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Activator and Repressor Functions of the Mot3 Transcription Factor in the Osmostress Response of *Saccharomyces cerevisiae*

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Mot3 and Rox1 are transcriptional repressors of hypoxic genes. Both factors recently have been found to be involved in the adaptive response to hyperosmotic stress, with an important function in the adjustment of ergosterol biosynthesis. Here, we determine the gene expression profile of a *mot3 rox1* double mutant under acute osmostress at the genomic scale in order to identify the target genes affected by both transcription factors upon stress. Unexpectedly, we find a specific subgroup of osmostress-inducible genes to be under positive control of Mot3. These Mot3-activated stress genes also depend on the general stress activators Msn2 and Msn4. We confirm that both Mot3 and Msn4 bind directly to some promoter regions of this gene group. Furthermore, osmostress-induced binding of the Msn2 and Msn4 factors to these target promoters is severely affected by the loss of Mot3 function. The genes repressed by Mot3 and Rox1 preferentially encode proteins of the cell wall and plasma membrane. Cell conjugation was the most significantly enriched biological process which was negatively regulated by both factors and by osmotic stress. The mating response was repressed by salt stress dependent on Mot3 and Rox1 function. Taking our findings together, the Mot3 transcriptional regulator has unanticipated diverse functions in the cellular adjustment to osmotic stress, including transcriptional activation and modulation of mating efficiency.

Changes in the osmolarity of the cell environment is a fundamental stimulus which triggers adaptive responses at many different physiological levels. Failure to respond properly to osmotic stress can cause cell death. In yeast, the adaptation to hyperosmotic conditions has been extensively studied, especially the molecular events leading to a massive reprogramming of gene expression upon osmostress (1–5). The high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway is the central signal transduction pathway activated by osmotic stress. Its terminal Hog1 MAP kinase is rapidly activated upon stress and, among other functions, is essential to coordinate a complex transcriptional program in the nucleus (6). Global transcription profiling experiments under hyperosmotic stress indicate that 3 to 7% of all yeast genes are strongly activated during the osmostress defense (7–9). Activated gene functions include osmolyte production, sugar metabolism, antioxidants, proteins involved in redox metabolism, mitochondrial functions, chaperones, cell surface proteins, and signal transduction molecules. Additionally, the expression of a large set of genes is negatively regulated upon osmostress, which include general growth-related functions, such as RNA metabolism or ribosomal functions, and specifically downregulated functions upon osmostress (8, 10). It is worth noting that kinetic surveys of gene expression indicate that more than 40% of the yeast genome is transcriptionally regulated, both positively or negatively, under osmotic stress conditions (11).

Over recent years, several specific transcription factors have been identified and characterized to be involved in the adaptive response to hyperosmotic stress. As suggested by the large number of differentially expressed genes upon osmoshock, many different DNA binding proteins are implicated in modulating gene expression upon osmostress (5). The Sko1 repressor/activator and the Hot1 and Smp1 transcriptional activators are directly regulated through phosphorylation by the Hog1 MAP kinase (12–16). Other DNA binding activators, such as Msn1, Msn2, and Msn4, are genetically linked to the HOG pathway (16, 17). Global location analyses of those transcription factors favor the idea that binding of each factor to a specific subset of target genes and the combination of these binding events allows for a fine-tuned control of gene expression upon osmostress (11).

Our recent work has added two more transcription factors with a function in yeast osmoadaptation, Mot3 and Rox1 (18). Both factors, together with the Hog1 kinase, are essential to downregulate the expression of specific *ERG* genes involved in the biosynthesis of ergosterol. As a consequence, total sterol levels of the cell decrease in response to osmotic stress, which is needed to efficiently adapt to hyperosmotic conditions (18). Rox1 and Mot3 are well-known repressors of hypoxic genes. These are genes whose expression is actively inhibited under aerobic conditions and activated upon oxygen limitation (19–21). Among the hypoxic genes, the expression of the *DAN* and *TIR* genes, encoding cell wall mannoproteins specific for anaerobic growth, or genes belonging to biochemical pathways which are limited by oxygen, such as sterol or heme biosynthesis, and others have been studied in detail (22–26). In many cases, Rox1 and Mot3 have been identified to contribute to the repression of these genes by direct binding to separate DNA motifs in the respective target promoters. Additionally, a synergistic mode of repression has been described for some specific Rox1- and Mot3-regulated hypoxic genes (24, 26, 27).

Here, we aimed at identifying the impact of Mot3 and Rox1 function on gene expression under acute osmotic stress by genome-wide transcript profiling of wild-type yeast and the *mot3*

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TABLE 1 *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Reference or source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
W303-1A	*MAT***a** *ade2-1 ura3-1 his3-11.-15 leu2,3-112 trp1*	R. Rothstein
mot3	BY4741 *mot3*::*KANMX4*	EUROSCARF
rox1	BY4741 *rox1*::*KANMX4*	EUROSCARF
MAP93	BY4741 *rox1*::*KANMX4 mot3*::*his5+* (*S. pombe*)	18
hog1	BY4741 *hog1*::*KANMX4*	EUROSCARF
msn2	BY4741 *msn2*::*KANMX4*	EUROSCARF
msn4	BY4741 *msn4*::*KANMX4*	EUROSCARF
Wmsn2msn4	W303-1A *msn2*::*HIS3 msn4*::*TRP1*	F. Estruch
hot1	BY4741 *hot1*::*KANMX4*	EUROSCARF
smp1	BY4741 *smp1*::*KANMX4*	EUROSCARF
sko1	BY4741 *sko1*::*KANMX4*	EUROSCARF
MAP80	W303-1A with *MSN2-3* × *HA-KAN*	This study
MAP81	W303-1A with *MSN4-3* × *HA-KAN*	This study
MAP119	W303-1A with *MSN2-3* × *HA-KAN mot3*::*URA3*	This study
MAP120	W303-1A with *MSN4-3* × *HA-KAN mot3*::*URA3*	This study
Rox1-TAP	BY4741 *ROX1-TAP*::*HIS3*	46
FY2081	FY1339 *MOT3-18myc*::*TRP1*	F. Winston
FY1339	*MAT***a** *ura3Δ1 his3Δ200 trp1Δ63*	F. Winston

rox1 mutant. Our data suggest that Mot3 and, to a much lesser extent, Rox1, contribute in a much broader way than anticipated to the adjustment of gene expression upon osmostress. Specifically, Mot3 activates the expression of a subset of osmoinducible genes, while it has repressor functions at other genes encoding, for example, plasma membrane transporters or mating-related proteins.

MATERIALS AND METHODS

Yeast strains and growth conditions. The yeast strains used in this study are listed in Table 1. Genomic tagging of Msn2 and Msn4 with 3× hemagglutinin (HA) was performed according to reference 28. Deletion of the *MOT3* gene was achieved by gene replacement with *URA3* (29). All yeast strains were grown at 28°C in YPD medium, which contained 2% glucose, 2% peptone, and 1% yeast extract. For sensitivity assays in continuous growth, fresh overnight precultures of the indicated strains were diluted in triplicate in multiwell plates to the same initial optical density (OD) in yeast extract-peptone-dextrose (YPD), YPD plus 1.2 M NaCl, or YPD plus 60 μg/ml hygromycin B. Growth was then constantly monitored in a Bioscreen C system (Thermo) for the indicated times. The expression of mating genes was induced by the addition of 5 μg/ml of α-factor to the growth cultures.

Northern blotting. Northern analysis was performed as described previously (14). Approximately 30 μg of hot phenol-extracted total RNA was resolved on agarose-formaldehyde gels and blotted onto nylon membranes. RNA was probed with gene-specific ^{32}P-labeled DNA fragments obtained by genomic PCR. Signals were quantified using a Fujifilm BAS-1500 phosphorimager. The gene-specific signals were normalized for the signal obtained for the *ACT1* control in each case and are represented as the relative mRNA level. At least two independent experiments were done to measure the expression level of each gene in this study.

Microarray analysis. For the comparison of the transcriptomes of the yeast wild type and the *mot3 rox1* mutant, the cells were grown in YPD medium until mid-log phase and then subjected to a brief osmotic shock (0.4 M NaCl for 20 min). Total RNA was prepared from three independent culture aliquots for each strain using the RNeasy kit (Qiagen). Biotin labeling of the samples, hybridization to Affymetrix GeneChip Yeast Genome 2.0 arrays, and data analysis were performed at the VIB Microarray Facility (Leuven, Belgium).

ChIP. Chromatin immunoprecipitation (ChIP) was performed as described previously (30). Quantitative PCR analyses at the indicated chromosomal loci were performed in real time using an Applied Biosystems 7500 sequence detector with the *POL1* coding sequence (+1796/+1996) as an internal control. Each ChIP was performed twice with different chromatin samples. All occupancy data are presented as fold IP efficiency over the *POL1* control sequence. All primer sequences are available upon request. Primers used for quantitative PCR analysis matched the following promoter regions: *GRE1* (−313/−203), *PHM8* (−398/−250), *SPS100* (−233/−119), *YHR140W* (−308/−204), *RTN2* (−195/−118), *PDR15* (−302/−161), *SIP18* (−315/−183), *YHR022C* (−202/−97), and *AGA1* (−308/−208).

Quantitative analysis of the mating response. Cultures of the indicated strains were grown to an A_{600} of 0.8. Cells from 4-ml aliquots were collected by centrifugation and resuspended in soybean casein digest (SCD) medium with or without alpha factor (5 μg/ml) in the presence or absence of 0.4 M NaCl. One-ml samples of cells were taken at 0, 30, 60, and 90 min and concentrated by centrifugation. Cells were visualized using a light transmission bright-field Axionplan 2 imaging microscope with a 63× objective. For each condition and strain, the appearance of shmoo-type cells was counted from a total of >300 individual cells. Error bars were calculated from at least two independent experiments.

RESULTS

Transcriptomic analysis of the *mot3 rox1* mutant. To gain insights into the biological functions of the Mot3 and Rox1 transcription factors upon osmotic stress, we determined and analyzed the transcription profiles of the *mot3 rox1* mutant and the wild-type strain under acute osmotic stress provoked by 0.4 M NaCl. Using the experimental design described in Materials and Methods, we first identified the genes whose expression in the double mutant was at least 1.5-fold higher or lower than that in the wild-type strain with a *P* value of <0.001. As expected from the previously described repressor function of Mot3 and Rox1, we found 151 genes to be upregulated in the mutant strain. Unexpectedly, however, we found 68 transcripts to be underrepresented in the *mot3 rox1* mutant, indicating a possible activator function of Mot3 and/or Rox1 upon osmotic stress (Fig. 1).

To characterize the biological processes represented by these differentially expressed genes, we applied the GO Term Finder tool. Among the overexpressed genes in the *mot3 rox1* mutant, several functional categories were significantly overrepresented, including conjugation ($P = 4.4E{-}5$), sexual reproduction ($P = 3.0E{-}4$), response to pheromone ($P = 0.001$), heme metabolism ($P = 0.005$), and sterol transport ($P = 0.01$). Additionally, the component cellular periphery was strongly overrepresented ($P = 2.2E{-}7$) among those Mot3 Rox1-repressed functions which were enriched in the cell wall ($P = 1.2E{-}6$) or the plasma membrane ($P = 2.0E{-}4$). On the other hand, we were not able to identify a significantly overrepresented biological function for the genes which had lower expression levels in the *mot3 rox1* mutant.

We next focused on the most deregulated genes in the *mot3 rox1* mutant to gain insights into the physiological functions of Mot3 and/or Rox1 upon osmostress. We found 22 genes which were strongly (≥2-fold) underrepresented in the transcriptome of the mutant. Interestingly, the expression of 10 of those genes is robustly activated upon NaCl stress according to published transcript profiling data sets (11). These osmostress-inducible and Mot3- and/or Rox1-dependent genes are depicted in Table 2. On the other hand, among the 56 strongly (≥2-fold) overrepresented genes in the *mot3 rox1* mutant (Table 3), we found 24 genes with a pronounced negative regulation of their expression in response to NaCl shock (6) (summarized in Table 3). Taking these results together, the transcript profile of the *mot3 rox1* mutant indicated

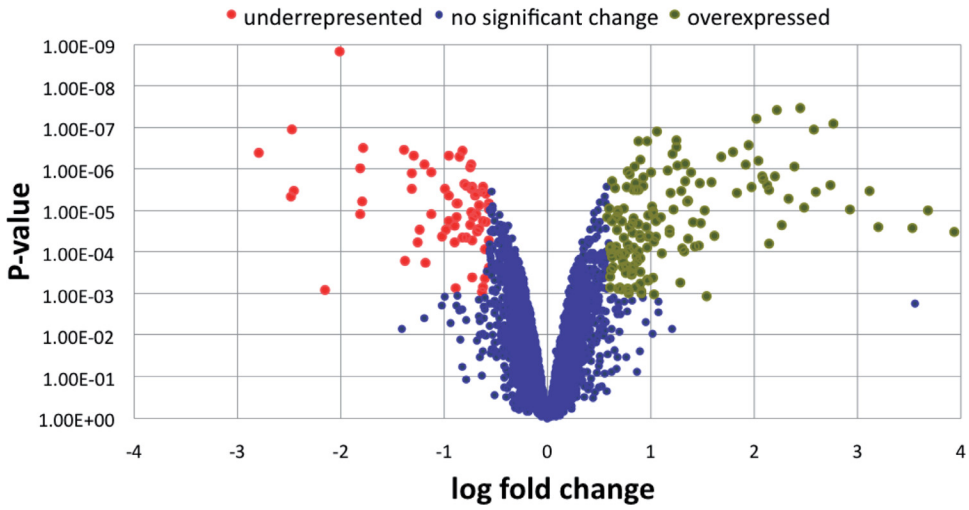

FIG 1 Graphical representation of over- and underrepresented transcripts in the *mot3 rox1* mutant upon osmostress. The genomic transcript profile of the mutant strains was compared to that of the wild type by microarray hybridization as described in Materials and Methods. Genes are marked as green dots when their expression was at least 1.5-fold greater than the wild-type level (with a *P* value of <0.001) and as red dots when their expression was at least 1.5-fold below the wild-type level (with a *P* value of <0.001).

that Mot3 and/or Rox1 have both positive and negative functions in the transcriptional program upon osmostress. We next tested whether the loss of function of Mot3 and Rox1 had phenotypic consequences under salt stress. As shown in Fig. 2, deletion of either *ROX1* or *MOT3* caused a slight growth delay upon NaCl stress and a clear sensitive phenotype in the presence of the highly toxic cation hygromycin B. Under both stress conditions, the phenotype was further enhanced in the *mot3 rox1* double mutant, indicating that Mot3 and Rox1 contribute to cation tolerance in yeast.

Mot3 is a transcriptional activator of a subset of osmostress-induced genes. We next focused on the analysis of the salt stress-inducible genes with a lower expression level in the *mot3 rox1* mutant. We performed Northern blot analysis of 7 individual members of this group in wild-type cells and the *mot3* and *rox1* single and double mutants. As shown in Fig. 3A, all genes tested showed a rapid and robust transcriptional activation upon NaCl shock in the wild type. This transcriptional activation was not affected by deletion of Rox1. However, we observed a substantial loss of osmostress-stimulated expression in the *mot3* and *mot3 rox1* mutant strains (Fig. 3A and B). This indicated that the Mot3 transcription factor (and not Rox1) has a positive function in the expression of the specific subset of osmostress-activated genes depicted in Table 2. As expected, we found this group of stress-responsive genes to be dependent on the MAP kinase Hog1 (Fig. 3A), with the exception of the *PHM8* gene.

Mot3 and Msn2,4 coordinately regulate specific osmostress-inducible genes. We have found that the Mot3 transcription factor contributes to the activation of specific salt-inducible genes. We next investigated the function of the previously known transcription factors Hot1, Sko1, Smp1, and Msn2 and Msn4 (termed Msn2,4), which have roles in the hyperosmotic stress response (13, 15–17), and tested their importance for the expression of the Mot3-regulated genes identified here. We compared the salt-induced expression profiles of *RTN2*, *GRE1*, *SPS100*, and *YHR140W* in the respective transcription factor mutants. As shown in Fig. 4, the expression of all four genes was dependent on the Msn2,4 activators upon NaCl shock. Additionally, Sko1 and Hot1 were important for the efficient activation of the *GRE1* and *SPS100* genes. We concluded that the general stress activators Msn2 and Msn4 played a dominant role in the expression of the Mot3-regulated gene cluster. We further examined this group of genes for the presence of potential Msn2,4 and Mot3 binding sites in the respective promoter regions. The CCCCT consensus motif for the Msn2 and Msn4 activators was found in 1 to 5 copies in the up-

TABLE 2 Osmostress-activated genes with reduced expression levels in the *mot3 rox1* mutant

Gene	Expression level (*mot3 rox1*/wild-type ratio)	*P* value	Osmostress regulation[a]	Function
SPS100 (*YHR139C*)	0.14	4.0E−07	104	Protein required for spore wall maturation
AQY1 (*YPR192W*)	0.18	4.7E−06	6.7	Spore-specific water channel
YHR140W	0.18	1.1E−07	5.3	Putative integral membrane protein
YHR022C	0.28	9.8E−07	18	Unknown
PHM8 (*YER037W*)	0.40	3.0E−06	4.6	Lysophosphatidic acid phosphatase
RTN2 (*YDL204W*)	0.40	1.3E−06	7.2	Unknown; similarity to mammalian reticulon proteins
YRO2 (*YBR054W*)	0.42	5.7E−05	2.7	Putative mitochondrial protein
PDR15 (*YDR406W*)	0.42	2.9E−05	3.5	Plasma membrane multidrug transporter of the ABC family
GRE1 (*YPL223C*)	0.44	7.8E−07	128	Stress-induced hydrophilic protein
SIP18 (*YMR175W*)	0.50	2.9E−05	82	Osmostress-induced phospholipid binding protein

[a] Fold induction upon NaCl stress. Data are taken from the datasets of genomic transcription profiling published previously (11).

TABLE 3 Genes with higher expression levels in the *mot3 rox1* mutant upon NaCl stress

Gene and repression level upon osmostress	Expression level (*mot3 rox1*/wild-type ratio)	*P* value	Osmostress regulation[a]	Function
Highly repressed				
DSF1 (*YEL070W*)	11.5	2.6E−05	0.4	Unknown
AAC3 (*YBR085W*)	7.6	9.4E−06	0.3	Mitochondrial ADP/ATP translocator, expressed under anaerobic conditions
FIG1 (*YBR040W*)	6.8	7.9E−08	0.2	Integral membrane protein required for mating
FET4 (*YMR319C*)	6.6	2.4E−06	0.4	Plasma membrane low-affinity Fe(II) transporter
YPR015C	5.9	1.1E−07	0.3	Unknown
YDL241W	5.6	8.2E−06	0.2	Unknown
YJL218W	5.2	8.6E−07	0.2	Unknown; expression induced by oleate
ZRT1 (*YGL255W*)	5.0	5.1E−06	0.1	Plasma membrane high-affinity zinc transporter
HEM13 (*YDR044W*)	4.8	2.3E−05	0.3	Enzyme of heme biosynthesis, repressed by Rox1 and HapI
TIR1 (*YER011W*)	4.4	6.3E−05	0.5	Cell wall mannoprotein, induced upon anaerobiosis
HXT9 (*YJL219W*)	4.4	3.2E−06	0.3	Putative hexose transporter
YGL262W	4.2	1.8E−06	0.4	Unknown
YOL014W	3.9	2.6E−07	0.1	Unknown
AGA1 (*YNR044W*)	2.8	2.1E−06	0.2	Anchorage subunit of a-agglutinin
THI11 (*YDL244W*)	2.6	5.6E−06	0.4	Protein involved in thiamine biosynthesis
ECM22 (*YLR228C*)	2.4	3.0E−07	0.4	Transcriptional activator of sterol biosynthetic genes
YCR102C	2.3	4.3E−07	0.4	Unknown; involved in copper metabolism
ATO3 (*YDR384C*)	2.3	3.4E−05	0.2	Plasma membrane protein, possible function in ammonia export
PHD1 (*YKL043W*)	2.2	1.1E−04	0.3	Transcriptional activator that enhances pseudohyphal growth
YAR068W	2.1	1.2E−07	0.3	Fungal specific protein
AGA2 (*YGL032C*)	2.0	1.0E−05	0.2	Adhesin subunit of a-agglutinin
SPS4 (*YOR313C*)	2.0	9.3E−05	0.3	Unknown; induced during sporulation
AAD4 (*YDL243C*)	2.0	7.6E−05	0.2	Putative aryl-alcohol dehydrogenase
BAR1 (*YIL015W*)	2.0	3.3E−05	0.3	Periplasmic aspartyl protease secreted by a-cells
Not regulated				
TIR4 (*YOR009W*)	15.3	3.2E−05		Cell wall mannoprotein, induced upon anaerobiosis
HUG1 (*YML058W*)	12.8	9.8E−06		Protein involved in DNA damage checkpoint, expression induced upon DNA damage
TIR3 (*YIL011W*)	9.2	2.5E−05		Cell wall mannoprotein, induced upon anaerobiosis
HMS1 (*YOR032C*)	8.6	3.4E−06		Basic helix-loop-helix protein, overexpression induces hyperfilamentous growth
YGR109W-A	6.0	3.5E−06		Transposable element gene
YML083C	4.7	3.7E−08		Unknown; expression strongly induced upon anaerobiosis
TIR2 (*YOR010C*)	4.3	2.4E−06		Cell wall mannoprotein, induced upon anaerobiosis
YIL082W-A	4.2	1.5E−06		Transposable element gene
PRM1 (*YNL279W*)	4.1	6.2E−08		Pheromone-regulated plasma membrane protein, localizes to the shmoo tip, regulated by Ste12
YGR109W-B	3.9	2.7E−06		Transposable element gene
PAU15 (*YCR104W*)	3.8	7.6E−07		Member of the seripauperin multigene family located in subtelomeric regions
SET4 (*YJL105W*)	3.6	3.7E−06		Unknown; contains a SET domain
PRM2 (*YIL037C*)	3.5	3.9E−07		Pheromone-regulated membrane protein, regulated by Ste12
PHO89 (*YBR296C*)	3.2	4.9E−07		Plasma membrane Na^+/Pi cotransporter
RTA1 (*YGR213C*)	2.8	2.0E−05		Membrane protein involved in 7-aminocholesterol resistance, expression induced upon anaerobiosis
SUT1 (*YGL162W*)	2.8	6.9E−05		Transcription factor involved in sterol uptake and the hypoxic induction of gene expression
SUR2 (*YDR297W*)	2.7	7.3E−05		Sphinganine C4-hydroxylase, catalyzes the conversion of sphinganine to phytosphingosine in sphingolipid biosynthesis
YHK8 (*YHR048W*)	2.7	1.9E−05		Presumed antiporter of the Dha1 family of multidrug resistance transporters
REE1 (*YJL217W*)	2.6	1.2E−06		Cytoplasmic protein involved in the regulation of enolase, expression induced by copper and calcium shortage
YCT1 (*YLL055W*)	2.6	4.7E−05		High-affinity cysteine transporter at the endoplasmic reticulum
GSC2 (*YGR032W*)	2.5	6.2E−06		Catalytic subunit of 1,3-beta-glucan synthase, involved in spore wall formation
PAU8 (*YAL068C*)	2.5	2.0E−06		Member of the seripauperin multigene family in subtelomeric regions
COS12 (*YGL263W*)	2.5	7.3E−07		Unknown; member of the DUP380 subfamily of conserved, often subtelomerically encoded proteins
PUT1 (*YLR142W*)	2.5	8.0E−05		Mitochondrial proline oxidase
DIF1 (*YLR437C*)	2.4	8.0E−07		Protein that regulates the nuclear localization of ribonucleotide reductase subunits
FUS1	2.4	2.0E−07		Membrane protein at the shmoo tip required for cell fusion, expression regulated by mating pheromone
PAU1 (*YIL176C*)	2.3	3.8E−06		Member of the seripauperin multigene family in subtelomeric regions
AUS1 (*YOR011W*)	2.3	1.1E−06		ATP-binding cassette transporter involved in sterol uptake and anaerobic growth
YAR066W	2.1	1.4E−05		Putative glycosylphosphatidylinositol protein
PRM9 (*YAR031W*)	2.1	1.8E−05		Pheromone-regulated membrane protein
IRC4 (*YDR540C*)	2.0	5.5E−05		Unknown
RSN1 (*YMR266W*)	2.0	1.1E−05		Membrane protein of unknown function, suppresses salt sensitivity of *sro7* mutants by restoring Ena1 localization to the plasma membrane

[a] Fold repression upon NaCl stress. Data are taken from the datasets of genomic transcription profiling published previously (11).

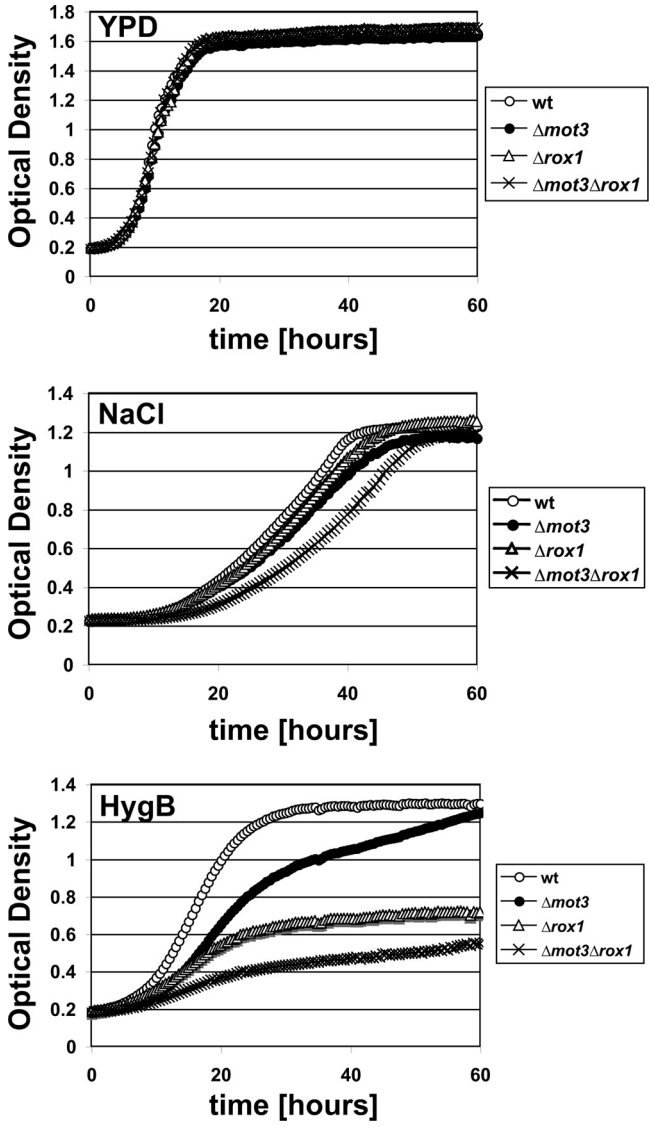

FIG 2 Mot3 and Rox1 are necessary for efficient growth upon ionic stress. The growth of the indicated strains was continuously recorded under normal conditions (YPD), salt stress (1.2 M NaCl), and in the presence of 60 μg/ml of the toxic cation hygromycin B. The growth curves are derived from three independent biological replicates with standard errors of <10%.

stream regulatory sequences up to bp −1000 of all genes represented in Table 2 except for the *GRE1* gene. The Mot3 binding motif (HAGGNW) is less restrictive, and we find multiple copies in all Mot3-activated gene promoters identified here.

Mot3 and Msn4 bind to a specific subset of osmoinducible genes. We next tested whether Mot3 bound directly to promoters of the subgroup of osmoinducible and Mot3-dependent genes identified here. We applied chromatin immunoprecipitation (ChIP) to a Mot3-myc-expressing yeast strain and detected significant binding at the *GRE1*, *AQY1*, and *SIP18* promoter regions. As shown in Fig. 5, Mot3 binding was absent from normal growth conditions and was rapidly induced upon NaCl stress in all cases. We next tested whether Msn2 and/or Msn4 bound to the same promoter regions using Msn2-HA- or Msn4-HA-expressing yeast strains. We obtained positive results only for Msn4, which was detectable by ChIP upon salt stress, similar to the Mot3 protein (Fig. 5). We concluded that both Msn4 and Mot3 target the same stress-inducible promoter regions within the *RTN2* cluster of osmoresponsive genes.

Mot3 function is required for efficient binding of Msn2 and Msn4 to stress-regulated promoters. We have shown that Mot3 and Msn2,4 are required for the induction of a specific subset of osmoinducible genes, and that at least Mot3 and Msn4 physically interact with some promoters of this group of genes. We next addressed the question of whether the Msn2 or Msn4 activator function was dependent on Mot3 in the small subset of osmostress genes positively regulated by Mot3. As shown in Fig. 6, we detected robust and transiently regulated binding of the Msn4 protein to 7 different promoters of this gene cluster upon salt shock. Significant binding of Msn2, however, could only be detected in the case of the *PHM8* gene promoter. We next repeated the ChIP analysis of Msn2 and Msn4 binding in strains lacking Mot3 function. As depicted in Fig. 6, in the absence of Mot3 function, the stress-induced binding of Msn2 and Msn4 was either severely diminished or, in most cases, completely lost. Therefore, we reasoned that Mot3 positively regulated the Msn2 and Msn4 general stress activators by favoring their binding to a subset of salt stress-inducible genes.

Repressor functions of Mot3 and Rox1 during the yeast osmostress response. As mentioned above, our transcriptomic analysis revealed that many genes are overexpressed in the absence of Mot3 and Rox1 function upon acute salt stress. As expected, we found many of those genes to be involved in heme and sterol metabolism and other functions regulated by oxygen. Specifically, we identify several members of the *TIR* and *PAU* gene family encoding cell wall mannoproteins and seripauperins previously known to be induced upon anaerobiosis. The expression of the majority of these genes is not affected by osmotic stress and is not further investigated here. Several genes which encode diverse transport functions of the cell were identified here to be overexpressed in the *mot3 rox1* mutant strain. These genes are *FET4*, *ZRT1*, *HXT9*, *ATO3*, *PHO89*, *YHK8*, *YCT1*, and *AUS1*, involved in iron, zinc, sugar, ammonia, phosphate, multidrug, cysteine, and sterol transport, respectively. In the case of the Fet4 alone, low-affinity iron permease, an oxygen-dependent regulation involving the Rox1 repressor function has been described previously (31, 32). Our results revealed a broad implication of Mot3 and/or Rox1 in adjusting transport activities mainly at the plasma membrane which might be important under salt stress conditions. The expression of the *FET4*, *ZRT1*, *HXT9*, and *ATO3* genes is strongly repressed upon NaCl shock (11). We quantified the expression of the *ZRT1* and *ATO3* genes along with salt shock by Northern blotting and compared the expression profiles of the wild type to the *mot3*, *rox1*, and *hog1* mutants. As shown in Fig. 7A and B, both genes were rapidly repressed upon salt stress in a wild-type strain. *ZRT1* transcript levels under normal growth conditions were increased in both *mot3* and *rox1* mutants and decreased with the salt shock, showing higher expression levels than those in the wild type. *ATO3* expression was similar without stress, but we observed a slower decrease of the *ATO3* transcript levels upon salt stress in the *mot3* and *rox1* mutants. These data confirmed a repressor function of both Mot3 and Rox1 at the *ZRT1* and *ATO3* genes. Mutation of both repressors did not abolish transcriptional inhibition of both genes upon stress, but we confirmed a contribution of Mot3 and Rox1 in the salt-induced repression of *ZRT1* and

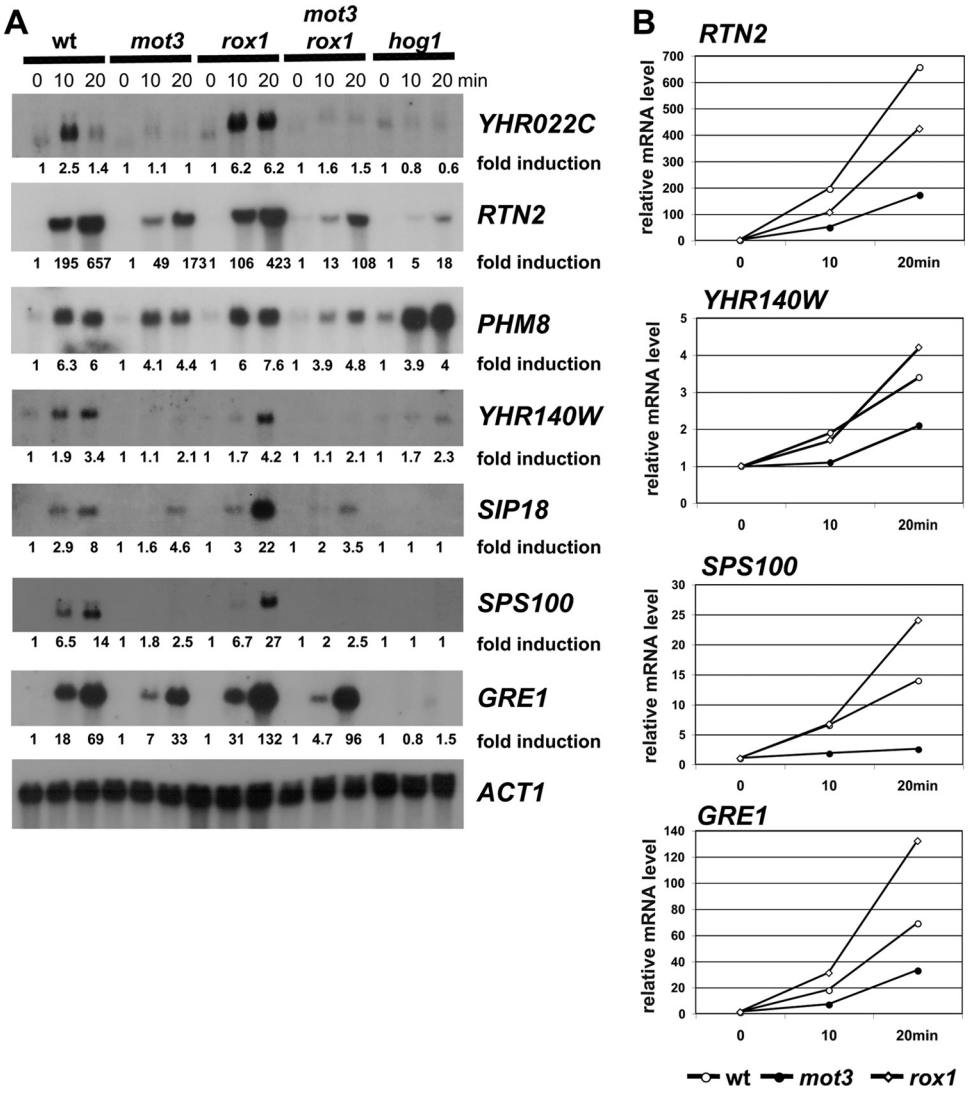

FIG 3 Mot3 is necessary for the activated expression of specific osmoinducible genes. (A) Expression analysis of the Mot3-dependent gene cluster. The indicated strains were grown in YPD and subjected to a brief osmotic shock (0.4 M NaCl for the indicated times). The expression levels of the specific genes were determined by Northern blotting. Below each panel the fold induction is given relative to the uninduced mRNA level. The *ACT1* messenger was used as an internal control. Representative blots are shown from at least two independent experiments with similar results. (B) Graphic representation of the expression levels of selected osmoinducible genes in yeast wild-type, *mot3*, and *rox1* mutant strains along with the osmotic shock caused by 0.4 M NaCl.

ATO3. The role of Hog1 in the negative control of both genes is different. While *ZRT1* repression was independent of the Hog1 MAP kinase, we observed a significant loss of repression of *ATO3* in a *hog1* mutant.

We additionally tested the salt stress-regulated expression of the *YJL218W* gene, which encodes a protein of unknown function and is overexpressed in a *mot3 rox1* mutant. We found that the expression of this gene was very regulated similarly to the mating-related genes. *YJL218W* expression was highly derepressed in *mot3* or *mot3 rox1* mutants, and although it was still negatively regulated upon NaCl treatment, it maintained elevated transcript levels during the salt shock.

Finally, we focused on the regulated expression of the *PHD1* gene, which encodes a central transcriptional activator of the developmental program for pseudohyphal growth in yeast (33). Our microarray analysis identified this gene to be under the negative control of the Mot3 and/or Rox1 factor. The Northern analysis shown in Fig. 7 demonstrated that the expression of *PHD1* was negatively regulated upon salt stress, and that both Mot3 and Rox1 act as repressors during salt treatment. In the *mot3 rox1* double mutant, we observed even higher *PHD1* transcript levels than those of the single mutants. The observed negative control of *PHD1* upon osmotic stress seemed to be regulated by the Hog1 kinase, since a *hog1* mutant strain showed almost constitutively high expression levels of *PHD1* upon NaCl shock.

Mot3 and Rox1 modulate the mating response upon stress. Another interesting group of genes with overexpressed transcript levels in the *mot3 rox1* mutant is related to the mating pheromone response. The mating-related genes were the functional group most significantly affected by the Mot3 and Rox1 repressors upon osmotic stress. *AGA1*, *AGA2*, *BAR1*, *PRM1*, *PRM2*, *FUS1*, *PRM9*, and *FIG1* belong to this gene cluster. Since the expression of

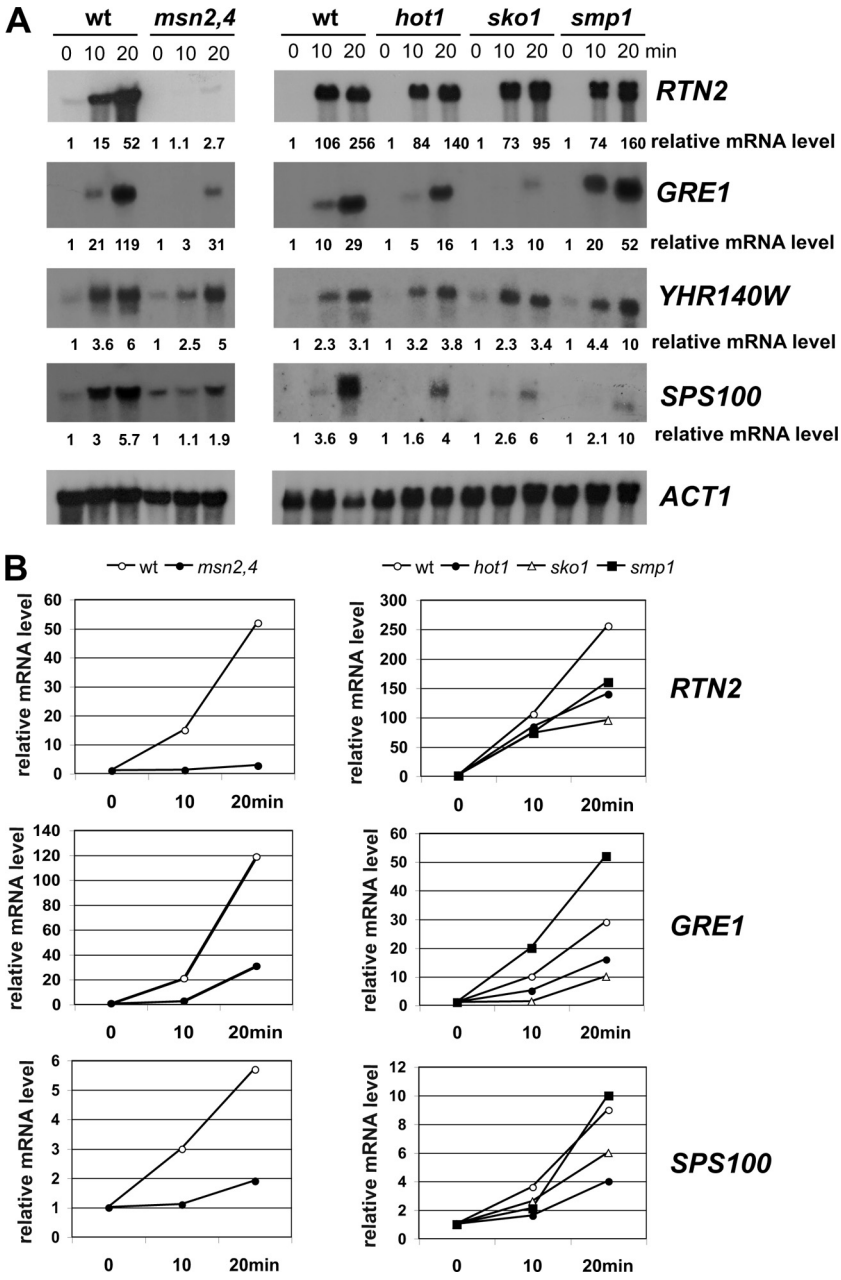

FIG 4 Mot3-dependent osmoinducible genes are regulated by the Msn2 and Msn4 activators. (A) Expression analysis of the indicated Mot3-dependent genes in yeast wild-type strain W303-1A and the *msn2 msn4* double mutant (left), as well as in yeast wild-type strain BY4741 and the *hot1*, *sko1*, and *smp1* mutants. The cells were grown in YPD and subjected to a brief osmotic shock (0.4 M NaCl for the indicated times). The expression levels relative to the *ACT1* control were determined as described for Fig. 2. (B) Graphic representation of the induction profiles for the *RTN2*, *GRE1*, and *SPS100* genes.

AGA1, *AGA2*, *BAR1*, and *FIG1* is strongly repressed by NaCl stress (11), we investigated the role of Mot3 and Rox1 in the transcriptional control of these genes. We analyzed by Northern blotting the expression levels of the *AGA1*, *AGA2*, and *FIG1* genes during salt shock and compared the wild type to the *mot3*, *rox1*, and *hog1* mutants. As shown in Fig. 7, the transcript levels of all three genes are reduced in response to salt stress. In the absence of Mot3, all three mating-related genes, and especially the *FIG1* gene, were overexpressed already under normal growth conditions. Although all three genes still showed negative regulation upon salt stress, we observed higher transcript levels during salt treatment for the *mot3* mutant than for the wild type. Rox1 only marginally, at the *AGA1* gene, contributed to the repression of the mating-related genes investigated here. We concluded that Mot3 is a major repressor of mating genes and that its function interferes with the shutdown of these genes upon salt stress. *AGA1*, *AGA2*, and *FIG1* were induced upon osmotic stress in a *hog1* mutant; however, this effect can be explained by the artificial activation of the mating pheromone pathway by salt in the absence of the Hog1 MAP kinase (34).

We tested whether the observed repression of these genes was the effect of direct binding of both factors to the respective pro-

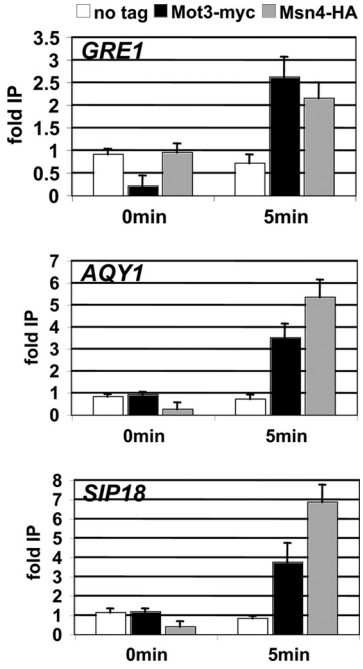

FIG 5 Mot3 and Msn4 bind to specific osmoinducible promoters. The association of Mot3-myc and Msn4-HA epitope-tagged proteins was determined by ChIP at the indicated promoter regions. Protein occupancy was calculated in each case relative to the unbound *POL1* control region. The no-tag parental strain is shown as a control. Cells were left untreated or were treated with 0.4 M NaCl for the indicated time. IP, immunoprecipitation.

moter regions. We tested by ChIP the association of Mot3 and Rox1 at the pheromone-regulated *AGA1* gene under nonstress and salt stress conditions. As shown in Fig. 7C, Mot3 and Rox1 bound to the *AGA1* promoter under normal growth conditions. The association of both factors was differently affected by salt stress. While Rox1 binding was lost at the *AGA1* promoter shortly after NaCl shock, Mot3 binding was still detectable under the same conditions.

We next addressed the question of whether Mot3 and Rox1 interfered with the induction of mating-related genes upon alpha factor treatment. We included in this study the *AGA1*, *AGA2*, *FIG1*, and *FUS1* genes and tested their transcriptional induction upon pheromone treatment in the respective transcription factor mutants. As shown in Fig. 8, the absence of either Mot3 or Rox1 leads to an overinduction of *AGA1* expression upon pheromone exposure compared to the wild type. A similar effect was observed for the *FUS1* gene; however, in this case an overstimulated expression was only detected in the *rox1* mutant or *mot3 rox1* double mutant. In the case of *AGA2* and *FIG1*, both genes were induced to similar levels independently of the presence of the Mot3 or Rox1 proteins. Finally, we aimed at establishing a link between the mating response and the Mot3 and Rox1 regulators upon stress. To this end, we quantified the mating response by the count of shmoo cells in response to α-factor exposure in the absence and presence of salt stress. As shown in Fig. 9, salt stress inhibited the formation of shmoos in wild-type cells. This inhibition was partially overcome in a *mot3* mutant and completely reverted in a *mot3 rox1* double deletion. These results indicated that Mot3 and Rox1 are important to repress the mating response under salt stress conditions by directly targeting specific mating-related genes.

DISCUSSION

In this work, we characterize the Mot3 transcription factor as an important regulator of the transcriptional program upon osmostress in yeast (Fig. 10). Mot3 plays both positive and negative roles in transcriptional regulation modulated by osmotic stress; therefore, it has to be considered a key regulator which acts coordinately with several other specific transcription factors, such as Sko1, Hot1, Smp1, Msn2, and Msn4. Here, we show that Mot3 is essential for the osmostress-activated expression of a specific subset of genes, the *RTN2* cluster. Apparently there is no unifying function within this gene cluster that is commonly activated by Mot3. Also, the function of these genes does not seem to be especially important for the resistance to hyperosmotic or salt stress, as we could not detect sensitive phenotypes in the respective mutants when we individually determined their growth rates on high-salinity media (data not shown). However, all genes of the *RTN2* cluster are directly targeted and regulated by the Msn2 and Msn4 transcription factors. Msn2 and Msn4 are pleiotropic activators in response to general stress and are known to bind and regulate many stress-responsive genes. Therefore, the genes identified here as coordinately activated by Msn2,4 and Mot3 represent a specific subset of Msn2,4-targeted genes. We experimentally confirm direct binding of Mot3 and Msn4 to three *RTN2* cluster genes; thus, we favor the idea that this gene cluster is generally directly regulated by the Msn2,4 and Mot3 factors. Importantly, Msn2 and Msn4 association with the *RTN2* cluster genes requires Mot3 function and suggests that Mot3 and the Msn2,4 proteins together form a transcriptionally active complex at this specific subset of stress genes. At these inducible genes, Mot3 and Msn2,4 are recruited upon osmostress. This is different from the Mot3- and Rox1-inhibited promoters, which are bound by the repressors under normal growth conditions.

It is important to note that a possible dual function for Mot3 as a repressor/activator has been suggested earlier based on the ability of Mot3 to activate gene expression from artificial reporter genes or by the reciprocal regulation of specific *CWP* genes in response to hypoxia (35, 36). Here, we confirm a dual activator/repressor function of Mot3 within the transcriptional response to hyperosmolarity. In this case, activation is achieved in cooperation with the general stress activators Msn2 and Msn4. Of note, other experimental data link Mot3 function to the osmostress response: expression of *MOT3* itself is stimulated by hyperosmotic stress through the HOG pathway (37), and the Sko1 transcription factor and Mot3 protein levels increase after osmotic shock (18).

According to the well-known repressor functions of both Mot3 and Rox1, we identify here many genes which are overexpressed under hyperosmotic conditions in yeast cells lacking Mot3 and Rox1 function. Gene products acting at the level of the cellular envelope are highly enriched among the Mot3- and Rox1-repressed genes. This suggests that both transcription factors contribute to the adjustment of cell wall and plasma membrane in response to salt and osmotic stress. A great number of different transport activities located at the plasma membrane are negatively regulated by Mot3 and Rox1, as revealed here by our transcriptomic analysis. Two specific genes of this transport-related cluster, *ZRT1* and *ATO3*, were closely investigated here, showing that both repressors, Mot3 and Rox1, contribute to the negative regu-

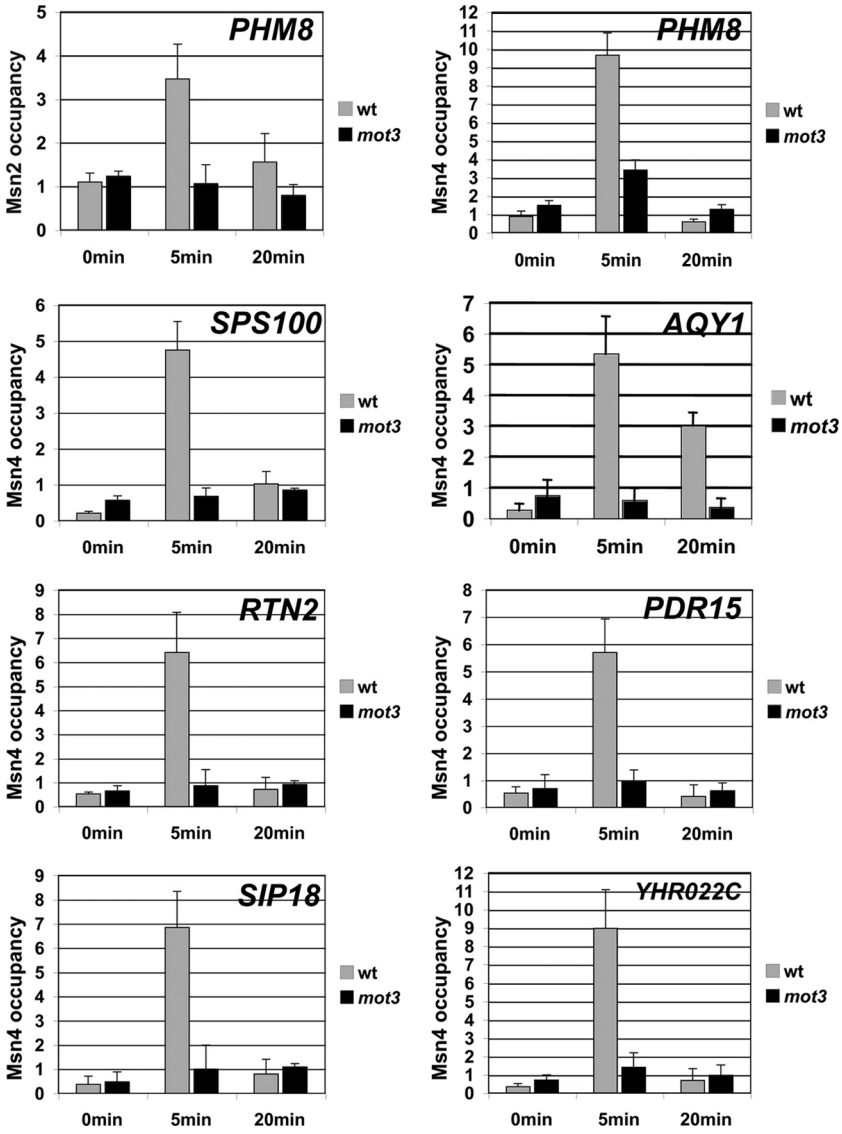

FIG 6 Msn2 and Msn4 binding to specific osmoinducible gene promoters depends on Mot3 function. Association of Msn2-HA and Msn4-HA epitope-tagged proteins was determined by ChIP in a wild-type or *mot3* deletion background. The strains were grown in YPD medium and then subjected to osmotic shock (0.4 M NaCl) for the indicated times. Protein occupancy was calculated in each case relative to the unbound *POL1* control region. Robust Msn2 binding was only detected at the *PHM8* promoter (upper left), while stress-induced Msn4 association was confirmed at all Mot3-activated gene promoters.

lation of these genes upon salt stress. Importantly, Mot3 and Rox1 seem to affect the transcription levels of these genes in general but not its stress-dependent shutdown. Accordingly, we detect Mot3 binding at several osmostress-repressed genes in the absence of stress (F. Martínez-Montañés, unpublished observations). We hypothesize that Mot3 and Rox1 are important to reduce specific transporter activities at the plasma membrane and that the defect in the *mot3 rox1* mutant contributes to the elevated sensitivity of this strain to diverse toxic cations reported recently (18). In line with this hypothesis is the observation that Mot3 and Rox1 functions are important to maintain low intracellular Na^+ concentrations during growth with elevated NaCl concentrations (18).

The sterol content of the plasma membrane is yet another important physiological determinant of salt stress resistance. We have recently shown that Mot3 and Rox1 repress specific *ERG* genes, such as *ERG2* and *ERG11*, involved in the biosynthesis of ergosterol in response to salt stress (18). Here, we identify more target genes repressed by Mot3 and Rox1 under acute salt stress which are related to sterol uptake and biosynthesis, such as *ECM22*, *RTA1*, *SUT1*, and *AUS1* (Fig. 7). This underlines the importance of the modulation of sterol synthesis as a way to efficiently deal with high-salinity stress. Plasma membrane sterols have important functions in adjusting membrane fluidity and are essential lipids to create specialized membrane domains or rafts (38, 39). Although to date we do not know how changes in the sterol composition of the membrane adapt specific transport activities to stress, we anticipate that the modulation of membrane rafts is highly regulated during the adaptation to salt and osmotic stress. Of note, we identify here the *SUR2* gene encoding a sphingolipid biosynthesis enzyme under negative control of Mot3

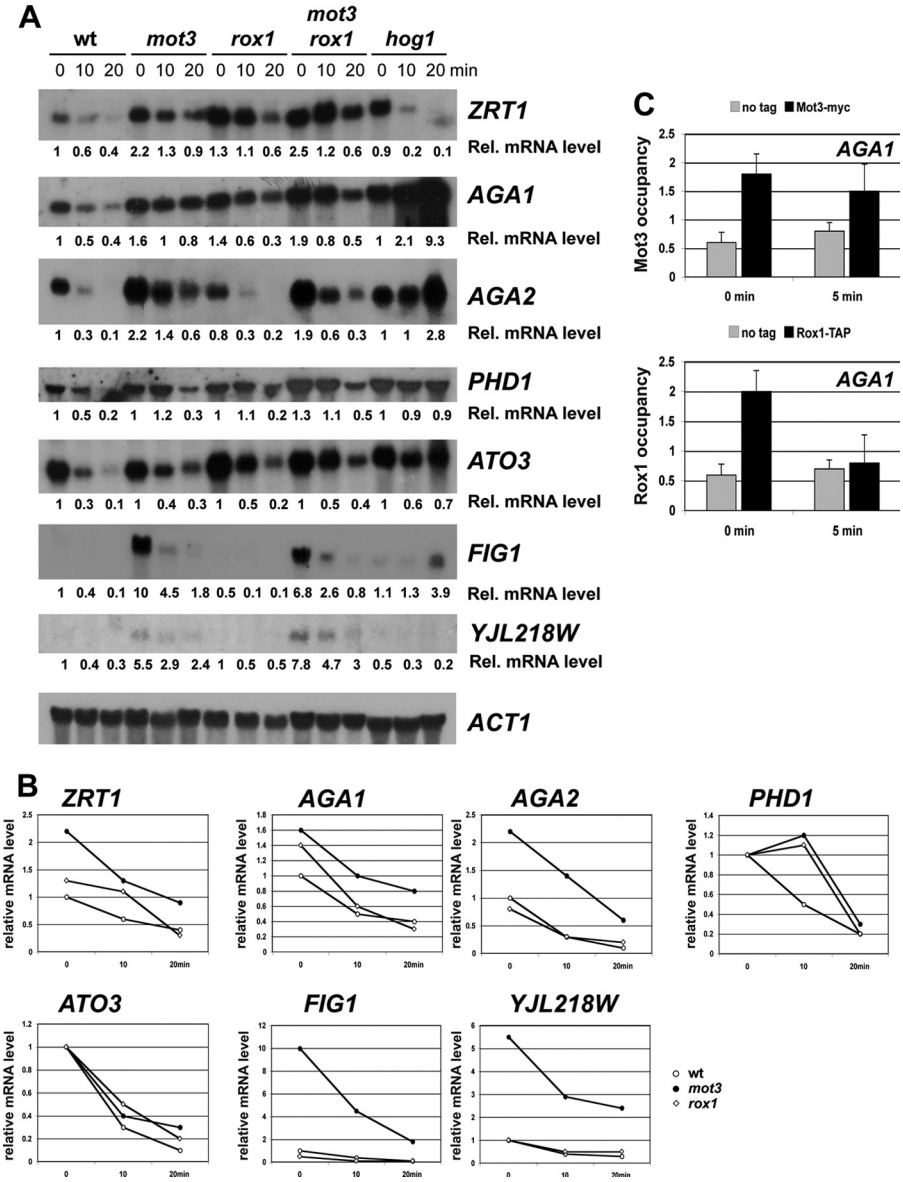

FIG 7 Expression analysis of osmostress-repressed genes targeted by Mot3 and Rox1. (A) The transcript levels of the indicated genes were determined by Northern blotting in the wild-type strain BY4741 and the *mot3*, *rox1*, *mot3 rox1*, and *hog1* deletion strains. Cells were grown in YPD medium and then subjected to osmotic shock (0.4 M NaCl) for the indicated times. The mRNA level was calculated for each gene relative (Rel.) to the *ACT1* control and was set to 1 for the wild type at time point 0. Representative blots are shown from at least two independent experiments with similar results. (B) Graphical representation of the different contribution of Mot3 and Rox1 to the repression of the osmostress-regulated genes tested here. (C) Mot3 and Rox1 binding to the *AGA1* promoter. Mot3-myc and Rox1-TAP epitope-tagged proteins were used to determine their association with the *AGA1* promoter by ChIP. Cells were left untreated or were treated with 0.4 M NaCl for 5 min. Protein occupancy was calculated in each case relative to the unbound *POL1* control region and is depicted in comparison to the no-tag wild-type control.

and/or Rox1. Since sphingolipids and sterols coordinately organize membrane microdomains (40), this implies that Mot3 and Rox1 are deeply involved in the homeostasis of membrane lipids regulated by stress.

Another biological process which appears to be shut down upon salt stress is the mating response. Eight different genes involved in the pheromone response are shown here to be under negative control of Mot3 and Rox1 (Fig. 10). Several of these genes are indeed heavily downregulated upon salt stress, such as the *AGA1*, *AGA2*, and *FIG1* genes. Here, Mot3 seems to be the main transcriptional repressor, and Rox1 only marginally contributes to their negative regulation. It is important to note that the essential cellular processes during yeast mating, such as shmoo formation and membrane fusion, depend on a normal membrane sterol composition, and several mutants in the ergosterol biosynthesis pathway have been characterized with mating defects (41–44). On the other hand, Mot3 and Rox1 downregulate sterol biosynthesis in response to salt stress, which might be incompatible with efficient mating. Therefore, the observed changes in gene expression might help adapt the membrane lipid composition and at the same time disable the mating process during the exposure to salinity stress. In line with this model, we observe that inhibition of

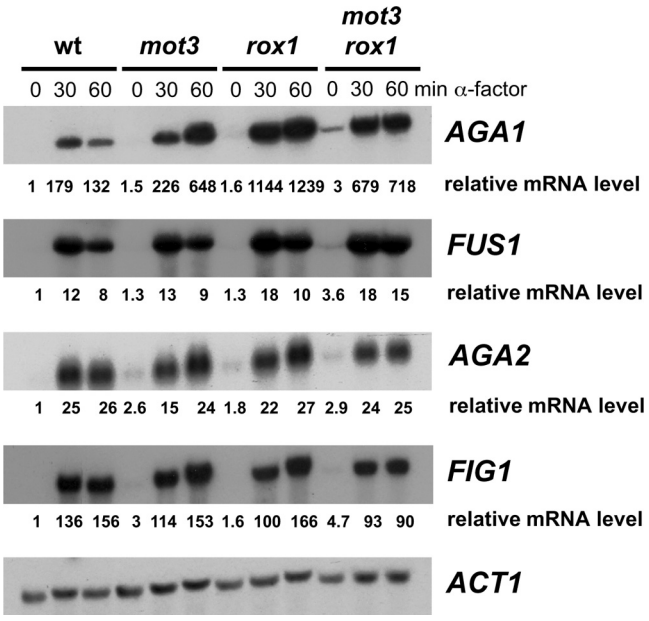

FIG 8 Loss of Mot3 and Rox1 function leads to overinduced gene expression of *AGA1* and *FUS1* in response to α-factor. The expression of the indicated pheromone-responsive genes was induced by α-factor treatment as described in Materials and Methods in yeast wild-type strain BY4741 and the *mot3*, *rox1*, and *mot3 rox1* deletion strains. The mRNA level was determined by Northern analysis, calculated for each gene relative to the *ACT1* control, and was set to 1 for the wild type at time point 0.

the mating response by salt stress can be suppressed by deletion of the Mot3 and Rox1 repressors. This is in agreement with the recently published observation that the mating response is suspended during the adaptation to osmotic stress (45). This is achieved in part through Hog1-mediated inhibition of Fus3, the MAP kinase of the mating pathway (45). Therefore, the shutdown of several genes necessary for mating during osmostress with the help of the Mot3 repressor is yet another layer of regulation to delay mating in favor of stress adaptation.

Taken together, our study reveals that the Mot3 transcriptional

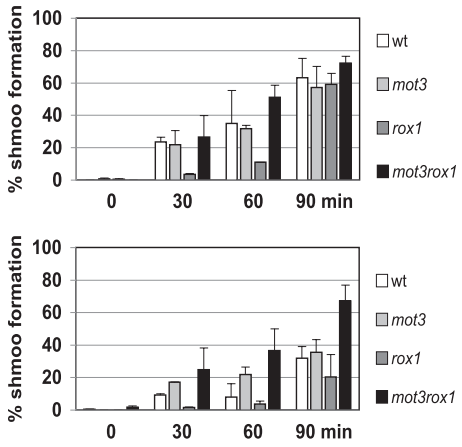

FIG 9 Mating response is inhibited by salt stress dependent on Mot3 and Rox1 function. The appearance of shmoo cells was determined in the indicated yeast strains after α-factor induction in the absence or presence of 0.4 M NaCl.

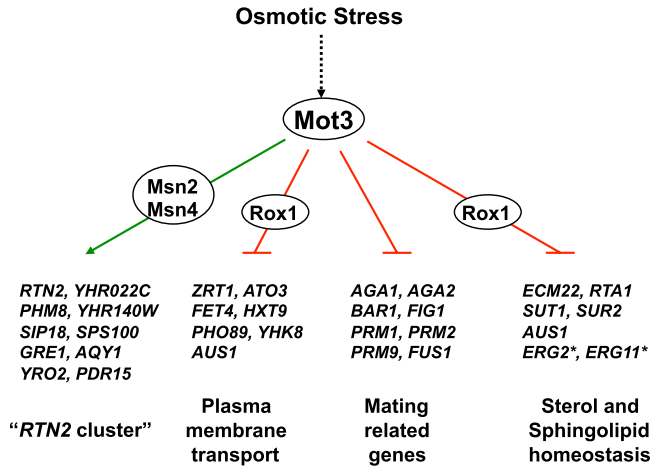

FIG 10 Overview of Mot3-regulated genes upon hyperosmotic stress. Mot3 contributes to the activated expression of a specific gene cluster (*RTN2* cluster) together with the Msn2 and Msn4 factors. Mot3 and Rox1 negatively regulate genes encoding plasma membrane transporters and genes involved in the biosynthesis and turnover of sterols and sphingolipids. Mating-related genes are repressed by Mot3 largely independently of Rox1. Genes marked with an asterisk were identified previously (18).

regulator has complex functions, both as a repressor and an activator, in the cellular adjustment to osmotic stress. Our study places Mot3 among the growing group of transcription factors involved in the osmostress response. Its functional overlap with the Msn2 and Msn4 activators at specific stress loci is an example for the complex interactive network operating upon osmostress which might be further unraveled in the future.

ACKNOWLEDGMENTS

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