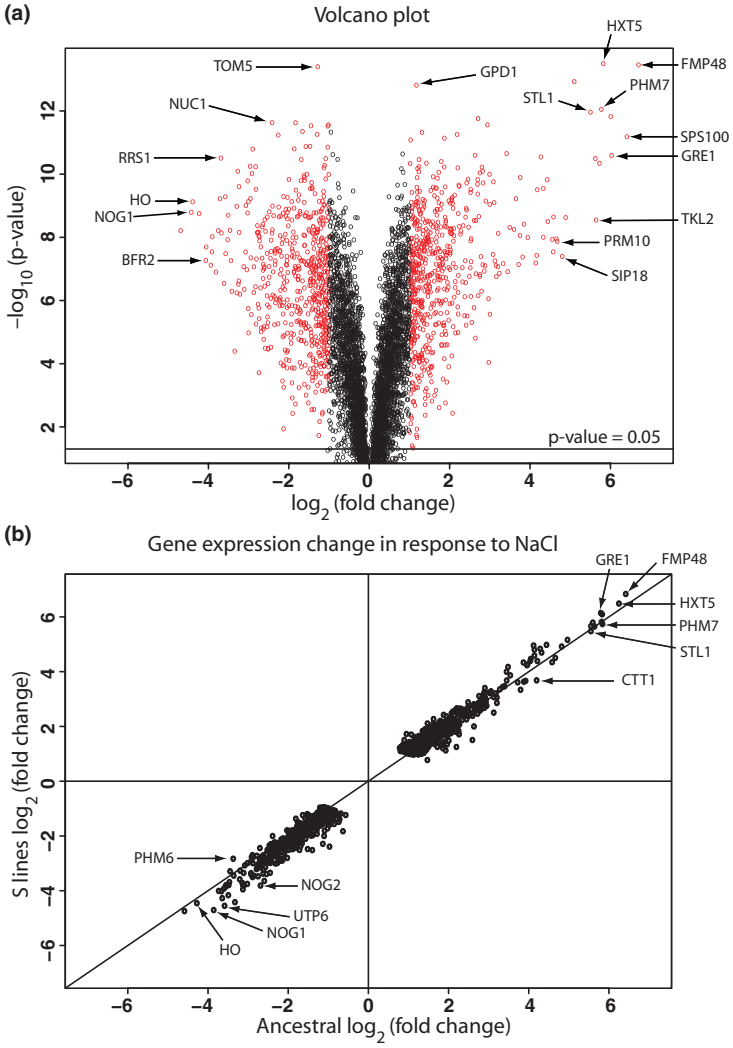


**Fig. 3** The genes showing similar (*t*-test, *P*-value < 0.05, false discovery rate < 10%) induction or repression by NaCl in the ancestral strain and in the evolved S lines. (a) Volcano plot, showing the *P*-values on the *Y*-axis and the fold expression change for these genes on the *X*-axis. The labelled genes include genes associated with the stress response (e.g. *GPD1, SIP18*), genes with high level of expression change (e.g. *FMP48, NOG1*) and genes in both categories (e.g. *GRE1*). (b) Scatter plot for the fold change of the common genes in the ancestral strain (*X*-axis) and in the evolved S lines (*Y*-axis). Although most of the common genes change expression to a similar extent in the ancestral strain and the S lines, a small number of genes change expression to a different extent.

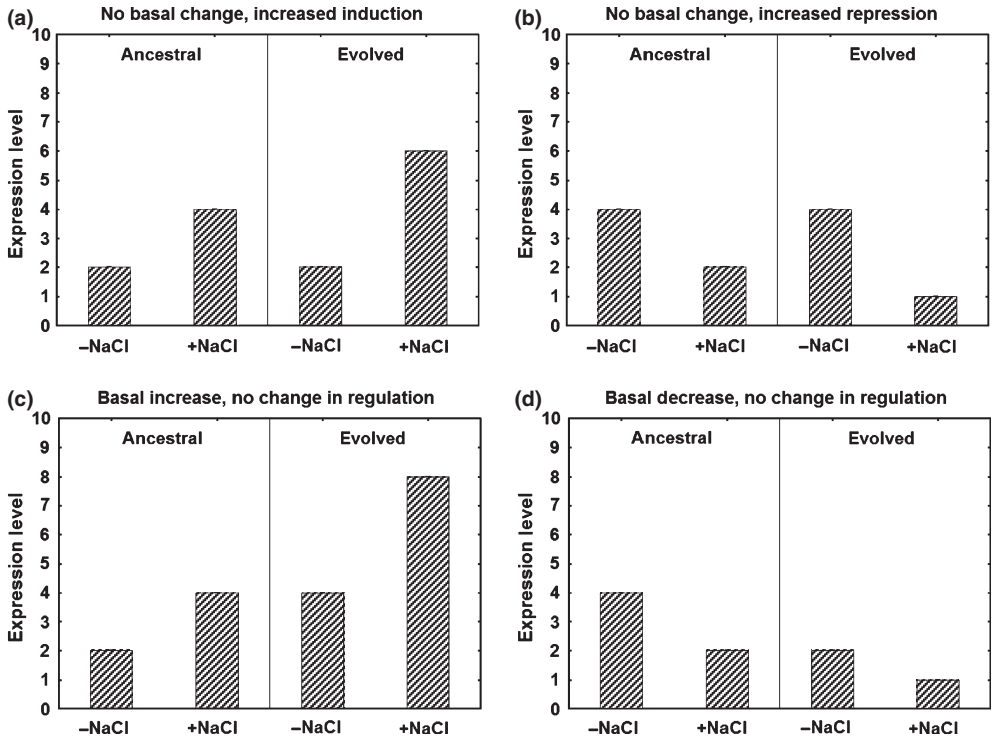


**Fig. 4** Schematic diagram for the type of expression change that can occur as a result of evolutionary adaptation in the evolved S lines compared with the ancestral strain. Each bar chart reflects the expression level of a hypothetical gene in the ancestral strain and the evolved line, in the absence and presence of salt. The scenarios that are depicted are as follows: (a) no basal change, increased regulation. (b) no basal change, increased repression. (c) basal increase in expression but no change in regulation. (d) basal decrease in expression but no change in regulation.

overrepresented among genes whose deletion reduced the growth rate of yeast in salt (Warringer *et al.*, 2003). A second class of genes are genes involved in protein synthesis. The *physiological* response to stress can cause repression of protein synthesis (Gasch *et al.*, 2000). In support of this observation, our data show that significantly fewer genes associated with protein synthesis are induced, and significantly more genes than expected are repressed (Appendices S1 and S3, Fig. S2). Again, this group of genes was significantly overrepresented among genes whose deletion increased salt resistance (Warringer *et al.*, 2003).

## Differentially expressed genes

While an analysis of genes regulated *similarly* in ancestral and evolved strains is instructive, we were more interested in genes that are expressed *differently*. These are genes whose expression adapted evolutionarily to salt stress. They can be subdivided into two main categories. The first comprises genes whose regulation has changed in the evolved lines. Figure 4a,b shows two hypothetical examples of genes in this category. The second category comprises genes whose basal level of expression changed in the evolved lines, even in the absence of salt stress (Fig. 4c,d). This second category of genes may be especially important, because our selection conditions imposed continuous salt stress. It is thus conceivable that cells whose expression is ancestrally regulated in response to salt stress simply increase or decrease their basal expression to the level of regulated expression in the ancestral strain in the absence of salt. We note that these two categories have multiple subcategories, and there can be genes where both the basal expression and regulation can change. Figure S3 (Appendix S3) shows an overview of all possibilities.

We distinguished genes in the main categories by calculating two different *Z*-scores for each gene, a *Z-score for change in basal expression* ($Z_b$) and a *Z-score for change in regulation* ($Z_r$) (see Methods) (Mukhopadhyay *et al.*, 2006). In this analysis, we considered genes with $|Z\text{-score}| \geq 1.5$ as differentially expressed.

## Genes whose regulation changes

In all, there are 62 genes whose regulation changes during our experiment. As we mentioned earlier, multiple types of such change are possible. First, a gene's induction in salt can increase. This will occur if the gene is induced more strongly in the evolved lines than the ancestral strain (Fig. S3b, Appendix S3). Second, a gene's induction in salt can be reduced (Fig. S3c, Appendix S3).

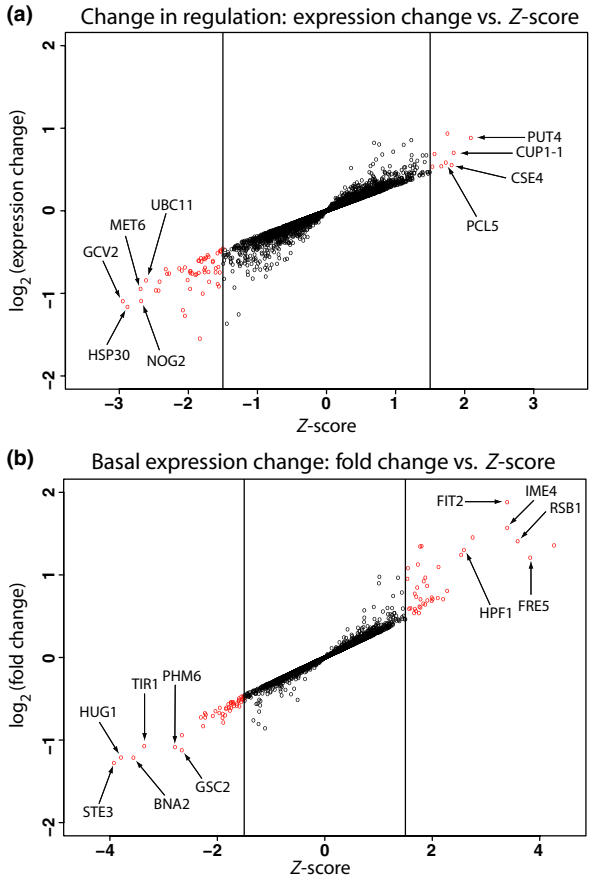


**Fig. 5** Genes with differential expression in the S lines compared with the ancestral strain (|*Z*-score| ≥ 1.5). The plots show the level of expression change (vertical axis) and the *Z*-score (horizontal axis) for genes with (a) changed regulation and (b) changed basal expression in the evolved lines. One should note that the basal change in expression is represented in terms of fold change, where the expression change is calculated as the ratio of the expressions in the evolved lines and the ancestral strain. The calculation of regulated expression change involves an additional subtraction of basal expression change and thus, cannot be represented as fold change.

Third, a gene's repression in salt can be increased, i.e. the gene becomes repressed to a greater extent in the evolved lines (Fig. S3d, Appendix S3). Fourth, a gene's repression in salt can be reduced (Fig. S3e, Appendix S3). Finally, a gene that was not regulated in the ancestral strain can become regulated in response to salt (Fig. S3f,g). Figure 5a plots the extent to which genes changed in their regulation against their $Z_r$ scores. Some of the genes with significant changes in the regulation are labelled. Figure 6a,b shows the five genes whose induction or repression changed most significantly (based on $Z_r$). We next discuss some of these genes.

There are only four genes in total which showed *increased induction* in the evolved lines. Among these four genes, two (*PUT4* and *PCL5*) also showed a decrease in basal expression in the evolved lines. Four further genes showed *new induction* in the evolved lines. One of them, *CUP1-1* (Fig. 5a), has previously been observed to be up-regulated in response to osmotic stress (Yale & Bohnert, 2001). Five genes showed *reduced induction* in our experiment. One of the genes, *HSP30*, encodes a stress-responsive protein that negatively regulates the H(+)-ATPase Pma1p. It is induced by heat shock, treatment with organic acid and ethanol, and glucose starvation (Panaretou & Piper, 1992; Piper *et al.*, 1994, 1997).

In contrast to the few genes that showed increased induction, a total of 37 genes showed *increased repression*. Of these 37 genes, one gene (*HLR1*) is directly involved in the stress response. This gene encodes a protein involved in the regulation of cell wall composition, as well as in the osmotic stress response (Alonso-Monge *et al.*, 2001). In our experiment, 12 genes showed *new repression*. Among these 12 genes, the genes *GCV2* and *GCV1* were previously found to be up-regulated in osmotic stress (Yale & Bohnert, 2001). We did not find any gene with *reduced repression*.

We next classified genes whose regulation changed in any of these ways according to their functions, using the CYGD classification of yeast genes (Güldener *et al.*, 2005) (Fig. S4, Appendix S3), and compared their distribution among functional categories with the proportions of the yeast genome in each category. The distribution of the number of genes among different classes is nonrandom for genes whose repression changes (49 genes in total; $\chi^2$ test, $P < 0.001$, d.f. = 17), whereas the distribution is not significantly different from random for the genes whose induction changes (13 genes; $P > 0.1$, d.f. = 17). We then asked whether genes whose regulation changes occur preferentially in specific functional categories. Interestingly, genes with change in induction level are (marginally) enriched only in the functional class of cell rescue, defense and virulence ($P = 0.0444$). Genes whose repression changes are enriched in the functional classes transcription ($P = 0.0006$), protein synthesis ($P = 0.0008$) and proteins with binding or catalytic function ($P = 0.0003$). The classes protein fate (folding, modification, destination; $P = 0.0232$), cellular transport ($P = 0.0048$), cell cycle/DNA processing ($P = 0.0134$) and cell rescue, defense and virulence ($P = 0.0230$) contain fewer genes whose repression changes than expected by chance alone (all *P*-values based on an exact binomial test).

## Genes with basal level expression change

Next we turn to genes that show a change in basal expression (Fig. S3a, Appendix S3). There are 82 such genes. Thirty-eight of them increased their basal expression, whereas 44 genes reduced their basal expression. Figure 5b plots the extent of change in basal expression ($\log_2$-transformed) of all the genes against their

**(a) Top five genes with change in induction level**

| Gene | $Log_2$ (expression change) | *Z*-score | Common name | Type of change | Previous studies |
|---|---|---|---|---|---|
| YOR348C | 0.8821 | 2.088 | PUT4 | Increased induction | Posas *et al*., 2000; Rep *et al*., 2000; Yale & Bonhert, 2001 |
| YHR053C | 0.7017 | 1.8401 | CUP1-1 | Increased induction | Yale & Bonhert, 2001 |
| YKL049C | 0.554 | 1.8101 | CSE4 | New induction | Yale & Bonhert, 2001 |
| YAR010C | 0.9355 | 1.7493 | --- | New induction | Yale & Bonhert, 2001 |
| YHR071W | 0.5834 | 1.726 | --- | Increased induction | Yale & Bonhert, 2001 |

**(b) Top five genes with change in repression level**

| Gene | $Log_2$ (expression change) | *Z*-score | Common name | Type of change | Previous studies |
|---|---|---|---|---|---|
| YMR189W | −1.0952 | −2.9498 | GCV2 | New repression | Yale & Bonhert, 2001 |
| YER091C | −0.9458 | −2.6921 | MET6 | Increased repression | Rep *et al*., 2000 |
| YNR053C | −1.0932 | −2.6838 | NOG2 | Increased repression | --- |
| YOR339C | −0.8424 | −2.613 | UBC11 | Increased repression | --- |
| YHR066W | −0.9644 | −2.4702 | SSF1 | Increased repression | --- |

**(c) Top five genes with increase in basal expression**

| Gene | $Log_2$ (fold change) | *Z*-score | Common name | Previous studies |
|---|---|---|---|---|
| YNL034W | 1.3572 | 4.2657 | --- | --- |
| YOR384W | 1.2084 | 3.8216 | FRE5 | --- |
| YOR049C | 1.4081 | 3.5885 | RSB1 | --- |
| YGL192W | 1.5698 | 3.3941 | IME4 | --- |
| YOR382W | 1.8809 | 3.3935 | FIT2 | Yale & Bonhert, 2001 |

**(d) Top five genes with decrease in basal expression**

| Gene | $Log_2$ (fold change) | *Z*-score | Common name | Previous studies |
|---|---|---|---|---|
| YKL178C | −1.2774 | −3.9263 | STE3 | --- |
| YML058W-A | −1.2118 | −3.7933 | HUG1 | --- |
| YJR078W | −1.2138 | −3.5631 | BNA2 | --- |
| YER011W | −1.0741 | −3.361 | TIR1 | --- |
| YDR281C | −1.0859 | −2.789 | PHM6 | --- |

**Fig. 6** The five genes with the highest *Z*-score in each of four categories of expression change in the evolved lines. Categories are (a) change in the level of induction, (b) change in the level of repression, (c) increase in basal expression and (d) decrease in basal expression. The columns show, from left to right, the systematic gene name, the extent of expression change, the *Z*-score, the gene's common name, the type of expression change (in (a) and (b) only), and a reference, if the gene had responded physiologically to hyperosmotic stress or saline stress in previous studies.

corresponding $Z_b$ values. Some of the genes with significant changes in the basal expression level are labelled. Figure 6c,d lists the top five genes (based on $Z_b$) with increased and decreased basal expression, respectively. The distribution of genes among different functional classes (Fig. S4, Appendix S3) is significantly different from what would be expected by chance alone for the genes with increase in basal expression ($P < 0.05$, d.f. = 17, $\chi^2$ test), and also for the genes with a decrease in basal expression ($P < 0.001$, d.f. = 17). For genes with an increase in basal expression, the class 'cell rescue and defense' (stress response) shows significantly more genes than expected ($P = 0.0059$, exact binomial test), and the class protein fate ($P = 0.0409$) shows significantly fewer genes than expected by chance alone. For genes with a decrease in basal expression, the class 'cell type differentiation' (cell wall, sporulation, spore wall, etc.) ($P = 0.0165$) shows more genes than expected, and the classes 'transcription' ($P = 0.0125$) as well as 'protein fate' (folding, modification, destination) ($P = 0.0014$) contain fewer genes than expected.

Two well-established stress response genes showed an increase in basal expression in the evolved lines. One of them is *CTT1*, whose basal expression increased by

approximately 1.6-fold compared with the ancestral strain. This gene was found to be induced in four previous genome-wide studies of *physiological* stress adaptation in yeast (Gasch *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001). The second known stress response gene is the transcription factor *MSN4*, whose expression increased by approximately 1.5-fold. A more detailed analysis of individual genes can be found in the Supporting Information (Appendix S1).

### Whole genome (re)sequencing analysis

Some laboratory evolution studies have identified few beneficial mutations of large fitness effects in evolving populations (Blanc & Adams, 2003; Velicer *et al.*, 2006; Stanek *et al.*, 2009). To find out if there are any such mutations in our study system, we subjected the ancestral strain and population samples from two of our evolved lines (S1 and S2) to deep sequencing at about approximately 10× coverage and the third line (S3) to approximately 50× coverage, using the Roche 454 Genome Sequencer (454 Life Sciences, Branford, CT 06405, USA). In our analysis of these sequence data, we aimed to identify only changes that may have swept to high frequency or fixation, and 10× coverage is sufficient for that purpose. We chose candidate single-nucleotide polymorphisms (SNPs) based on the criteria for the changed (derived) nucleotide described in Appendix S2, and our approach is deliberately conservative. We identified 56 candidate SNPs and seven deletions in total from all the three evolved (S) lines.

We sequenced all 56 candidate SNPs and seven candidate deletions, but only one of them turned out to be a true change. This change was a SNP unique to line S2, and occurred at a frequency exceeding 50 per cent, based both on next generation sequencing data and PCR sequencing data. This SNP is a nonsynonymous G to A mutation (amino acid change: G230D) in the *MOT2* (YER068W) gene. The gene *MOT2* is a subunit of the CCR4-NOT complex which has roles in transcription regulation, mRNA degradation and post-transcriptional modifications (Cade & Errede, 1994; Liu *et al.*, 1998; Badarinarayana *et al.*, 2000; Denis *et al.*, 2001; Panasenko *et al.*, 2006; Mersman *et al.*, 2009), with no known role in salt tolerance. To test whether the *MOT2* SNP alone provided any fitness benefit to yeast in salt, we replaced the wild-type variant of *MOT2* in the genome of the ancestral strain with the mutant (see Methods). Three replicate competition assays using FACS (see Methods) showed a relative population fitness $w = 1.043 \pm 0.008$ for the mutant *MOT2* allele (Fig. 7a) in medium with NaCl, compared with $w = 0.999 \pm 0.014$ for the wild-type *MOT2* allele. However, the fitness increase of the mutant *MOT2* could only explain 25.3 per cent of the total amount of fitness increase in line S2, whose mean relative fitness was $w = 1.17$, indicating that there are other factors that contribute to the fitness increase of the line S2. In addition, we note that the *MOT2* mutation also conferred a substantial fitness increase in the absence of NaCl (Fig. 7b), indicating that this mutation is not specifically adaptive to salt stress.

### Duplication and PFGE

We next turned to copy number variations as sources of evolutionary adaptation. As large-scale chromosomal rearrangements and aneuploidies are frequent in yeast laboratory evolution (Adams *et al.*, 1992; Dunham *et al.*, 2002; Rancati *et al.*, 2008), and are also observed in yeast gene knock-out strains (Hughes *et al.*, 2000), we first wanted to know whether such rearrangements were numerous in our evolved lines. To this end, we first performed pulsed field gel electrophoresis (PFGE) analysis of whole chromosomes for the ancestral strain and for two clones from each of the evolved lines. This experiment revealed no changes in any of the lines, except for one additional band (at approximately 500 kb) that occurred exclusively in line S3 (Fig. 8a). PFGE for

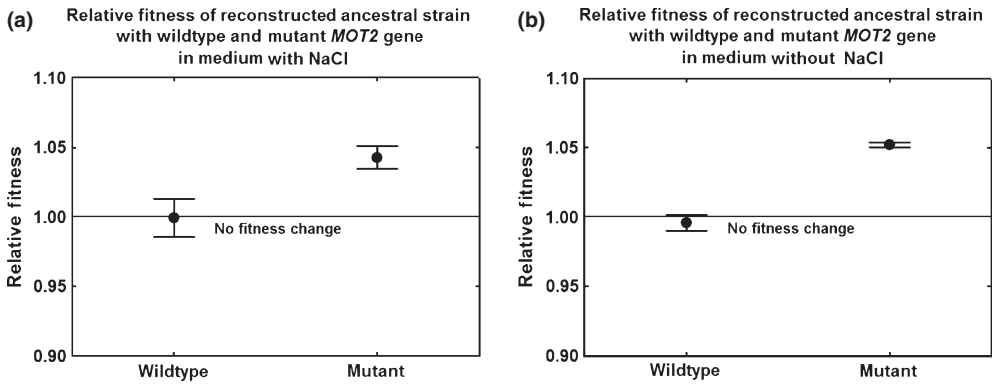


**Fig. 7** Fitness analysis (using FACS) of the ancestral strain into which the mutant *MOT2* gene of line S2 was introduced, and the corresponding wild-type *MOT2* gene as a control. (a) Fitness in medium with NaCl and (b) fitness in medium without NaCl. The data show that the mutant *MOT2* gene increases the fitness in salt medium, as well as in medium without salt, and to similar extents.

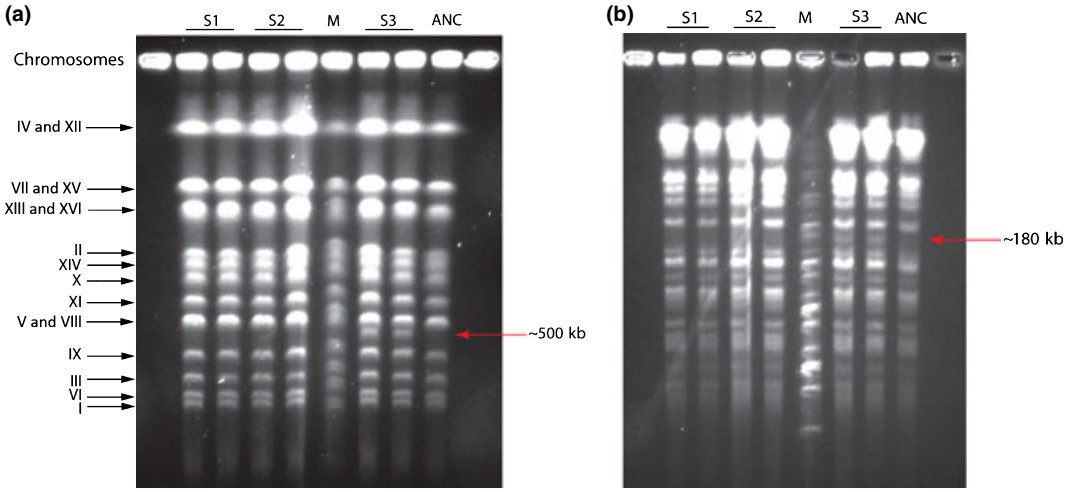


**Fig. 8** Pulsed field gel electrophoresis (PFGE) analysis of the three replicate salt evolved lines S1, S2 & S3 and the ancestral strain. Two clones from each of the lines S1, S2 and S3 were subjected to PFGE analysis along with the ancestral strain. (a) The PFGE for the yeast chromosomes from the evolved lines S1, S2 and S3 and the ancestral strain. The chromosomes are shown on the left side. M represents the yeast chromosome PFG marker (NEB Catalog# N0345S). Only line S3 shows a new band (red arrow) of size approximately 500 kb. (b) PFGE analysis of the *Not*I digested chromosomes from the lines S1, S2, S3 and the ancestral strain. M represents the mid-range PFG marker I (NEB Catalog# N3551S), whose fragments range between 15 and 291 kb. Again, only line S3 shows a new band (red arrow) in the gel, which has a size of approximately 180 kb.

chromosomes digested with the *Not*I restriction enzyme also revealed a single novel band (approximately 180 kb), which was again unique to line S3 (Fig. 8b). Taken together, these observations suggest that high-frequency copy number changes on a chromosomal scale are not rampant in our evolved lines, and the same arrangements do not occur across lines. All other distinguishable fragments in the gel, apart from the novel band of size approximately 180 kb in line S3, were as predicted for a haploid yeast genome from a computational restriction digest of the genome with *Not*I. Additionally, chromosome-wide read coverage data from next generation sequencing support the notion that there are no copy number changes involving large chromosome fragments (Fig. S5, Appendix S3). However, a change in ploidy in the evolved lines that would affect entire chromosomes is not detectable by any of these two methods.

## Gene amplifications and deletions from next generation sequencing data

We next turned to smaller scale copy number changes that we could detect in our next generation sequencing data. To detect gene duplication and deletions, a segmentation algorithm (see Appendix S2) was used along with analysis of DNA breakpoints from the reads (see Appendix S2). The results of both analyses are described in detail in the Appendix S1.

We did not observe any large amplification or deletion in the genomes of any of the lines (and specifically in line S3) that could explain the appearance of novel bands in the pulsed field electrophoresis data from Fig. 8. This observation, together with read coverage data of individual chromosomes (Fig. S5, Appendix S3), suggests that these novel bands were results of a nonduplicative translocation event in the line S3. However, for a haploid genome, a translocation event would displace one of the original bands in the PFGE, which we did not observe. Thus, such a translocation event is only consistent with the PFGE data if the affected chromosome occurs in more than one copy. In other words, it is consistent with a ploidy change in line S3.

## Ploidy of the evolved yeast lines

Changes in ploidy of haploid yeast strains under salt stress have been observed before (Gerstein *et al.*, 2006). Neither PFGE nor chromosome-wide read coverage data would be able to detect such changes. To estimate the ploidy level of evolved lines in comparison with the ancestral strain, we grew ancestral strain and population samples of the evolved lines for 24 h at 30 °C and estimated the cell densities. We then isolated genomic DNA from a defined number of cells and quantified the DNA amount, which allowed us to calculate the DNA content per yeast cell for all the lines. We found that the DNA content per cell of lines S1 and S2 had increased by 78.7 per cent and 85.4 per cent, respectively, from that of the ancestral strain. For the line S3, the DNA content per cell was more than double that of the ancestral strain (119.91 per cent increase from the ancestral strain)

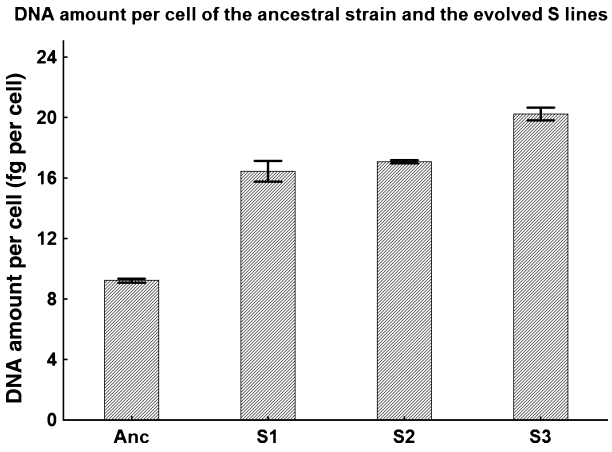


**Fig. 9** DNA content per yeast cell of the ancestral strain and the evolved lines. The ancestral strain and population samples of the evolved lines were grown for 24 h at 30 °C. For each of the cultures, the cell density was estimated. Genomic DNA was isolated from a defined number of cells, and DNA quantification was carried out with a NanoDrop ND-1000 spectrophotometer. The DNA content per cell was then calculated for each line. The figure suggests that all the evolved lines have become massively aneuploid.

(Fig. 9). These observations suggest that all the evolved lines have become massively aneuploid.

### A consistent cell size increase during our experiment

Increases in ploidy are often associated with cell size increases. Our lines are not exception, as exemplified by the histogram of Fig. 10a, which shows the distribution of cell diameters of the ancestral strain and the evolved line S2. This increase is also microscopically visible (Fig. 10b). The mean cell diameter increased from 2.35 $\mu$m (±0.70 $\mu$m) to 6.73 $\mu$m (±2.34 $\mu$m). Along with a significant increase in the mean cell diameter (*t*-test, *P*-value $< 2.2 \times 10^{-16}$), the coefficient of variation ($C_v$), as defined by the ratio of standard deviation and mean, also increased significantly (*t*-test for distributions of $C_v$ for samples drawn from two distributions, $P < 10^{-6}$). In other words, not only did cells become larger during laboratory evolution, they also became more variable in size. Cell size changes in laboratory evolution experiments are not unprecedented. They have been observed in evolving *Escherichia coli* populations (Lenski & Travisano, 1994; Lenski & Mongold, 2000; Philippe *et al.*, 2009), as well as in *Staphylococcus aureus* populations in NaCl medium (Vijaranakul *et al.*, 1995). However, the effect of cell size on survival and fitness is generally poorly understood.

## Discussion

To investigate evolutionary adaptation to long-term osmotic stress, we evolved yeast cells in the laboratory

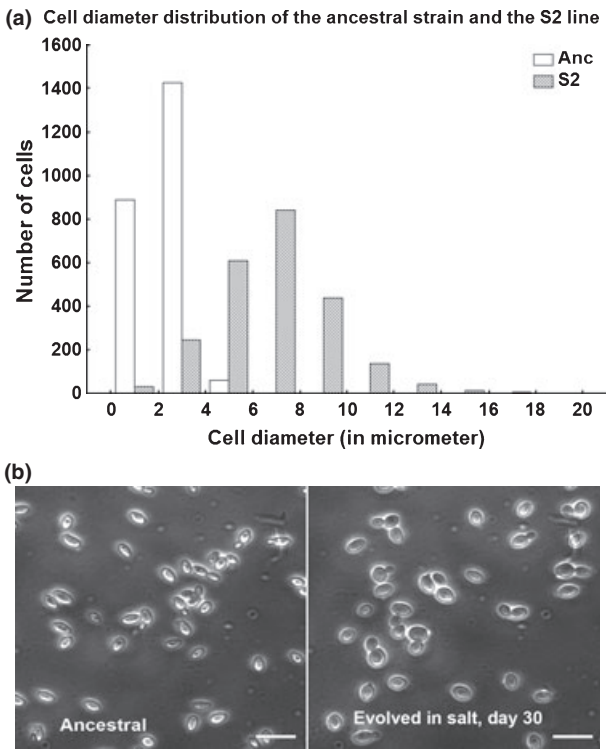


**Fig. 10** Cell size comparison between ancestral strain and one evolved line. (a) Histogram of cell diameters of the ancestral strain and the S2 line, showing that the salt evolved cells are bigger than the ancestral cells. (b) Microphotograph of cell samples from the ancestral strain and the salt evolved lines.

for 300 generations in high salt (NaCl) medium. We observed that salt reduces the growth rate of our ancestral strain by 11 per cent. Consequently, the final cell density after 24 h of growth is approximately 3 times lower compared with cells grown in the same medium but without salt. Our three replicate evolved lines that grow approximately 8–12 per cent faster than the ancestral strain in high salt medium, and part of this increase reflects adaptation specific to salt.

We analysed the gene expression levels in all the evolved lines as well as in the ancestral strain. Although there were many shared genes that respond to salt in similar manner in the evolved lines and in the ancestral strain, we also observed multiple differentially expressed genes in the evolved lines compared with the ancestral strain. These differentially expressed genes can be divided into two categories. The first category comprises genes with changes in their basal expression level in the evolved lines compared with the ancestor (i.e. even in the absence of salt). The second category comprises genes regulated differently in response to salt in the evolved lines.

Multiple genes whose expression shows an *evolutionary* response in our experiments were also affected by

hyperosmotic stress or salt stress in previous studies of the *physiological* stress response. Changed expression of many genes in the *physiological* stress response in general and in the hyperosmotic stress response in particular is transient (Gasch *et al.*, 2000; Causton *et al.*, 2001), and may depend on the time and conditions in which it is measured. It is thus expected that different studies show limited comparability with respect to the identity of these genes.

Some of the genes whose expression evolved are known to be associated with the stress response. For example, two genes, *CTT1* and *GAC1*, showed an increase in basal expression in the evolved lines compared with the ancestral strain. Both these genes were also up-regulated in response to salt in our ancestral strain. *CTT1* encodes a cytosolic catalase and protects the cell from oxidative damage by reactive oxygen species (Jamieson, 1998; Lushchak & Gospodaryov, 2005; Herrero *et al.*, 2008). This gene has been shown to be up-regulated in four previous studies of *physiological* stress response and salt stress response (Gasch *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001). The gene *GAC1* is associated with glycogen synthesis (Wu *et al.*, 2001) and is also induced during osmoadaptation (Posas *et al.*, 2000). Both these genes contain stress-responsive elements (STRE) in their promoters (Schuller *et al.*, 1994; Moskvina *et al.*, 1998) and are known to be activated under various stress conditions. A third gene, *CUP1-1*, showed increased induction in the evolved lines and also was up-regulated in response to NaCl in our ancestral strain. This gene was also shown to be up-regulated in one previous study of *physiological* stress response (Yale & Bohnert, 2001). Yet another gene, *HLR1*, showed increased repression in the evolved lines, and was down-regulated in the ancestral strain in NaCl medium. This gene encodes a protein involved in maintaining cell wall composition and is also involved in response to osmotic stress (Alonso-Monge *et al.*, 2001).

There are three main *physiological* components of the salt stress response. They affect adaptation time to salt, growth rate in salt and efficiency of growth in salt (Warringer *et al.*, 2003). In our experiments, *evolutionary* adaptation to salt must affect one or more of these three components, because the viability itself of yeast cells does not change between the ancestral strain and the evolved lines. There are various ways in which these components could change during evolution. First, some of the genes whose basal expression changes might be directly involved in the initial adaptation to salt. Increasing or decreasing the basal expression of those genes to the level needed for salt adaptation might enable the cells respond to salt much more quickly as opposed to changing their expression state. For example, some ion transporter genes (e.g. *FRE5*) have an increased basal expression in the evolved lines in our experiment. Higher levels of these transporter proteins at the initial stages of the salt stress response would help cells achieve ion homeostasis much more quickly. Second, some of the differentially expressed genes might not be directly related to the salt stress response but encode transcription factors that control salt stress response genes. For example, the transcription factor *MSN4*, that controls many stress response genes, has increased basal expression levels in our evolved yeast lines. Higher levels of this transcription factor during the initial adaptation phase could ensure faster induction of the genes required for salt stress response. Third, there are genes that show evolutionary change in regulation in the evolved lines. Higher level of induction or repression of these genes might increase the growth rate and/or efficiency of growth in salt medium.

We found 16 genes that gained regulation (new induction and new repression) in the evolution experiment, suggesting that new ways of salt stress adaptation could also arise during evolution. However, we also observed loss of regulation, at least to some extent, for five genes (genes with reduced induction and reduced repression) in the evolved lines. Because we performed our evolution experiment under constant environmental stress, losing regulation might be advantageous for evolutionary adaptation to salt stress. If the affected genes are involved in adaptation to multiple stressors, losing regulation could actually be beneficial both under constant or fluctuating environmental stressors. However, if the affected genes are specific for a particular stress, a loss in regulation may also have a cost, because cells would become physiologically less flexible under fluctuating environmental stresses. Among the five genes with loss of regulation, one gene, *HSP30*, can be induced by several stressors (Piper *et al.*, 1997). On the contrary, the mRNA level of this gene was shown to be diminished in NaCl medium by Rep *et al.* (2000).

The hyperosmotic and salt stress responses are complex and influenced by many genes. It is an open question whether a small number of genetic changes with large effects could dramatically increase salt stress resistance, and thus explain the fitness increase in our evolved lines. To find out, we also sequenced the ancestral strain and population samples of the evolved lines using deep sequencing with genome coverages between 10- and 50-fold to identify whether any high-frequency polymorphisms arose in the evolved lines during our evolution experiment. For two reasons, we chose deep population sequencing over clone sequencing. First, population sequencing gives a comprehensive view of polymorphisms in a population. Second, it can also permit estimation of polymorphism frequencies, although any such estimate would be more qualitative than quantitative at our sequence coverage. At the very least, population sequencing can detect high-frequency polymorphisms. Such polymorphisms are of the greatest interest to us, because they would correspond to adaptive mutations with strong fitness effects that rose to high frequencies. We note that the effective population sizes in our experiment are so large ($> 2 \times 10^6$) that no neutral

polymorphisms would be expected to rise to high frequencies during the experiment's short duration except perhaps through hitchhiking with an advantageous mutation. (Clone sequencing can detect low-frequency polymorphisms, but for a heterogeneous population, this method requires sequencing of many clones and is thus currently prohibitively costly.)

Only one of our evolved lines (S2) contained a high-frequency SNP. This SNP occurs in more than 50 per cent of the population and causes an amino acid substitution in the protein encoded by the *MOT2* gene. When introduced into the ancestral strain, this mutation causes a fitness increase that explains 25.3 per cent of the increase in fitness $w$ in the evolved strain. However, the mutation causes a fitness increase also in the absence of salt, which means that its effects do not reflect a specific adaptation to salt.

Multiple genetic changes of low population frequency could be present in our evolved lines, and these changes might explain a part of the fitness increase we see. Such low-frequency SNPs might be present for several reasons. First, individual mutations may confer only modest fitness benefits and thus increase in frequency slowly. Second, some mutations with very strong fitness effects may have occurred, but late in the experiment, and thus might simply not have had enough time to rise to high frequency. Third, the fitness effect of alleles might be determined by epistatic interactions with other mutations elsewhere in the genome (Elena & Lenski, 1997, 2001; Morgan & Feldman, 2001). Eventually genotypes with such alleles might rise to high frequency, but at time scales much longer than those of our experiment, because multiple mutations might have to occur before any one epistatic combination with strong benefits arises. Finally, and perhaps most importantly, there could be clonal interference between multiple beneficial polymorphisms in the population, and this might prevent any particular polymorphism from rising to high frequency in the population (Kao & Sherlock, 2008).

Similar to our analysis on SNPs, our analysis on copy number variations, both based on PFGE and next generation sequencing, did not reveal any large-scale copy number changes shared across strains. However, we observed ploidy increases in all the evolved lines, and this could be the reason behind the increase in cell size of these lines.

Evidence from plants suggests that an increase in ploidy and the resultant increase in cell size can be advantageous under salt-stressed conditions. For example, within a species, plants with higher ploidy can cope with salt stress better than plants with lower ploidy (Saleh *et al.*, 2008). Polyploid plants have higher water content and lower osmotic pressure than diploid plant (Noggle, 1946). Their increased water content is because of a decrease in the surface-to-volume ratio of cells (Stebbins, 1950). In addition, any water content decrease because of high salinity is smaller in polyploid plants than that in diploid plants (Tal & Gardi, 1976). Gerstein *et al.* (2006) observed that initially haploid yeast lines increased genome size significantly faster under salt stress than in unstressed conditions, suggesting an advantage for higher ploidy or increased cell size also in yeast.

Changes in ploidy could help yeast cells adapt faster physiologically, for example by affecting expression of genes important for salt stress tolerance. Could all the changes in gene expression that we observed in our experiment be caused solely by ploidy changes or by the concomitant increase in cell size? To find out, we compared the set of genes that changed expression in our evolved lines with the set of genes that are known to change expression after cell size increases (Wu *et al.*, 2010) and ploidy changes (Galitski *et al.*, 1999). Remarkably, only three genes that evolve changed expression in our experiment (of 144 differentially expressed genes in our dataset) are among the genes affected by cell size increase or ploidy changes. Two of these genes showed increased basal expression in our experiment. One of them, YIL169C, was also observed to be induced with increase in cell size (Wu *et al.*, 2010). The other gene, *COS8*, was observed to change expression with a change in ploidy level (Galitski *et al.*, 1999). The third gene, YLR042C, showed a basal decrease in expression in our evolved lines. It was also observed to be repressed in cells with increased size (Wu *et al.*, 2010).

Overall, the vast majority of changes that we observe in gene expression cannot be caused by changed ploidy or cell size. Because our whole genome sequencing analysis revealed no small genetic changes of strong phenotypic effects, the gene expression changes we observe could be caused by multiple genetic changes of modest individual effects. Some of these changes might affect a gene's expression in *cis*, others in *trans*, for example through changes in the amino acid sequence of transcriptional regulators (Wittkopp *et al.*, 2004; Emerson & Li, 2010). In addition, some expression changes may be caused by epigenetic change (e.g. changes in DNA methylation) or alterations in cellular memory (Turner, 2002; Ringrose & Paro, 2004; Zhang *et al.*, 2005; Zachariodakis *et al.*, 2007).

We found several genes with modest changes in expression level in our evolved lines, and no gene with very strong expression change. Our observations stand in contrast to the only laboratory evolution study we know of that investigated evolutionary adaptation to a stressor. The study asked how *E. coli* cells adapt to heat stress. It observed very high level of evolutionary expression change in two proteins, GroEL and GroES, which are known as heat-shock genes (Rudolph *et al.*, 2010). The reason for this difference to our work is probably that adaptation to salt stress requires several molecular functions simultaneously, namely ionic detoxification to achieve ionic equilibrium, maintaining cellular water activity and osmolyte synthesis. Each of the evolutionarily

altered genes in our experiment may perform or control some of these functions and thus, may have only a modest individual fitness contribution when differentially expressed. In addition, gene interactions (Phillips, 2008; He *et al.*, 2010) could also contribute to a fitness increase. This scenario resembles recent observations in genome-wide association studies (GWAS) for complex human traits and diseases, where researchers have observed many genetic variants with modest individual effects on a phenotype (Frayling, 2007; Barrett *et al.*, 2008; Cooper *et al.*, 2008; Visscher, 2008; Zeggini *et al.*, 2008; Hindorff *et al.*, 2009; Manolio *et al.*, 2009; Visscher & Montgomery, 2009; Park *et al.*, 2010; Yang *et al.*, 2010).

In sum, adaptation to salt stress is associated with gene expression changes, and with a DNA content increase in all three evolved lines. It is also associated with one high-frequency mutation in one of the evolved lines, and with one chromosomal rearrangement in another. Previous studies suggest that the DNA content increase we observe might facilitate evolutionary adaptation to high salt. However, it is not sufficient to explain the expression changes in 144 genes that our evolved lines show. These expression changes may be caused by multiple genetic changes of low frequency (that might vary among subpopulations) or by epigenetic changes. Several of the genes that change expression through evolutionary adaptation are also involved in the physiological stress response, which supports a causal role for these genes in evolutionary adaptation to salt stress. Although the fine-tuning of several genes helps shape the complex, polygenic trait we study, some of their expression changes might also reflect cellular constraints on evolution. Future work will allow us to disentangle these two contributors to evolutionary adaptation in gene expression.

## Methods

### Strains and media

All laboratory evolution experiments started from the same clone of haploid yeast strain BY4741, which is referred to as the *ancestral* strain. The three replicate yeast lines evolved in NaCl are referred to as lines S1, S2 and S3. The growth rates of the evolved lines as well as the ancestral strain were measured relative to a BY4741 strain in which the *CWP2* gene was GFP-tagged (termed as the *reference* strain). For serial transfers, cells were cultured in YP and 2% galactose (YPG) and YPG supplemented with 0.5 M NaCl (YPGN).

### Serial transfer

Six parallel serial transfer experiments were started from one single clone of the ancestral strain. In each parallel experiment, 50-ml yeast culture was grown at 30 °C. Every 24 h, 50 $\mu$l of grown culture was transferred into fresh culture medium; 30 such transfer cycles were carried out for a total of approximately 300 generations (Each transfer cycle involved approximately $\log_2 1000 \approx 10$ cell generations). In three of the parallel experiments, the culture medium was YPG, whereas in the other three parallel experiments, the medium was YPGN.

### Viability assays

To estimate cell viabilities, cultures of growing yeast cells were sampled after 16 h (during exponential growth phase), as well as after 24 h (during stationary phase), diluted and plated. The plates were incubated at 30 °C for 5 days, and the number of colonies was counted. The relative viabilities of both the ancestral strain and the evolved lines were estimated from the ratio of colonies forming on agar plates containing YPG+ 0.5 M NaCl to that of plates containing only YPG. All the measurements were carried out in three biological replicates.

### Competition assays

To compare growth rates of the evolved lines with that of the ancestral strain, competition assays were carried out in triplicates using fluorescence activated cell sorting (FACS). Cells from frozen glycerol stocks were grown overnight in 4 ml of YPD medium (30 °C, 220 rpm) until late logarithmic phase ($< 1.5 \times 10^8$ cells ml$^{-1}$). For the competition assay, approximately equal cell numbers of the reference strain and of the competing strain were mixed and grown for 24 h at 30 °C. The relative cell numbers at the beginning and at the end of the competition experiment were determined using FACS (Appendix S2), and growth rate differences were estimated as described in Appendix S2. For calculation of fitness, it was taken into consideration that the cell numbers at the beginning of the assay may have differed between the reference strain and the competing strain.

### Whole genome transcriptome analysis

The mRNA expression levels in the ancestral strain and in the evolved lines were analysed using a GeneChip Yeast Genome 2.0 Array (Affymetrix). Equal number of cells from the ancestral strain and the evolved lines were grown in YPG medium for 16 h. The cells were then either induced with 0.5 M NaCl or grown uninduced for 20 further minutes. The microarray analysis was carried out for two replicates each for the ancestral strain in YPG and YPGN (4 in total) and for four replicate population samples (one for S1, two for S2 and one for S3) each in YPG and YPGN for the S lines (8 in total). 'Shared' genes, genes that respond to NaCl in a similar manner between the ancestral strain and the evolved lines, with significant up-regulation or down-regulation were identified using a *t*-test at $P = 0.05$, false discovery rate (FDR) < 10% and $|\log_2 \text{(fold change)}| \geq 1$.

Genes with changes in basal expression or change in regulation were identified based on two *Z*-scores, $Z_r$ and $Z_b$ (see Appendix S2). Genes whose absolute *Z*-scores exceeded a value of 1.5 were considered to be differentially expressed. The differentially expressed genes were then grouped into different classes using the CYGD functional classification for yeast genes (Güldener *et al.*, 2005).

### Whole genome sequencing and SNP identification

The ancestral strain, as well as population samples of the NaCl evolved lines at generation 300, was sequenced at approximately 10× coverage using next generation pyrosequencing (Margulies *et al.*, 2005) (genome sequencer FLX LR system, Roche). The line S3 was further sequenced to a total of approximately 50× coverage. Candidate SNPs and indels were identified using blastn (Altschul *et al.*, 1990) followed by MUSCLE (Edgar, 2004) based on the criteria described in Appendix S2. PCR sequencing was done to confirm candidate SNPs and indels.

### Pulsed field gel electrophoresis

To identify large-scale chromosomal rearrangements, two clones from each of the evolved lines (S1, S2 and S3) and the ancestral strain were analysed by PFGE. Agarose plugs were prepared with the CHEF Yeast Genomic DNA Plug Kit (Bio-Rad, Reinach, Switzerland) according to the manufacturer's protocol. For restriction enzyme digestion, agarose plugs were incubated with *Not*I restriction enzyme for 16 h at 37 °C mixture in an appropriate restriction buffer. The digested or undigested plugs were then loaded into the wells of 1% agarose gels. Electrophoresis was carried out in a CHEF-DRIII system (Bio-Rad). The gels were stained using ethidium bromide (EtBr) and photographed.

### Gene amplification and deletions from next generation sequencing data

In addition to identifying very short indels within individual reads, larger gene duplications and deletions in the evolved lines, compared with the ancestral strain, were identified using read coverage information from the sequencing data. Furthermore, DNA breakpoint analysis was performed to confirm those candidate copy number changes.

### Ploidy determination of the evolved lines

To estimate the ploidy level of the evolved lines in comparison with the ancestral strain, the ancestral strain and population samples of the evolved lines were grown for 24 h at 30 °C. Genomic DNA was isolated from a defined number of cells following the protocol described in Sambrook & Russell, 2001 (Chapter 6).

### Cell size measurement

Population samples of ancestral and evolved cells were grown in YPG medium up to mid-log phase. The cell density was adjusted to be the same for all the samples (approximately $1 \times 10^7$ cells $ml^{-1}$). Twenty microlitres of cells were then loaded onto disposable counting chambers, and the cell sizes were measured using a Cellometer M10 (Nexcelom Bioscience, Lawrence, MA, USA), following the manufacturer's protocol.

## Acknowledgments

We acknowledge the help of the Functional Genomics Center Zurich for carrying out next generation sequencing and microarray experiments. AW acknowledges support through SNF grants 315200-116814, 315200-119697 and 315230-129708, as well as through the YeastX project of SystemsX.ch, and the University Research Priority Program in systems biology at the University of Zurich.

## References

Adams, J. & Oeller, P.W. 1986. Structure of evolving populations of *Saccharomyces cerevisiae*: adaptive changes are frequently associated with sequence alterations involving mobile elements belonging to the Ty family. *Proc. Natl Acad. Sci. USA* **83**: 7124–7127.

Adams, J., Puskas-Rozsa, S., Simlar, J. & Wilke, C.M. 1992. Adaptation and major chromosomal changes in populations of *Saccharomyces cerevisiae*. *Curr. Genet.* **22**: 13–19.

Alonso-Monge, R., Real, E., Wojda, I., Bebelman, J.P., Mager, W.H. & Siderius, M. 2001. Hyperosmotic stress response and regulation of cell wall integrity in *Saccharomyces cerevisiae* share common functional aspects. *Mol. Microbiol.* **41**: 717–730.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**: 403–410.

Apse, M.P., Aharon, G.S., Snedden, W.A. & Blumwald, E. 1999. Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in *Arabidopsis*. *Science* **285**: 1256–1258.

Attfield, P.V. 1997. Stress tolerance: the key to effective strains of industrial baker's yeast. *Nat. Biotech.* **15**: 1351–1357.

Badarinarayana, V., Chiang, Y.C. & Denis, C.L. 2000. Functional interaction of CCR4-NOT proteins with TATAA-binding protein (TBP) and its associated factors in yeast. *Genetics* **155**: 1045–1054.

Barrett, J.C., Hansoul, S., Nicolae, D.L., Cho, J.H., Duerr, R.H., Rioux, J.D. *et al.* 2008. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat. Genet.* **40**: 955–962.

Benfey, P.N. & Protopapas, A.D. 2005. *Essentials of Genomics*. Prentice-Hall, Upper Saddle River, NJ.

Betz, C., Zajonc, D., Moll, M. & Schweizer, E. 2002. ISC1-encoded inositol phosphosphingolipid phospholipase C is involved in Na+/Li+ halotolerance of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **269**: 4033–4039.

Blanc, V.M. & Adams, J. 2003. Evolution in *Saccharomyces cerevisiae*: identification of mutations increasing fitness in laboratory populations. *Genetics* **165**: 975–983.

Bressan, R.A., Hasegawa, P.M. & Pardo, J.M. 1998. Plants use calcium to resolve salt stress. *Trends Plant Sci.* **3**: 411–412.

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E. & Gustin, M.C. 1993. An osmosensing signal transduction pathway in yeast. *Science* **259**: 1760–1763.

Cade, R.M. & Errede, B. 1994. MOT2 encodes a negative regulator of gene expression that affects basal expression of pheromone-responsive genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**: 3139–3149.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G. *et al.* 2001. Remodeling of yeast genome expression in response to environmental changes. *Mol. Biol. Cell* **2**: 323–337.

Cooper, J.D., Smyth, D.J., Smiles, A.M., Plagnol, V., Walker, N.M., Allen, J.E. *et al.* 2008. Meta-analysis of genome-wide association study data identifies additional type 1 diabetes risk loci. *Nat. Genet.* **40**: 1399–1401.

Denis, C.L., Chiang, Y.C., Cui, Y. & Chen, J. 2001. Genetic evidence supports a role for the yeast CCR4-NOT complex in transcriptional elongation. *Genetics* **158**: 627–634.

Dihazi, H., Kessler, R. & Eschrich, K. 2004. High Osmolarity Glycerol (HOG) pathway-induced phosphorylation and activation of 6-phosphofructo-2-kinase are essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress. *J. Biol. Chem.* **279**: 23961–23968.

Dunham, M.J., Badrane, H., Ferea, T., Adams, J., Brown, P.O., Rosenzweig, F. *et al.* 2002. Characteristic genome rearrangements in experimental evolution of *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA* **99**: 16144–16149.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**: 1792–1797.

Elena, S.F. & Lenski, R.E. 1997. Test of synergistic interactions among deleterious mutations in bacteria. *Nature* **390**: 395–398.

Elena, S.F. & Lenski, R.E. 2001. Epistasis between new mutations and genetic background and a test of genetic canalization. *Evolution* **55**: 1746–1752.

Emerson, J.J. & Li, W.-H. 2010. The genetic basis of evolutionary change in gene expression levels. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **365**: 2581–2590.

Espinosa-Ruiz, A., Belles, J.M., Serrano, R. & Culianez-Macla, V. 1999. *Arabidopsis thaliana* AtHAL3: a flavoprotein related to salt and osmotic tolerance and plant growth. *Plant J.* **20**: 529–539.

Ferea, T.L., Botstein, D., Brown, P.O. & Rosenzweig, R.F. 1999. Systematic changes in gene expression patterns following adaptive evolution in yeast. *Proc. Natl Acad. Sci. USA* **96**: 9721–9726.

Ferrando, A., Kron, S.J., Rios, G., Fink, G.R. & Serrano, R. 1995. Regulation of cation transport in *Saccharomyces cerevisiae* by the salt tolerance gene HAL3. *Mol. Cell. Biol.* **15**: 5470–5481.

Frayling, T.M. 2007. Genome-wide association studies provide new insights into type 2 diabetes aetiology. *Nat. Rev. Genet.* **8**: 657–662.

Galcheva-Gargova, Z., Derijard, B., Wu, I.H. & Davis, R.J. 1994. An osmosensing signal transduction pathway in mammalian cells. *Science* **265**: 806–808.

Galitski, T., Saldanha, A.J., Styles, C.A., Lander, E.S. & Fink, G.R. 1999. Ploidy regulation of gene expression. *Science* **285**: 251–254.

Ganster, R.W., McCartney, R.R. & Schmidt, M.C. 1998. Identification of a calcineurin-independent pathway required for sodium ion stress response in *Saccharomyces cerevisiae*. *Genetics* **150**: 31–42.

Garciadeblas, B., Rubio, F., Quintero, F.J., Bañuelos, M.A., Haro, R. & Rodríguez-Navarro, A. 1993. Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **236**: 363–368.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G. *et al.* 2000. Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **11**: 4241–4257.

Gaxiola, R., de Larrinoa, I.F., Villalba, J.M. & Serrano, R. 1992. A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. *EMBO J.* **11**: 3157–3164.

Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper, S.L. & Fink, G.R. 1999. The *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. *Proc. Natl Acad. Sci. USA* **96**: 1480–1485.

Gerstein, A.C., Chun, H.J.E., Grant, A. & Otto, S.P. 2006. Genomic convergence toward diploidy in *Saccharomyces cerevisiae*. *PLoS Genet.* **2**: e145.

Gisbert, C., Rus, A.M., Bolarín, M.C., Lopez-Coronado, J.M., Arrillaga, I., Montesinos, C. *et al.* 2000. The yeast HAL1 gene improves salt tolerance of transgenic tomato. *Plant Physiol.* **123**: 393–402.

Goossens, A., Forment, J. & Serrano, R. 2002. Involvement of Nst1p/YNL091w and Msl1p, a U2B'' splicing factor, in *Saccharomyces cerevisiae* salt tolerance. *Yeast* **19**: 193–202.

Gorner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B. *et al.* 1998. Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev.* **12**: 586–597.

Gresham, D., Desai, M.M., Tucker, C.M., Jenq, H.T., Pai, D.A., Ward, A. *et al.* 2008. The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. *PLoS Genet.* **4**: e1000303.

Güldener, U., Münsterkötter, M., Kastenmüller, G., Strack, N., van Helden, J., Lemer, C. *et al.* 2005. CYGD: the comprehensive yeast genome database. *Nucleic Acids Res.* **33**: D364–D368.

Han, J., Lee, J.D., Bibbs, L. & Ulevitch, R.J. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265**: 808–811.

Haro, R., Garciadeblas, B. & Rodríguez-Navarro, A. 1991. A novel P-type ATPase from yeast involved in sodium transport. *FEBS Lett.* **291**: 189–191.

Hasegawa, P.M. & Bressan, R.A. 2000. Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* **51**: 463–499.

He, X., Qian, W., Wang, Z., Li, Y. & Zhang, J. 2010. Prevalent positive epistasis in *Escherichia coli* and *Saccharomyces cerevisiae* metabolic networks. *Nat. Genet.* **42**: 272–276.

Heath, V.L., Shaw, S.L., Roy, S. & Cyert, M.S. 2004. Hph1p and Hph2p, novel components of calcineurin-mediated stress responses in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **3**: 695–704.

Herrero, E., Ros, J., Bellí, G. & Cabiscol, E. 2008. Redox control and oxidative stress in yeast cells. *Biochim. Biophys. Acta* **1780**: 1217–1235.

Hindorff, L.A., Sethupathy, P., Junkins, H.A., Ramos, E.M., Mehta, J.P., Collins, F.S. *et al.* 2009. Potential etiologic and functional implications of genome-wide association loci for

human diseases and traits. *Proc. Natl Acad. Sci. USA* **106**: 9362–9367.

Hirata, Y., Andoh, T., Asahara, T. & Kikuchi, A. 2003. Yeast glycogen synthase kinase-3 activates Msn2p-dependent transcription of stress responsive genes. *Mol. Biol. Cell* **14**: 302–312.

Hohmann, S. 2002. Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* **66**: 300–372.

Hughes, T.R., Roberts, C.J., Dai, H., Jones, A.R., Meyer, M.R., Slade, D. *et al.* 2000. Widespread aneuploidy revealed by DNA microarray expression profiling. *Nat. Genet.* **25**: 333–337.

Jamieson, D.J. 1998. Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast* **14**: 1511–1527.

Jonak, C., Kiegerl, S., Ligterink, W., Barker, P.J., Huskisson, N.S. & Hirt, H. 1996. Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Natl Acad. Sci. USA* **93**: 11274–11279.

Jonak, C., Ligterink, W. & Hirt, H. 1999. MAP kinases in plant signal transduction. *Cell. Mol. Life Sci.* **55**: 204–213.

Kao, K.C. & Sherlock, G. 2008. Molecular characterization of clonal interference during adaptive evolution in asexual populations of *Saccharomyces cerevisiae*. *Nat. Genet.* **40**: 1499–1504.

Kosako, H., Nishida, E. & Gotoh, Y. 1993. cDNA cloning of MAP kinase kinase reveals kinase cascade pathways in yeasts to vertebrates. *EMBO J.* **12**: 787–794.

Kovtun, Y., Chiu, W.-L., Tena, G. & Sheen, J. 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl Acad. Sci. USA* **97**: 2940–2945.

Kyriakis, J.M. & Avruch, J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* **81**: 807–869.

Lee, J.H., Montagu, M.V. & Verbruggen, N. 1999. A highly conserved kinase is an essential component for stress tolerance in yeast and plant cells. *Proc. Natl Acad. Sci. USA* **96**: 5873–5877.

Lenski, R.E. & Mongold, J.A. 2000. Cell size, shape, and fitness in evolving populations of bacteria. In: *Scaling in Biology* (J.H. Brown & G.B. West, eds), pp. 221–235. Oxford University Press, Oxford.

Lenski, R.E. & Travisano, M. 1994. Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc. Natl Acad. Sci. USA* **91**: 6808–6814.

Liu, H.Y., Badarinarayana, V., Audino, D.C., Rappsilber, J., Mann, M. & Denis, C.L. 1998. The NOT proteins are part of the CCR4 transcriptional complex and affect gene expression both positively and negatively. *EMBO J.* **17**: 1096–1106.

Lushchak, V.I. & Gospodaryov, D.V. 2005. Catalases protect cellular proteins from oxidative modification in *Saccharomyces cerevisiae*. *Cell Biol. Int.* **29**: 187–192.

Maathuis, F.J.M. & Amtmann, A. 1999. K+ nutrition and Na+ toxicity: the basis of cellular K+/Na+ ratios. *Ann. Bot.* **84**: 123–133.

Mai, B. & Breeden, L. 1997. Xbp1, a stress-induced transcriptional repressor of the *Saccharomyces cerevisiae* Swi4/Mbp1 family. *Mol. Cell. Biol.* **17**: 6491–6501.

Manolio, T.A., Collins, F.S., Cox, N.J., Goldstein, D.B., Hindorff, L.A., Hunter, D.J. *et al.* 2009. Finding the missing heritability of complex diseases. *Nature* **461**: 747–753.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A. *et al.* 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**: 376–380.

Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H. & Estruch, F. 1996. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *EMBO J.* **15**: 2227–2235.

Mendizabal, I., Rios, G., Mulet, J.M., Serrano, R. & de Larrinoa, I.F. 1998. Yeast putative transcription factors involved in salt tolerance. *FEBS Lett.* **425**: 323–328.

Mendoza, I., Rubioli, F., Rodriguez-Navarroli, A. & Pardo, J.M. 1994. The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **269**: 8792–8796.

Mersman, D.P., Du, H.N., Fingerman, I.M., South, P.F. & Briggs, S.D. 2009. Polyubiquitination of the demethylase Jhd2 controls histone methylation and gene expression. *Genes Dev.* **23**: 951–962.

Morgan, L.W. & Feldman, J.F. 2001. Epistatic and synergistic interactions between circadian clock mutations in *Neurospora crassa*. *Genetics* **159**: 537–543.

Moskvina, E., Schuller, C., Maurer, C.T.C., Mager, W.H. & Ruis, H. 1998. A search in the genome of *Saccharomyces cerevisiae* for genes regulated via stress response elements. *Yeast* **14**: 1041–1050.

Mukhopadhyay, A., He, Z., Alm, E.J., Arkin, A.P., Baidoo, E.E., Borglin, S.C. *et al.* 2006. Salt stress in *Desulfovibrio vulgaris* Hildenborough: an integrated genomics approach. *J. Bacteriol.* **188**: 4068–4078.

Mulet, J.M., Leube, M.P., Kron, S.J., Rios, G., Fink, G.R. & Serrano, R. 1999. A novel mechanism of ion homeostasis and salt tolerance in yeast: the Hal4 and Hal5 protein kinases modulate the Trk1–Trk2 potassium transporter. *Mol. Cell. Biol.* **19**: 3328–3337.

Murguía, J.R., Bellés, J.M. & Serrano, R. 1996. The yeast HAL2 nucleotidase is an *in vivo* target of salt toxicity. *J. Biol. Chem.* **271**: 29029–29033.

Neiman, A.M. 1993. Conservation and reiteration of a kinase cascade. *Trends Genet.* **9**: 390–394.

Noggle, R. 1946. The physiology of polyploidy in plants. I. Review of the literature. *Lloydia* **9**: 153–173.

Ostrander, D.B. & Gorman, J.A. 1999. The extracellular domain of the *Saccharomyces cerevisiae* Sln1p membrane osmolarity sensor is necessary for kinase activity. *J. Bacteriol.* **181**: 2527–2534.

Panaretou, B. & Piper, P.W. 1992. The plasma membrane of yeast acquires a novel heat-shock protein (hsp30) and displays a decline in proton-pumping ATPase levels in response to both heat shock and the entry to stationary phase. *Eur. J. Biochem.* **206**: 635–640.

Panasenko, O., Landrieux, E., Feuermann, M., Finka, A., Paquet, N. & Collart, M.A. 2006. The yeast Ccr4-Not complex controls ubiquitination of the nascent-associated polypeptide (NAC-EGD) complex. *J. Biol. Chem.* **281**: 31389–31398.

Pardo, J.M., Reddy, M.P., Yang, S., Maggio, A., Huh, G.-H., Matsumoto, T. *et al.* 1998. Stress signaling through $Ca^{2+}$/calmodulin-dependent protein phosphatase calcineurin mediates salt adaptation in plants. *Proc. Natl Acad. Sci. USA* **95**: 9681–9686.

Park, J.H., Wacholder, S., Gail, M.H., Peters, U., Jacobs, K.B., Chanock, S.J. *et al.* 2010. Estimation of effect size distribution

from genome-wide association studies and implications for future discoveries. *Nat. Genet.* **42**: 570–575.

Philippe, N., Pelosi, L., Lenski, R.E. & Schneider, D. 2009. Evolution of penicillin-binding protein 2 concentration and cell shape during a long-term experiment with *Escherichia coli*. *J. Bacteriol.* **191**: 909–921.

Phillips, P.C. 2008. Epistasis – the essential role of gene interactions in the structure and evolution of genetic systems. *Nat. Rev. Genet.* **9**: 855–867.

Piper, P.W., Talreja, K., Panaretou, B., Moradas-Ferreira, P., Byrne, K., Praekelt, U.M. *et al.* 1994. Induction of major heat-shock proteins of *Saccharomyces cerevisiae*, including plasma membrane Hsp30, by ethanol levels above a critical threshold. *Microbiology* **140**: 3031–3038.

Piper, P.W., Ortiz-Calderon, C., Holyoak, C., Coote, P. & Cole, M. 1997. Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H(+)-ATPase. *Cell Stress Chaperones* **2**: 12–24.

Posas, F., Chambers, J.R., Heyman, J.A., Hoeffler, J.P., de Nadal, E. & Arino, J. 2000. The transcriptional response of yeast to saline stress. *J. Biol. Chem.* **275**: 17249–17255.

Quintero, F.J., Ohta, M., Shi, H., Zhu, J.-K. & Pardo, J.M. 2002. Reconstitution in yeast of the *Arabidopsis* SOS signaling pathway for $Na^+$ homeostasis. *Proc. Natl Acad. Sci. USA* **99**: 9061–9066.

Quinteroa, F.J., Blatt, M.R. & Pardo, J.M. 2000. Functional conservation between yeast and plant endosomal Na+/H+ antiporters. *FEBS Lett.* **471**: 224–228.

Rancati, G., Pavelka, N., Fleharty, B., Noll, A., Trimble, R., Walton, K. *et al.* 2008. Aneuploidy underlies rapid adaptive evolution of yeast cells deprived of a conserved cytokinesis motor. *Cell* **135**: 879–893.

Reiser, V., Salah, S.M. & Ammerer, G. 2000. Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42. *Nat. Cell Biol.* **2**: 620–627.

Rep, M., Reiser, V., Gartner, U., Thevelein, J.M., Hohmann, S., Ammerer, G. *et al.* 1999. Osmotic Stress-Induced Gene Expression in *Saccharomyces cerevisiae* Requires Msn1p and the Novel Nuclear Factor Hot1p. *Mol. Cell. Biol.* **19**: 5474–5485.

Rep, M., Krantz, M., Thevelein, J.M. & Hohmann, S. 2000. The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. *J. Biol. Chem.* **275**: 8290–8300.

Ringrose, L. & Paro, R. 2004. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. *Annu. Rev. Genet.* **38**: 413–443.

Rios, G., Ferrando, A. & Serrano, R. 1997. Mechanisms of salt tolerance conferred by overexpression of the HAL1 gene in *Saccharomyces cerevisiae*. *Yeast* **13**: 515–528.

Rudolph, B., Gebendorfer, K.M., Buchner, J. & Winter, J. 2010. Evolution of *Escherichia coli* for growth at high temperatures. *J. Biol. Chem.* **285**: 19029–19034.

Saito, H. & Tatebayashi, K. 2004. Regulation of the osmoregulatory HOG MAPK cascade in yeast. *J. Biochem.* **136**: 267–272.

Saleh, B., Allario, T., Dambier, D., Ollitrault, P. & Morillon, R. 2008. Tetraploid citrus rootstocks are more tolerant to salt stress than diploid. *C. R. Biologies*, **331**: 703–710.

Samani, P. & Bell, G. 2010. Adaptation of experimental yeast populations to stressful conditions in relation to population size. *J. Evol. Biol.* **23**: 791–796.

Sambrook, J. & Russell, D.W. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, New York.

Sanders, D. 2000. Plant biology: the salty tale of *Arabidopsis*. *Curr. Biol.* **10**: R486–R488.

Schmitt, A.P. & McEntee, K. 1996. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA* **93**: 5777–5782.

Schuller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C. & Ruis, H. 1994. The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* CTT1 gene. *EMBO J.* **13**: 4382–4389.

Serrano, R. 1996. Salt tolerance in plants and microorganisms: toxicity targets and defense responses. *Int. Rev. Cytol.* **165**: 1–52.

Serrano, R., Mulet, J.M., Rios, G., Marquez, J.A., de Larrinoa, I.F., Leube, M.P. *et al.* 1999. A glimpse of the mechanisms of ion homeostasis during salt stress. *J. Exp. Bot.* **50**: 1023–1036.

Stanek, M.T., Cooper, T.F. & Lenski, R.E. 2009. Identification and dynamics of a beneficial mutation in a long-term evolution experiment with *Escherichia coli*. *BMC Evol. Biol.* **9**: 302.

Stebbins, G.L. 1950. *Variation and Evolution in Plants*. Columbia University Press, New York.

Tal, M. & Gardi, I. 1976. Physiology of polyploid plants: water balance in autotetraploid and diploid tomato under low and high salinity. *Physiol. Plant* **38**: 257–261.

Teige, M., Scheikl, E., Eulgem, T., Doczi, R., Ichimura, K., Shinozaki, K. *et al.* 2004. The MKK2 pathway mediates cold and salt stress signaling in *Arabidopsis*. *Mol. Cell* **15**: 141–152.

Trainotti, N. & Stambuk, B.U. 2001. NaCl stress inhibits maltose fermentation by *Saccharomyces cerevisiae*. *Biotech. Lett.* **23**: 1703–1707.

Tsujimoto, Y., Izawa, S. & Inoue, Y. 2000. Cooperative regulation of DOG2, encoding 2-deoxyglucose-6-phosphate phosphatase, by Snf1 kinase and the high-osmolarity glycerol–mitogen-activated protein kinase cascade in stress responses of *Saccharomyces cerevisiae*. *J. Bacteriol.* **182**: 5121–5126.

Turner, B.M. 2002. Cellular memory and the histone code. *Cell* **111**: 285–291.

Velicer, G.J., Raddatz, G., Keller, H., Deiss, S., Lanz, C., Dinkelacker, I. *et al.* 2006. Comprehensive mutation identification in an evolved bacterial cooperator and its cheating ancestor. *Proc. Natl Acad. Sci. USA* **103**: 8107–8112.

Vijaranakul, U., Nadakavukaren, M.J., de Jonge, B.L., Wilkinson, B.J. & Jayaswal, R.K. 1995. Increased cell size and shortened peptidoglycan interpeptide bridge of NaCl-stressed *Staphylococcus aureus* and their reversal by glycine betaine. *J. Bacteriol.* **177**: 5116–5121.

Visscher, P.M. 2008. Sizing up human height variation. *Nat. Genet.* **40**: 489–490.

Visscher, P.M. & Montgomery, G.W. 2009. Genome-wide association studies and human disease: from trickle to flood. *J. Am. Med. Assoc.* **302**: 2028–2029.

Warringer, J., Ericson, E., Fernandez, L., Nerman, O. & Blomberg, A. 2003. High-resolution yeast phenomics resolves different physiological features in the saline response. *Proc. Natl Acad. Sci. USA* **100**: 15724–15729.

Waskiewicz, A.J. & Cooper, J.A. 1995. Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell Biol.* **7**: 798–805.

Wittkopp, P.J., Haerum, B.K. & Clark, A.G. 2004. Evolutionary changes in cis and trans gene regulation. *Nature* **430**: 85–88.

Wu, X., Hart, H., Cheng, C., Roach, P.J. & Tatchell, K. 2001. Characterization of Gac1p, a regulatory subunit of protein phosphatase type I involved in glycogen accumulation in *Saccharomyces cerevisiae*. *Mol. Genet. Genomics* **265**: 622–635.

Wu, C.-Y., Rolfe, P.A., Gifford, D.K. & Fink, G.R. 2010. Control of transcription by cell size. *PLoS Biol.* **8**: e1000523.

Yale, J. & Bohnert, H.J. 2001. Transcript expression in *Saccharomyces cerevisiae* at high salinity. *J. Biol. Chem.* **276**: 15996–16007.

Yang, S.X., Zhao, Y.X., Zhang, Q., He, Y.K., Zhang, H. & Luo, D. 2001. HAL1 mediate salt adaptation in *Arabidopsis thaliana*. *Cell Res.* **11**: 142–148.

Yang, J., Benyamin, B., McEvoy, B.P., Gordon, S., Henders, A.K., Nyholt, D.R. *et al.* 2010. Common SNPs explain a large proportion of the heritability for human height. *Nat. Genet.* **42**: 565–569.

Zacharioudakis, I., Gligoris, T. & Tzamarias, D. 2007. A yeast catabolic enzyme controls transcriptional memory. *Curr. Biol.* **17**: 2041–2046.

Zeggini, E., Scott, L.J., Saxena, R., Voight, B.F., Marchini, J.L., Hu, T. *et al.* 2008. Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nat. Genet.* **40**: 638–645.

Zeyl, C., Vanderford, T. & Carter, M. 2003. An evolutionary advantage of haploidy in large yeast populations. *Science* **299**: 555–558.

Zhang, Y., Fatima, N. & Dufau, M.L. 2005. Coordinated changes in DNA methylation and histone modifications regulate silencing/derepression of luteinizing hormone receptor gene transcription. *Mol. Cell. Biol.* **25**: 7929–7939.

Zheng, D.Q., Wu, X.C., Tao, X.L., Wang, P.M., Li, P., Chi, X.Q. *et al.* 2011. Screening and construction of *Saccharomyces cerevisiae* strains with improved multi-tolerance and bioethanol fermentation performance. *Bioresour. Technol.* **102**: 3020–3027.

Zhu, J.-K. 2001. Plant salt tolerance. *Trends Plant Sci.* **6**: 66–71.

Zhu, J.-K. 2002. Salt and Drought Stress Signal Transduction in Plants. *Annu. Rev. Plant. Biol.* **53**: 247–273.

## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Supplementary text.

**Appendix S2** Supplementary methods.

**Appendix S3** Supplementary figures.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

*Received 24 November 2010; revised 27 January 2011; accepted 28 January 2011*