universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Visualisation of stress response pathways in yeast *Saccharomyces cerevisiae* strain for the integrative study using fluorescent *in vivo* assays

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Magister/Magistra der Naturwissenschaften (Mag[a]. rer.nat.)

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SUMMARY

Among the most interesting discoveries in molecular and cellular biology are signal transduction cascades triggered by extracellular stimuli, carrying signals from the external membrane all the way into the cell nucleus. It enables cells to sense environmental changes and to respond by changing their transcriptional activity. One of the most prominent cascades, the Mitogen-Activated Protein Kinase cascade (MAPK), regulates diverse biological processes from proliferation and differentiation to apoptosis. The budding yeast *Saccharomyces cerevisiae* has at least five MAPK cascade signal pathways: the cell-wall integrity pathway, the spore wall assembly pathway, the filamentous/invasive growth pathway, the pheromone response pathway, and the high osmolarity glycerol pathway.

In the past, signal transduction was usually explained as a linear connection between receptors and regulators of gene expression. Today, signalling pathways are observed as a network of pathways interacting with each other. To understand the complex behaviour and cross-talk of signalling networks, we propose to decipher the quantitative interaction between different stress response pathways in *S. cerevisiae*. Therefore, five different stress-specific reporter genes were integrated into the yeast genome and measured corresponding promoter activity upon stimulation by various triggers.

ZUSAMMENFASSUNG

Eine der interessantesten Entdeckungen der Molekular- und Zellbiologie waren die Signaltransduktionskaskade welche, initiiert durch einen extrazellulären Stimulus, Signale von der Zellmembran bis in den Kern leiten. Der Zelle wird damit ermöglicht, Veränderungen in der Umgebung wahrzunehmen und durch transkriptionelle Anpassung darauf zu reagieren. Einer der wichtigsten Transduktionswege ist dabei der „Mitogen-Activated Protein Kinase (MAPK) Pathway“. Dieser reguliert zahlreiche zelluläre Prozesse von Stressantwort über Proliferation bis hin zur Apoptose. In der Bäckerhefe *Saccharomyces cerevisiae* wurden bisher fünf MAPK Signalwege beschrieben: der „Cell-wall integrity“-, der „Spore wall assembly“-, der „Filamentous/invasive growth“-, der „Pheromone response“-und der „High osmolarity glycerol“-Signalweg.

Bis vor nicht kurzem wurden Signaltransduktionwege als lineare Verbindungen zwischen Rezeptoren und Regulatoren der Genexpression angesehen. Doch heutzutage weiß man, dass diese als integrative Netzwerke miteinander verbinden sind. Um diese komplexen Zusammenhänge besser zu verstehen, haben wir uns zum Ziel gemacht, die quantitativen Interaktionen der fünf Signalwege die an der Stressantwort in *S. cerevisiae* beteiligt sind,zu untersuchen. Dafür wurden fünf verschiedene, stress-spezifische Reportergene in das Hefegenom integriert und die korrespondierenden Promotoraktivitäten zum Zeitpunkt unterschiedlicher Stressstimulationen erfasst.

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I. INTRODUCTION

I.1. General features of stress response

The power to adapt to environmental changes is a universal precondition for survival and evolution. Yeast consistently resists changing availability of nutrients, temperatures, osmolarity and acidity in their environment. Likewise, the presence of toxic agents is readily tolerable. Once environmental conditions change, cells must quickly adjust their expression program to adapt to these new conditions, as they affect growth. The presence of molecular mechanisms of response, repair and adaptation, which are greatly conserved across nature, provide the cell with the plasticity to adjust to its environment (Ruis and Schueller 1995). Through the sensing and transduction of the stress signals into the nucleus, a genetic reprogramming and response occurs. On the one hand, it decreases expression of housekeeping genes, and, at the same time, drives increased expression of stress-adaptor genes. These genes encode molecular chaperones responsible for maintaining protein folding; transcription factors that further modulate gene expression, as well as membrane transporters and proteins involved in different cell metabolism and growth control (Moskvina et al. 1999). Although specific stress conditions trigger distinct cellular responses, a specific set of signature genes representing a common denominator to all environmental stress responses are also modulated (Estruch 2000).

The ability of yeast cells for sensing and responding to complex environmental variations is only beginning to become explored. Genes whose transcription is responsive to a variety of stresses have been implicated in a general yeast response, while there are also genes, which appear to be specific to particular

adverse stress conditions (Gasch et al. 2000, Mager and De Kruijff 1995, Ruis and Schueller 1995).

I.2. Signalling pathways

One of the most exciting findings in molecular and cell biology has been the discovery of signalling cascades. Signalling pathways enable cells to sense changes in their environment and to respond to them by changes in transcriptional activity (Toone and Jones 1998). Among the most important cascade in eukaryotic cells is the Mitogen-Activated Protein Kinase (MAPK) cascade (Marshall 1994). MAPK pathways regulate diverse processes, ranging from general stress response to proliferation, differentiation and even to apoptosis (Figure 1).

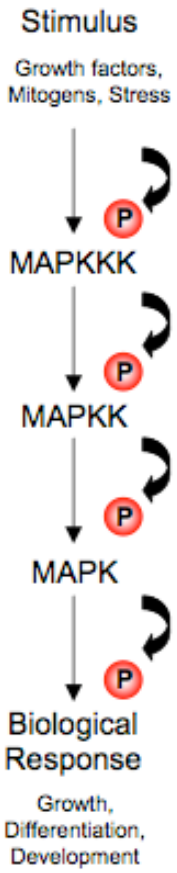


Figure 1. Schematic diagram of MAPK signaling pathway in *S. cerevisiae*. The hierarchical MAPK pathway transmits a chain reaction signal. Extracellular signals activate MAPK kinase kinase (MAPKKK), which upon activation phosphorylates two serine or threonine residues on its target MAPK kinase (MAPKK). Activated MAPKK phosphorylates further MAPK. Courtesy of O.Majer

Each MAPK cascade is activated through transitions of a signal, either by a small GTP-binding protein or by lipids (e.g. Ste20, PKC) (Marshall 1994). The core cascade is usually composed of MAPKKK, MAPKK and MAPK. The stimulus triggers a signal such as phosphorylation, which can be passed on directly to a MAPKKK or through a mediator kinase (MAPKKKK). Hence, the signal is transmitted down

the cascade by enzymes, which are referred to as MAP kinases (MAPKK), culminating in the phosphorylation of the key MAPK. The existence of three or more levels in each of the MAPK pathways is probably essential for signal amplification, specificity determination and tight regulation of the transmitted signal. Pathway specificity itself is regulated at several levels: kinase-kinase and kinase-substrate interactions, co-localisation of kinases by scaffold proteins, and inhibition of output by the MAPK themselves (Gustin et al. 1998).

In addition to MAPK cascade, other signalling pathways exist in cells, including those involving G-protein-linked receptors (Hao et al. 2007). G-protein-linked receptors belong to the cell-surface receptor family and are found in all eukaryotes. They mediate the responses to an enormous diversity of signal molecules. Recognition by a receptor triggers a conformational change and activates GTP-binding proteins (G-proteins). In turn, G-proteins can either activate or inhibit enzymes that generate specific second messengers. Some G-protein-linked receptors change the intracellular concentration of the mediator cyclic AMP (Hirata et al. 1995). Others increase the Ca^{2+} concentration in the cytosol that affects cells mainly by stimulating Ca^{2+}/calmodulin-dependent protein kinases (Hirata et al. 1995).

I.2.1.Yeast MAPKs

Genetic and biochemical studies in yeast revealed five MAP kinase pathways encoded in the *S. cerevisiae* genome (Errede et al. 1995, Gavrias et al. 1996) triggered by extracellular signals (Figure 2), such as pheromones, starvation, high osmolarity, hypotonic shock, and carbon/nitrogen deprivation (Hunter and Plowman 1997). Each of these MAP kinases mediates specific cellular responses to cell-wall damage, spore wall assembly, filamentous/invasive growth, mating pheromone response, and high osmolarity (Posas et al. 1998).

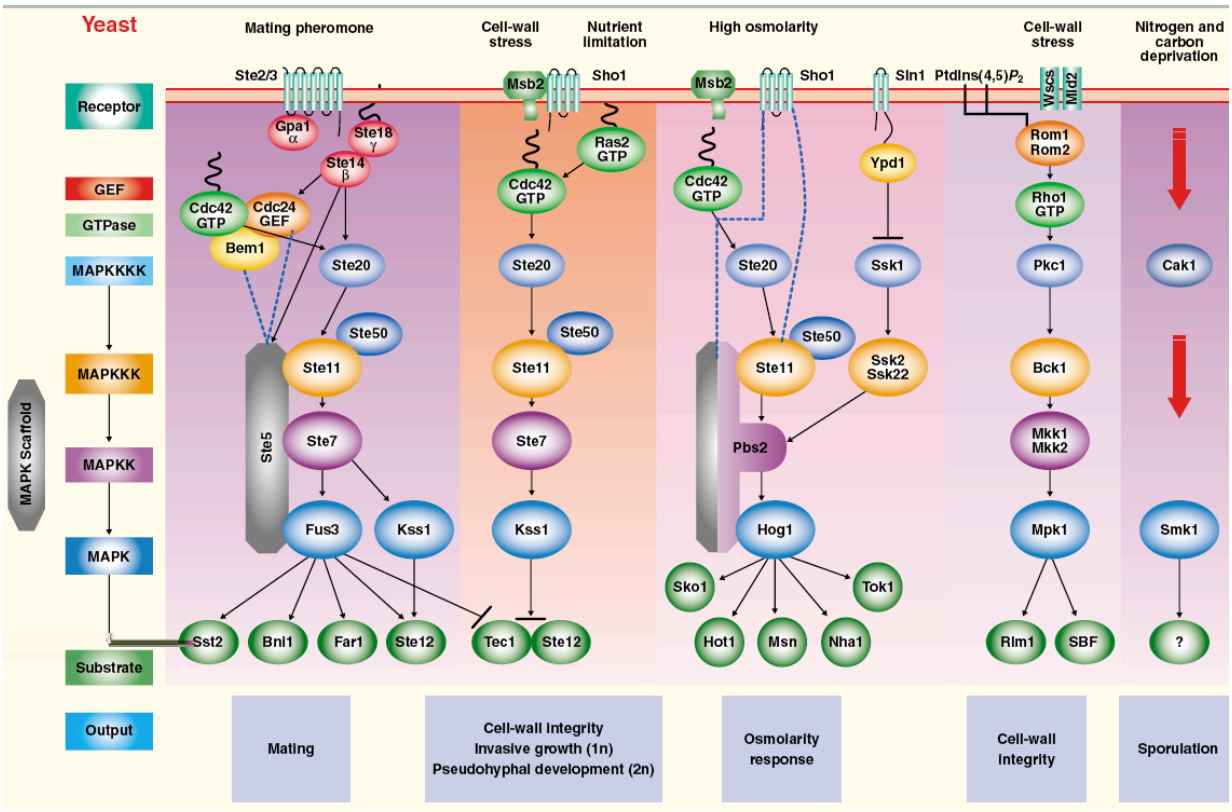


Figure 2: **MAPK pathways in yeast, *S.cerevisiae***. There are five MAPK pathways in yeast. Each cascade has a unique MAPK (dark blue). In addition, some protein kinases act in more than one pathway: the MAPKK Ste7 (pink) in two pathways (mating pheromones and cell-wall stress), the MAPKKK Ste11 (orange) and the upstream MAPK cascade activator kinases Ste20 and Ste50 (light blue) in three pathways (mating pheromones, cell-wall stress and high osmolarity) taken from (Qi and Elion 2005).

I.2.2. The yeast high osmolarity glycerol pathway

Increasing external osmolarity is a common stress for a yeast cell in a variety of environments (e.g. a split-open grape drying under the sun, a agar plate left open in the incubator, or fermentation start after sugar is added). Hyperosmotic stress causes extrusion of water from the cell resulting in cell shrinking, loss of cell pressure and increased concentration of cellular solutes. Conversely, hypo-osmolar

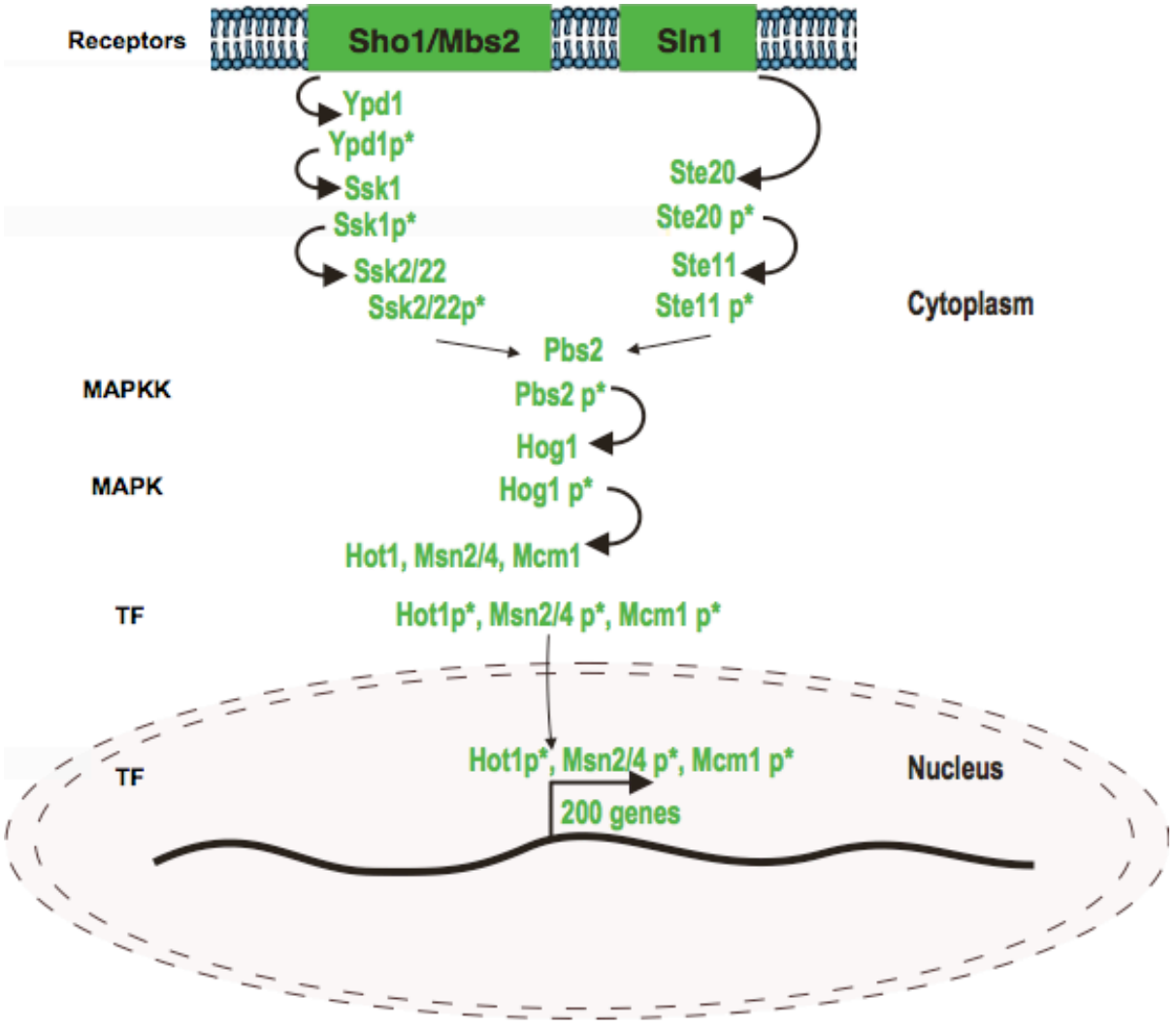


Figure 3: Schematic illustration of the HOG pathway of *S. cerevisiae*. Two independent upstream branches of the HOG pathway can each activate Pbs2 and Hog1. The Sho1 branch regulates the activity of the two MAPKKKs, Ssk2 and Ssk22. The Sln1 branch recruits Pbs2 to the membrane, after which Ste20 phosphorylates the MAPKKK Ste11. Under osmostress, activated Pbs2 activates the Hog1 MAPK, which induces a set of osmoadaptive responses. p* indicates Thr/Tyr phosphorylation events; see text for details.

environment allows the water movement into cells causing cell swelling, high pressure and diluted intracellular milieu (Blomberg and Adler 1992, Hohmann 2002). To counteract these adverse effects, cells actively accumulate or reduce osmolytes (e.g. glycerol and trehalose), which protect cells against the effects of osmotic variations by altering the intracellular osmotic pressure (Reed et al. 1987, Yancey et al. 1982). Therefore, many changes in gene expression upon osmotic challenges are dedicated to altering the metabolism and cell permeability to these compounds.

In yeast, two incoming and partially redundant branches of high osmolarity glycerol (HOG) pathway are activated upon increase of external osmolarity (Maeda et al. 1995). One branch is fed by the predicted membrane protein Sho1 and the MAPKKK Ste11. The other upstream branch of the HOG pathway contains a three-component signalling protein complex composed of the putative Sln1 sensor, Ypd1 and Ssk1. These three proteins regulate the function of the closely related and partially redundant MAPKKK Ssk22 (Bender and Pringle 1991). Thus, two independent MAPKKK, Ssk22 and Ste11 (Maeda et al. 1995), can activate a single downstream MAPKK, Pbs2 (Belazzi et al. 1991, Maeda et al. 1994). Upon Pbs2 phosphorylation, Hog1 kinase is activated through Thr／Tyr phosphorylation, promoting its rapid translocation into the nucleus and increasing its kinase activity (Proft et al. 2001). Hog1 interacts with Msn2⁄4 (Posas and Saito 1997), and Hot1 and Msn1 (Reiser et al. 1999) that cause signal amplification, often with overlapping functions, leading to modulation of target gene expression (Gasch et al. 2000).

I.2.3. The yeast cell wall integrity pathway

Another very important MAPK cascade operating in yeast is the cell wall integrity pathway. In yeast, cell wall integrity is necessary for proper maintenance of cell shape (Cid et al. 1995, Klis 1994), budding, as well as for communication with the exterior. Thus, yeast must remodel cell wall rigid structures to accommodate cell expansion during vegetative proliferation, mating pheromone-induced morphogenesis or nutrient-induced filamentation. The cell wall also responds to several external stress stimuli (e.g. temperature, damage, changes in external osmolarity, mating pheromone and various drugs). Signalling proteins on the cell wall integrity (PKC) pathway (Figure 4) include the GTP binding protein Rho1 (Kamada et al. 1996, Nonaka et al. 1995), the protein kinase C Pkc1 (Li et al. 1992), the MAPKKK Bck1 (Lee and Levin 1992), the pair of MAPKKs Mkk1 and Mkk2 (Irie et al. 1993), the MAPK Slt2 (Torres et al. 1991), and the transcription factors Rlm1 (Dodou and Treisman 1997, Watanabe et al. 1995) and Swi4/6 (Dirick et al. 1992).

S. cerevisiae Pkc1 protein was identified by its homology to proteins from the PKC family in animal cells (Levin et al. 1990). Cells lacking Pkc1 lyse with a small bud, and this phenotype is partially suppressed by an osmotic stabilizer, suggesting a defective cell wall (Paravicini et al. 1992). It is activated directly by Rho GTPases (Kamada et al. 1995, Nonaka et al. 1995) through phosphorylation of a conserved threonine residue within the activation loop of the essential kinase domain. Rho1 plays a key function in this pathway since is a positive regulator of the (1,3) β-D-

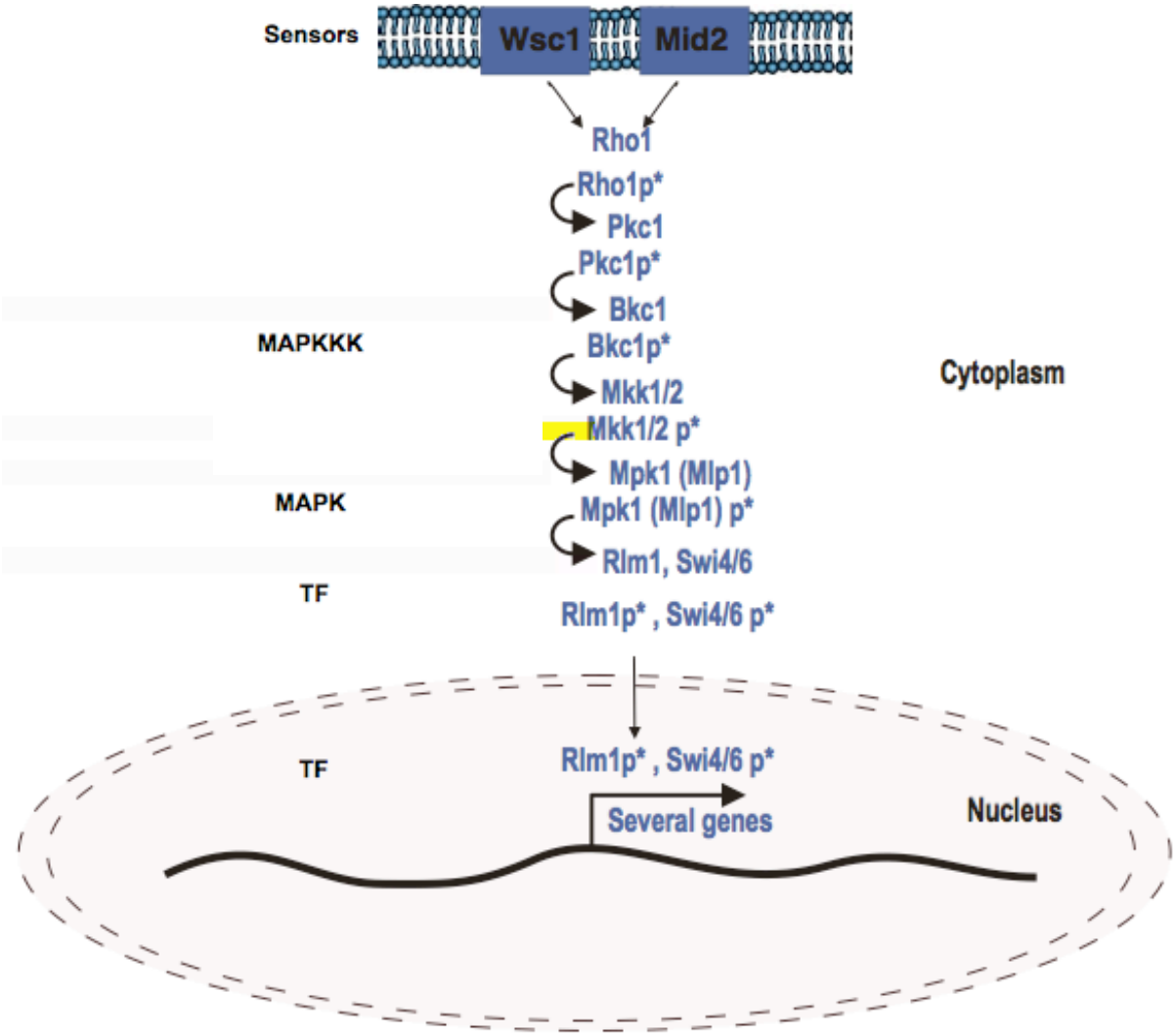


Figure 4: Cartoon describing the cell integrity pathway of *S. cerevisiae*. This pathway is regulated by several different signals (e.g. temperature, osmolarity, pheromone, and drugs). Signals are initiated through the putative cell surface sensors Wsc1 and Mid2. The sensors stimulate Rho1 that activates Pkc1, which in turn activates the MAP kinase cascade comprised of Bck1, Mkk1/2, and Mpk1. Two transcription factors, Rlm1 and the SBF complex (Swi4/Swi6), induce a set of cell-wall integrity response. p* indicates phosphorylation events; see text for details.

glucan synthase, responsible for the synthesis of the *S. cerevisiae* main cell wall component. A family of membrane proteins, Wsc1-4 and Mid2, sensing cell wall homeostasis and remodelling required for vegetative growth. In addition, Mid2 is implicated driving the mating process to ensure cell wall integrity during projection formation (Rajavel et al. 1999, Verna et al. 1997). Wsc1 and Mid2 interact and activate Rho1 through a GDP exchange factor. The main target of Pkc1 activation is the MAPK cascade module consisting of Bck1, Mkk1 and Mkk2, and Slt2/ Mpk1 (Figure 4). Rlm1 and SBF are the main downstream transcription factors responsible for Slt2/Mpk1 mediated cell wall regulation (Iyer et al. 2001). Mutants in the MAPK signalling cascade undergo cell lysis because of a deficiency in cell wall biogenesis and remodelling (Heinisch et al. 1999).

I.2.4. The yeast mating response pathway

Yeast cells are by default haploid. Haploid cells of the opposite mating type (**a** /α) can mate and form a diploid cell. This process is stimulated by the release of mating pheromones, **a**-factor (from *MAT***a**) and factor α (from *MAT*α) that act on cells of the opposite mating type (Wang and Dohlman 2004). Cellular responses to mating pheromones include polarized growth toward a mating partner, cell cycle arrest in G1, increased expression of proteins needed for cell adhesion, cell fusion, and nuclear fusion (Chang and Herskowitz 1990). Furthermore, pheromone activates a signalling MAPK cascade pathway (Figure 5) that upon recognition by receptors activates downstream MAPK cascade. The α-factor pheromone binds to the Ste2 receptor and **a**-factor to Ste3, respectively. These activates Ste5 and Ste20 proteins,

and in turn stimulate the Ste11-Ste7-Fus3 cascade (Cairns et al. 1992, Stevenson et al. 1992). The MAPK Fus3 phosphorylates several downstream targets, among them the Ste12 (Chang and Herskowitz 1990) that mediate various responses required for successful mating. *FUS1* is one of the most highly induced genes in yeast (McCaffrey et al. 1987, Trueheart et al. 1987).

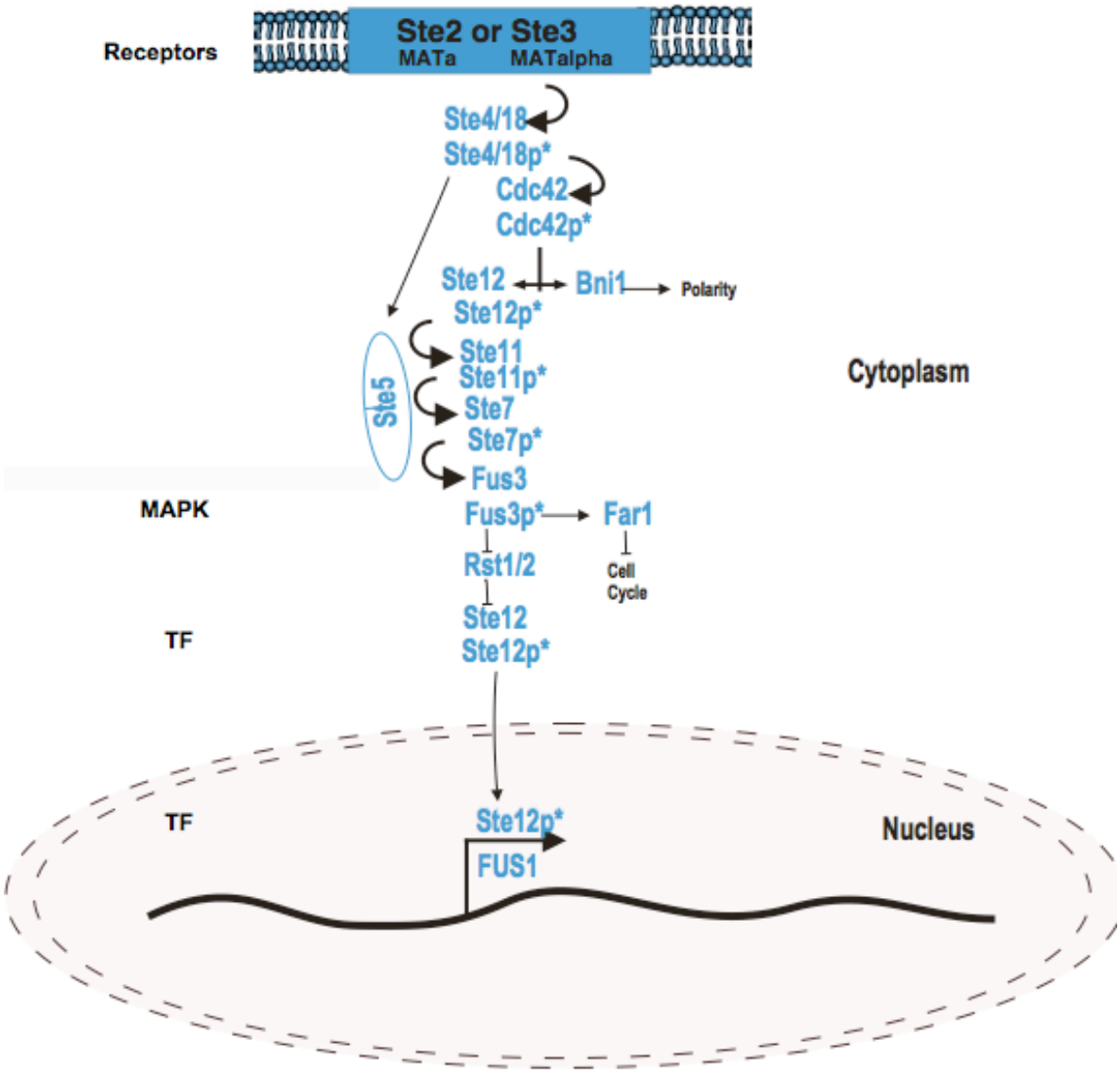


Figure 5: Signal transduction of the mating pathway to adaptation to pheromone stress. Both, Ste2 and Ste3 are receptors in a cell membrane for a *MAT***a** and *MAT*,α respectively, TF transcription factor. p* activated kinases.

I.2.5. The yeast response to extracellular pH

Environmental pH has dramatic effects on cells (e.g. at the plasma membrane, on protein activity, maintenance of the proton gradient, and nutrient availability). The ability of yeast to respond to alkaline environments is mediated by conserved signal transduction pathways, which control pH responses through the activity of zinc finger transcription factor Rim101 (Su and Mitchell 1993b), through its proteolytic activation (Figure 6). At acidic pH, Rim101 is found predominantly in the full-length (non-functional) form, whereas at alkaline pH, it is found predominantly in the processed (active) form (Li and Mitchell 1997, Orejas et al. 1995). Proteolytic processing involves removal of the C-terminal D/E-rich domain, involving the activities of several upstream gene products (e.g. Rim13, Rim20, Rim8, Rim21, and Rim9) (Su and Mitchell 1993a). Rim13 has homology to proteases and is thought to be the protease processing Rim101. Rim20 interacts with the C-terminal domain of Rim101 and may act as a scaffolding protein to bring Rim13 into position to allow for cleavage of full-length Rim101 (Xu and Mitchell 2001).

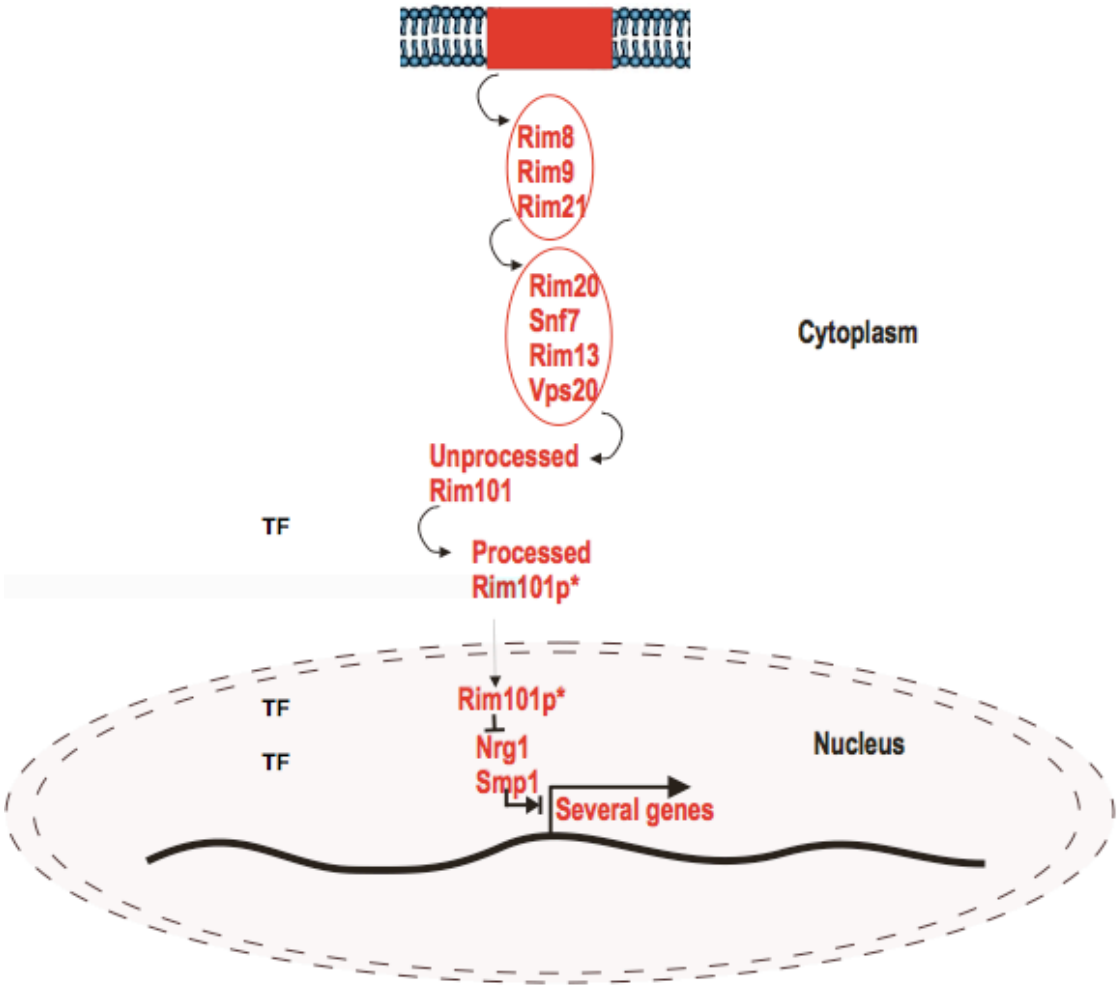


Figure 6: Signal transduction of the RIM pathway as adaptation to the alkaline pH stress. Not a MAPK pathway. TF;transcription factor. Rim101p* as activated repressor that in turn repressing another repressor activate gene expression.

The function of *S. cerevisiae* Rim101 is mediated by the repression of two transcriptional repressors, *NRG1* and *SMP1* (Lamb and Mitchell 2003). Nrg1 is a transcriptional repressor that interacts with the global repressor Tup1-Ssn6 (Park et al. 1999). Finally, environmental pH serves as a potent stimulator of the Rim101 pathway. However, the nature of the triggering mechanism remains to be elucidated.

I.2.6. The yeast response to calcium

Nearly all eukaryotic cells use calcium-mediated signalling mechanisms to regulate a wide variety of cellular processes, as Ca^{2+} acts as a vital second messenger in many signal transduction events. The budding yeast maintains cytosolic free Ca^{2+} concentrations at extremely low levels. However, extreme gradients of

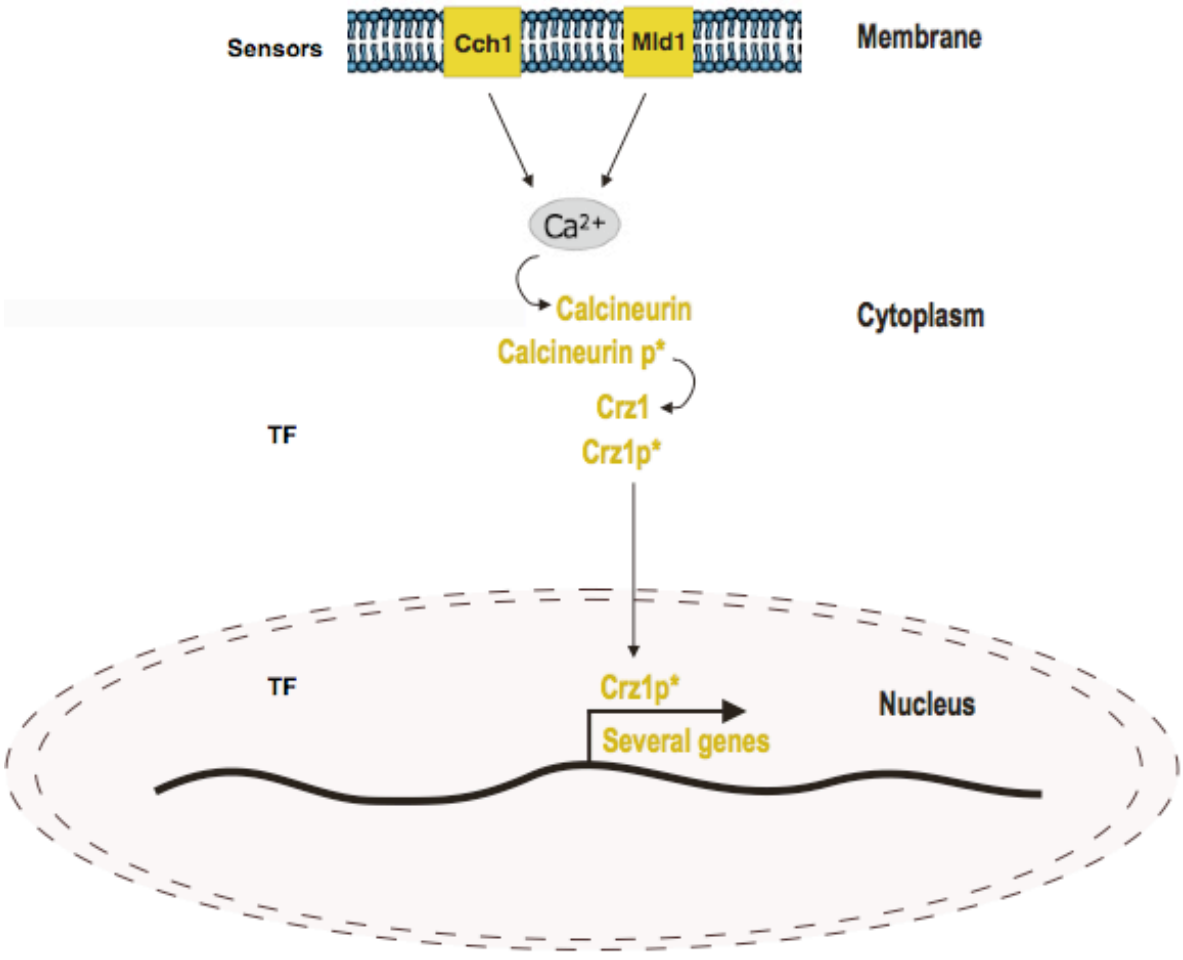


Figure 7: Signal transduction of a pathway corresponding to an adaptation to the calcium stress. This is not a MAPK pathway. TF = transcription factor. Calcineurin p* and Crz1p* present activated enzymes.

Ca^{2+} ion across the plasma membrane and across intracellular membranes exists. Transient increases in cytosolic calcium results in the activation of the phosphatase calcineurin (Stathopoulos and Cyert 1997). In budding yeast, calcineurin dephosphorylates Crz1/Tcn1 (Figure 7), a zinc-finger transcription factor, resulting in Crz1 translocation into the nucleus, which in turn regulates expression of numerous genes (Stathopoulos and Cyert 1997).

Calcineurin itself is a heterodimer of catalytic and regulatory subunits (Nakamura et al. 1993). Cells lacking catalytic or regulatory subunit lack calcineurin activity (Nakamura et al. 1993). Exposure of yeast cells to a number of stimuli or other cellular conditions (e.g. mating (Iida et al. 1990), glucose (Nakajima-Shimada et al. 1991), NaCl (Hirata et al. 1995, Mendoza et al. 1994), or cell wall remodelling (Garrett-Engele et al. 1995) trigger the increase of cytoplasmic calcium. This calcium increase results in activation of calcineurin, and is a consequence of external calcium influx or the release from internal stores.

I.3. Cross-talk of signalling pathways (MAPK-sharing)

Activation of intracellular signalling proteins (Ste20, Ste11, Ste7, and Ste12, See Figure 2) in different cells can lead to similar or different cellular responses. Likewise, activation of different MAPK by different stimuli (Yashar et al. 1995) may cause pathway-overlapping activities. The mechanisms controlling response specificity of MAP kinases are still poorly understood, but it is clear that a cross-talk must be tightly regulated. For instance, the MAPKKK Ste11 act in signalling pathways that regulate mating, filamentous growth and osmoregulation (Posas and

Saito 1997). Nevertheless, each pathway activates only one downstream MAPK: Fus3 in the mating pathway, Kss1 in the filamentation pathway and Hog1 in the osmoregulation pathway. Another MAPKK, Ste7, can trigger different MAPK from separate pathways (Elion 2001). Given this sharing of components among different MAPK pathways, raises the question how the specificity of the responses to particular signals is maintained? Recent studies of the MAP kinase pathways in yeast suggest possible answers to this question: scaffold protein can increase the specifity of the kinase cascade by bloking inappropriate interactions with other related or unspecific kinase cascades. Ste11 can activate Hog1 through the Pbs2 (Ferrell and Cimprich 2003) scaffold to the plasma membrane sensor Sho1 (Sprague 1998). Ste5 and Pbs2 provide specificity by segregating shared kinases with pathway-specific kinases and receptors that sense stimuli. Ste5 allows separate binding sites for Ste11, Ste7 and Fus3 and stimulates phospho-relay by proximity effects, oligomerisation and conformational changes (Elion et al. 2005, Sprague 1998). The Pbs2 scaffold protein joins Ste11 with Hog1, and it links Ste11 to Cdc42-GTP-bound Ste20 through binding of its proline-rich domain to an SH3 domain of Sho1, which also binds to Ste11.

I.4. Aims of my diploma thesis

Signalling pathways enable cells to sense changes in their environment, and to respond to them by changes in transcriptional activity. The proper function of these pathways is crucial for adaptation and survival under varying growth conditions. In the past, signal transduction was usually explained as a linear connection between receptors and regulators of downstream gene expression. Recently, evidence indicates that pathways interact with each other, forming a signalling network through cross signalling (Schwartz and Baron 1999). The aim of my diploma thesis was to validate interactions between different stress pathways in budding yeast using a quantitative multi-pathway reporter system.

The first question to answer was to identify particularly regulated genes in each pathway upon specific stress stimuli. For this purpose, using real time PCR, I identified for each stress response the most highly induced target gene.

Next, I utilized promoters of these target genes for designing a multi-pathway, multi-fluorescent reporter strain of *S. cerevisiae* as a tool to study stress response. To understand the complex behaviour of signalling networks, five different reporter genes were integrated into yeast genome in one single strain.

Finally, the measurement of stress-specific promoter activity, by qualitative microscopy as well as quantitative multilabel counter plate reader (Victor^3) assay was established to analyse cross signalling in yeast stress response pathways.

II. MATERIALS AND METHODS

II.1. Basic bacteriological methods

II.1.1 Media for *E.coli*

	1xLB	2xLB
Tryptone	10g/l	10g/l
Yeast extract (DIFCO)	5g/l	5g/l
NaCl	10g/l	10g/l

Autoclave medium for 20min at 121°C. For LB-plates 250ml 4% (w/v) autoclaved bacteriological agar is added to 250 ml autoclaved 2xLB. For LB + Amp medium or plates, Ampicillin is added at a final concentration of 0.1mg/ml after cooling to 50°C. Medium and plates containing Ampicillin are stored at 4°C.

II.1.2 Competent bacteria and transformation

Competent bacteria

1. Inoculate overnight culture to in 200ml LB-medium and grow at 37°C to an OD_{600} of 0.4
2. Chill bacterial culture on ice for 10min
3. Harvest cells by centrifugation at 4°C for 7min at 3100rpm in sterile Falcon tubes
4. Resuspend pellet in 40ml ice-cold $CaCl_2$-solution
5. Centrifuge cells at 4°C for 5min at 2600rpm
6. Keep cells resuspend on ice for 30min
7. Centrifuge cells at 4°C for 5min at 2400rpm
8. Resuspend pellet gently in 8ml ice-cold $CaCl_2$-solution
9. Aliquot and shock-freeze with liquid nitrogen
10. Store at –80°C

Transformation

1. Add DNA (100-50ng plasmid, 5µl ligation mix) to 200µl competent bacteria
2. Incubate transformation mix for 30min on ice and plate cells on selective LB-plates

$CaCl_2$-solution: 60mM $CaCl_2$
10% Glycerol (w/v)
10mM PIPES
Adjust pH to 7.0 and autoclave

II.2. Basic yeast methods

II.2.1 Media for yeast

1xYPD		2xYPD	
Yeast extract (DIFCO)	10g/ll	Yeast extract (DIFCO)	20g/l
Peptone	20g/l	Peptone	40g/l
Glucose	2% (w/v)	Glucose	4% (w/v)

Liquid medium: Yeast extract and peptone are dissolved in 900ml H_2O and autoclaved for 20min at 121°C. A 20% glucose stock-solution is autoclaved separately and added at final concentration of 2% (100ml) to 900ml sterile YP-medium after cooling.

YPD-plates: Yeast extract and peptone are dissolved in 200ml H_2O and autoclaved for 20min at 121°C. 50 ml sterile 20% glucose stock-solution and 250ml 4% autoclaved bacteriological agar (w/v) are added to 200ml 2xYP after cooling to 50°C.

Minimal medium

Bacto-YNB w/o amino acids (DIFCO)	6.7g/l
Glucose	2% (w/v)

Bacto-YNB w/o amino acids and a 20% (w/v) glucose stock-solution are autoclaved separately. Glucose is then added at a final concentration of 2%. For plates bacteriological agar is added at a final concentration of 2% (w/v) as described above for YPD plates.

Drop-out media <u>2xSC</u>

Bacto-YNB w/o amino acids and $(NH_4)_2SO_4$	3.4g/l
$(NH_4)_2SO_4$	10.0g/l
Amino acid mix	2.86g/l

Bacto-YNB, $(NH_4)_2SO_4$ and amino acid mix are dissolved in 740ml H_2O and autoclaved for 20min at 121°C. For 500ml SC selective media, 250ml sterile water, 50ml 20% glucose stock-solution (w/v) and 5ml of the required amino acids from the 100x stocks are added. For plates, 250ml autoclaved 4% bacteriological agar (w/v) is added instead of H_2O.

100xURA:	0.4g of L-uracil	in 100ml H_2O
100xHIS:	0.6g of L-histidine	in 100ml H_2O
100xLEU:	2.6g of L-leucine	in 100ml H_2O

These 3 stocks can be autoclaved at 121°C for 20 minutes

100xTRP:	0.8g of L-tryptophane	in 100ml H_2O

0.22µm filter-sterilize, store at 4°C in the dark

Amino acid mix (g/29g):

- 0.4g Arginine
- 0.6g Tyrosine
- 0.6g Iso-leucine
- 0.8g Adenine
- 1.0g Phenylalanine
- 2.0g Glutamic acid
- 2.0g Aspartic acid
- 3.0g Valine
- 3.0g Methionine
- 3.6g Lysine

4.0g Threonine
8.0g Serine
mix well until a homogenens powder is obtained.

Sporulation plates

KAc	10g/l
Yeast extract (DIFCO)	1g/l
Glucose	0.5g/l
Agar	2% (w/v)

II.2.2 Yeast transformation by heat shock

Competent cells

1. Grow single yeast colony from a fresh plate in 5ml YPD at 30°C overnight
2. Dilute preculture in 50ml YPD to OD_{600} 0.2 and grow to OD_{600} 0.8-1
3. Harvest cells in centrifuge by spinning at 5min at 3000 rpm at 4°C, resuspend cells in 20ml sterile water
4. Spin again and resuspend cells in 1ml 0.1M LiAc/ 1xTE
5. Spin again and resuspend cells in 200µl 0.1M LiAc/ 1xTE
6. Cells can be stored at 4°C for about one week

Yeast transformation

7. Add 5-10µl DNA to 50µl competent cells and incubate for 20min at 30°C
8. Add 300µl 0.1M LiAc/ 1xTE/ 40% PEG 3350 (w/v) and incubate for another 20min at 30°C
9. Vortex and incubate for 20min at 42°C
10. Spin 5min at 2000rpm, resuspend cells in 2ml YPD, shake 1h at 30°C (regeneration)
11. Spin 5min at 2000rpm, resuspend cells in about 200µl YPD and plate on selective plate
12. Incubate at 30°C

1xTE : 10mM Tris/HCl pH 7.5
1mM EDTA

II.2.3 Colony-PCR

A Single colony is picked with a sterile toothpick and lysed by heating for 90 sec in the microwave at 750W, and put immediately on ice before resuspending in:

2,5µl primer 1 [10pmol/µl]
2,5µl primer 2 [10pmol/µl]
3µl dNTPs [2.5mM]
3µl PCR-Puffer (containing Mg^{2+})
1µl Taq polymerase [5U/µl] (lab made)
to a total volume of 20µl H_2O

PCR-program

2´	94°C	denaturation	
1´	94°C	denaturation	29 cycles
1´	52°C	primer annealing	
x´	72°C (1min for 1kb)	extension	
10´	72°C	final extension	
	10°C	hold	

Name	Length bp	Annealing Tm	Sequence
FUSLacZC1	20	46	5´- GCAAATTTCGTCAAAAATGC – 3´
FUSLacZC2	16	49	5´- CATCGGAAGAGGTGGC-3´
FUSLacZC3	16	46	5´- CAGTTGGTCTGGTGTC-3´
FUSLacZC4	21	47	5´- CCTGTGAAATTAATAACAGAC-3´
pRS-all-C3	18	62	5´- GGCGGGTGTCGGGGCTGG-3´
pRS-all-C2	28	61	5-GGTATTTTCTCCTTACGCATCTGTGCGG-3´
303PTCit-C4	29	60	5´- CAACTAACTTTTTCCCGTTCCTCCATCTC-3´
303PTCit-C1	30	59	5´-CTATTGCTTTGCTGTGGGAAAAACTTATCG-3´
304FTCFP-C4	30	55	5´-CAGATTTTATGTTTAGATCTTTTATGCTTGC-3´
304FTCFP-C1	18	62	5´-CGCCCGTCTGGACGCGCC-3´
306FTmRFP-C1	25	56	5´-GACCATCAAAGAAGGTTAATGTGGC-3´
306FTmRPF-C4	26	55	5´-CGTCATTATAGAAATCATTACGACCG-3´

II.2.4 Crossing yeast strains

1. Grow two strains of opposite mating type in 5ml YPD at 30°C overnight
2. Mix OD_{600} 1 cells of each type in YPD at 30°C for 4h
3. Briefly, spin down cells and resuspend them in 200µl YPD and let them grow on YPD plate
4. To select diploids cells streak colonies from YPD plate out on selective plates

II.2.5 Sporulation and tetrad dissection

Sporulation

1. Grow a single diploid yeast colony in 5ml YPD at 30°C overnight
2. Leave culture at room temperature for 2 days without shaking
3. Harvest cells by centrifugation at 2500rpm for 3 min and wash with H_2O
4. Resuspend cells in 500µl H_2O and drop cell suspension on a sporulation plate

Incubate plates for 2 days at 30°C
Check for tetrad formation under microscope frequently.

Tetrad dissection

1. Resuspend sporulated cells from the sporulation plate in 200µl sterile water
2. Add 1µl Zymolyase [20mg/ml] and incubate for 5min at room temperature
3. Drop 30µl of this suspension at the edge of a YPD plate and let dry

4. Separate spores physically with the needle of the micromanipulator (Singer MSM) and place spores one by one on the YPD plate
5. Incubate the plate for 3 days at 30°C
6. Streak single spores on patches on fresh YPD plates and incubate for another 2 days at 30°C

II.2.6 Testing of mating type

For mating type tests, two tester strains of opposite mating type are available containing a mutation that inhibits their growth on minimal medium (JJ-1A and JJ-1C). Only diploid cells are able to grow because the genes from the mating partner complement the mutations.

1. Patch colonies to be tested on YPD and incubated overnight at 30°C
2. Plate the tester strains L-1542 and L-1544 on YPD plates and incubate overnight at 30°C
3. Replica-plate cells to be tested together with each of the two tester-strains on minimal medium and incubate overnight at 30°C
4. Cells that are able to grow on minimal medium together with the *MAT***a** tester-strain are *MAT*α and vice versa

II.3. DNA Methods

II.3.1 Agarose gel electrophoresis

1. DNA samples (5-50µl) are mixed with an appropriate amount of 10x loading buffer
2. Load samples on a gel containing 0.7-1.5% agarose in TAE buffer and 0.5µg/ml ethidium bromide, in electrophoresis chamber filled with electrophoresis buffer
3. Load 5µl molecular weight standard on the gel (Bioline Hyperladder I)
4. Separate DNA fragments in an electric field with 80-100 Volts
5. Visualize nucleic acid fragments under UV-light and compare fragment size to molecular weight standard

TAE (50x stock solution): 2M Tris/HCl pH8
1M acetic acid
50mM EDTA

Electrophoresis buffer: 1xTAE

Loading buffer: 1.2ml 10xTAE
0.6ml 5% bromphenol blue
0.6ml 5% xylene cyanol
4.8ml 100% glycerol
4.8ml H_2O

II.3.2 QIAGEN plasmid midi isolation protocol

1. Grow E. coli cells containing the desired plasmid in 50ml LB+Ampicillin at 37°C overnight
2. Harvest cells by centrifugation at 6000 x g for 15min at 4°C

3. Resuspend cell pellet in 4ml Buffer P1 (QIAGEN)
4. Add 4ml of Buffer P2 (QIAGEN), mix gently by inverting several times and incubate at room temperature for 5min
5. Add 4ml of chilled Buffer P3 (QIAGEN), mix thoroughly by inverting several times and incubate 15min on ice
6. Centrifuge at 20000 x g for 30min at 4°C and re-centrifuge supernatant again for 15min
7. Equilibrate a QIAGEN-tip 100 by applying 4ml Buffer QTB (QIAGEN) and allow the column to empty by gravity flow
8. Apply the supernatant from the step before to the QIAGEN-tip and allow it to enter the resin by gravity flow
9. Wash the tip twice with 10ml Buffer QC (QIAGEN)
10. Elute DNA with 5ml Buffer QF (QIAGEN)
11. Precipitate DNA by adding 3.5ml isopropanol at room temperature.
12. Mix and centrifuge immediately at 15000 x g for 30min at 4°C.
13. Decant supernatant and wash pellet with 2ml 70% ethanol. Centrifuge again at 15000 x g for 10min at 4°C.
14. Decant supernatant, air-dry the pellet for 15min and redissolve DNA in 100µl sterile H_2O.

II.3.3 Plasmid mini preparation (thermic lysis)

Adapted from Holmes and Quigley, 1981

1. Harvest 2ml of an overnight culture by centrifugation at 6000rpm for 2min in eppendorf tubes
2. Resuspend pellet in 500µl STET-buffer and put on ice
3. Add 20µl of lysozyme (20mg/ml), mix by inverting several times and incubate on ice for 5min
4. For lysis incubate suspension at 95°C for 70sec, put on ice afterwards
5. Centrifuge at 14000rpm for 10min
6. Remove the pellet from the eppendorf tube using a sterile toothpick
7. Add 500µl isopropanol, mix by inverting several times and incubate at room temperature for 15min
8. Centrifuge at 14000rpm for 10min, wash pellet with 70% ethanol and centrifuge again
9. Dry pellet and resuspend in 100µl TE.
10. Add RNaseA to a final concentration of 50µg/ml

STET-buffer (100ml): 8g Saccharose
0.5ml Triton X-100
10ml 0.5M EDTA
5ml 1M Tris/HCl pH 8.0
0.45µm filter-sterilize

II.3.4 Cloning procedures

Restriction enzyme digest

1. Digest 1-3µg DNA in a total volume of 30µl using the buffer supplied with the enzyme and under the conditions recommended by the manufacturer.
2. Phosphatase treatment:
3. After restriction digest, add 1µl alkaline phosphatase to reaction mix
4. Incubate 45 min at 37°C
5. Load reaction mix on a gel to elute the fragment

Gel elution

Cut the desired DNA fragment out of the gel
Elute DNA from gel using the Qiagen QiaexII kit according to manufacturers protocol

Ligation of DNA fragments

Ligation was carried out with the rapid ligation kit (Promega), using vector and insert at a molar ration of 1:3

Ligation mix: x µl Insert
x µl Vector
5µl Buffer
1µ Ligase
10µl total

Incubate ligation mix for 1 hour at room temperature before transforming 5-10µl into *E.coli* cells

II.3.5 DNA-sequencing

Done in IMBA Sequencing Service

II.3.6 Determination of DNA concentration

1. Dilute DNA in H_2O
2. Measure absorption at 260nm in a spectrophotometer
3. Calculate DNA-concentration according to following formula:

$$\frac{\text{extinction}_{260nm} \times 50 \times \text{dilution}}{1\,000} = [\mu g/\mu l]$$

II.3.7 Synthesis PCR

Reaction mix:
150µg template
2,5µl primer [10pmol/µl]
2,5µl primer [10pmol/µl]
5µl dNTPs [2.5mMl]
5µl PCR-Puffer (containing Mg^{2+})
1µl Taq polymerase [5U/µl] (lab made)
Xµl H_2O

50µl total volume

PCR program:

2´	94°C	denaturation	
1´	94°C	denaturation	29 cycles
1´	52°C	primer annealing	
x´	72°C (1min for 1kb)	extension	
10´	72°C	final extension	
	10°C	hold	

II.4 RNA Methods

II.4.1. RNA extraction from yeast cells

1. Growth cells at 30°C overnight in 100ml.
2. On the next day dilute them to OD600=0,2 into 1,5L YPD medium and let them grow till OD_{600}=0,8-1.
3. Take 50ml culture as unstressed sample, and the rest of culture divide into 4 cultures (250ml each stressed and 150ml unstressed) and add corresponding stimulus:

50ml 5M Sorbitol	+	200ml culture (1M Sorbitol final con.)
60ml 5M NaCl	+	190ml culture (1.2M NaCl final conc.)
50ml 1M $CaCl_2$	+	200ml culture (0.2M $CaCl_2$ final conc.)
625µl 1M DTT	+	187,5ml culture (2,5mM DTT final conc.)
25ml 1M KOH	+	225ml culture (100mM KOH final conc.)
133,2µl alpha-Factor1		(50nM final conc.)

4. At specific time points harvest 50ml culture, put them immediately on ICE, (at 4°C, 3000 rpm for 3 min) and wash with ice-cold water
5. Freeze pallet in liquid Na_2 and keep it in –80°C or proceed immediately with RNA isolation:
6. Resuspend pellet in 400µl 50mM NaAc pH=5,3 /10mM EDTA
7. Transfer to tube and add 40µl 10% SDS, vortex and add 400µl Phenol
8. Vortex again and incubate on 65°C for 15min
9. Rapidly chill in ice
10. Centrifuge for 5 min at 14000rpm, then take upper phase and add 400µl PCI
11. Incubate for 5min at room temperature, then centrifuge for 5min at 14000rpm
12. Repeat extraction with PCI
13. Add 400µl Chloroform to aqueous phase and incubate for 5 min at room temperature, then centrifuge for 5min at 14000rpm
14. Repeat extraction with Chloroform then add 40µl 3M NaAc pH=5,3 to aqueous phase, invert 2 times tube and add 800µl EtOH abs. and incubate for 1h at -20°C
15. Centrifuge 15 min at 14000rpm and wash pellet with 70% EtOH
16. Air-dry pellet and resuspend in 50-100 µl H_2O by shaking 30 min at 56°C. Measure RNA concentration diluted in 1xTE
17. Measure RNA degradation in chip: Load into chip 200ng/µl in 1 µl

II.4.2. Agilent RNA6000 nano assay protocol

Gel preparation

1. Pipette 550µl of RNA 6000 Nano gel matrix into spin filter
2. Cantrifuge at 15000rpm for 10 min at room temperature
3. Aliquot 65µl filtered gel into 0.5ml RNAse-free tube.

Gel-dye mix preparation

1. Allow the RNA6000 nano dye concentrate to equilibrate to room temperature for 30 min
2. Vortex RNA6000 nano dye concentrate for 10 sec., spin down and add 1µl of dye into 65µl aliquot of filtered gel
3. Vortex solution well. Spin tube at 13000 rpm for 10 min at room temperature

Loading the gel-dye mix

1. Put the new RNA6000 nano chip on the chip priming station
2. Pipette 9µl of gel-dye mix into the well marked **G**
3. Close chip priming station
4. Press plunger until it is held by the clip
5. Wait for exactly 30 sec. then release chip
6. Pipette 9µl of gel-dye mix in the well **G**
7. Discard the remaining gel-dye mix

Loading the agilent RNA6000 nano marker

Pipette 5µl of RNA6000 nano marker in all 12 samples well and in the well marked as ladder

Loading the ladder and samples

1. Pipette 1µl of prepared ladder in well marked as ladder
2. Pipette 1µl of sample in each of the 12 sample wells
3. Pipette 1µl of RNA6000 nano marker in each unused well
4. Put the chip horizontally in adapter and vortex for 1 min at 2000 rpm
5. Run the chip in the Agilent 2100 bioanalyzer within 5 min

II.4.3. Nothern blot

Prehybridisation of nylon membrane in prehybridisation buffer:

2ml 50x Denhardt reagent
1ml 20x SSC
500µl 20% SDS
6,5ml sterile water
40µl ssDNA
10ml total volume
at 65°C for at least 3h

Labeling of DNA probes (Megaprime DNA Labeling Systems Amersham):

Reaction mix: 20-100ng DNA
5µl primer solution
x µl sterile water
33l total volume
at 95°C for 10 minutes, put on ice
add: 10µl labeling buffer
5µl P^{32} dCTP
2µl Klenow polymerase
Leave at 37°C for at least 2 hours

Purification of labeled DNA

1. Remove cap of NICK column, remove liquid
2. Rinse column once with 1xTE
3. Equilibrate column with 3ml 1xTE
4. (HOT ROOM) add sample (labelled DNA) and add 400µl 1xTE
5. Collect in tube#1
6. Add 450µl 1xTE and collect in tube #2
7. Add 1ml 1xTE and collect in tube #3
8. Measure activity of 3 elutes using a scintillation
9. Denaturate probe for 5 min at 95°C

Hybridisation

Add labelled probe to the pre-hybridisation buffer and incubate on at 65°C

Washing

Wash 2 times with 2xSSC 1%SDS for an hour
Wash 2 times with 1xSSC 1%SDS for an hour
Expose for 2 h at –80°C, make a film, and then again for overexpose over night

II.4.4 Real-time PCR

II.4.4.1. Synthesis of first strand cDNA suitable for PCR

(Fermentas, Hanover, MD, USA)
Prepare the following reaction mixture in a tube on ice:
template RNA*: total RNA 0.1-5µg
primer: oligo(dT)18 primer (0.5µg/µl) 1µl
DEPC-treated water till 11µl

1. Mix gently and spin down for 3-5sec. in a microcentrifuge.
2. Incubate the mixture at 70°C for 5min, chill on ice and collect drops by brief centrifugation.
3. Place the tube on ice and add the following components in the indicated order:
4. 5x reaction buffer 4µl
5. RiboLock™ Ribonuclease Inhibitor (20u/µl) 1µl
6. 10mM dNTP mix 2µl
7. Mix gently and collect drops by brief centrifugation.
8. Incubate at 37°C for 5min

9. Add M-MuLV reverse transcriptase (20u/µl)	2µl
10. Final volume	20µl

Incubate the mixture at 37°C for 60min
Stop the reaction by heating at 70°C for 10min. Chill on ice.
The first strand cDNA synthesized can be used directly for amplification by PCR

Real time PCR reaction mix (Roche kit) pro sample:

12.5µl kit mix
5µl template (1:10 diluted)
0.2µl primer1 [10pmol/µl]
0.2µl primer2 [10pmol/µl]
till 25µl water

Reactions are submitted to amplifications under the following conditions: initial denaturation 95°C for 4min, followed by 40 cycles (95°C for 10s, 60°C for 15s, 72°C for 15s, 80°C for 10s), and a melting curve analysis from 60 to 95 °C in 30min. Each real-time PCR assay data point was performed in triplicate. For relative quantification, data are analyzed according to the ΔΔCt method and are expressed as the fold expression (R) of the gene of interest (GOI) versus the expression of a house-keeping control gene (in our conditions, β-actin) in treated (t) versus untreated (ut) conditions, based on the following equation

$R = 2^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = (\Delta Ct_{GOI}t - \Delta Ct_{actin}t) - (\Delta Ct_{GOI}ut - \Delta Ct_{actin}ut)$.

II.4.4.2. Real time PCR oligonucleotides

Name	Annealing Tm	Length bp	Sequence
GLO-QRT_fp5	62	21	5´-GCGAAGAGCTAGAATCTCAGG-3´
GLO-QRT_rp3	62	21	5´-CCTAAAGCAAACGCAATGTCC-3´
HSP-QRT_fp5	61	21	5´-ATACGCTGAACAAGGTAAGGA-3´
HSP-QRT_rp3	61	20	5´-GCCAAAGATTCACCTTGACC-3´
HOR-QRT_fp5	62	20	5´-AAATGGTTCGAGCATCTGGG-3´
HOR-QRT_rp3	62	20	5´-TCCTGCCCTTCAGATATGGT-3´
STL-QRT_fp5	61	21	5´-CGAAGCTGTTATGGAGTTGAG-3´
STL-QRT_rp3	61	21	5´-GTCCTCAATTTGCCTATCGAC-3´
GDP-QRT_fp5	61	21	5´-TTCACACGTCAGAGCTATCTC-3´
GDP-QRT_rp3	61	21	5´-AGAGCACCACATTGAATACCT-3´
FLO-QRT_fp5	61	21	5´-TGACAACCCAACTGATTTCAC-3´
FLO-QRT_rp3	62	20	5´-CATATTGAGCGGCACTACCT-3´
FUS-QRT_fp5	62	21	5´-TTTGGCAGAGTAAGTGTGAGG-3´
FUS-QRT_rp3	62	20	5´-ATGTTATCACCCGAGGTCCA-3´
RCN-QRT_fp5	61	20	5´-GAAGCTAGCCAACTATTGCC-3´
RCN-QRT_rp3	61	21	5´-TCGTGGGACATCTATCAATGG-3´
CMK-QRT_fp5	61	20	5´-AGGGAAGTTCACAGAAGTGG-3´
CMK-QRT_rp3	61	21	5´-CGTTCTCAGGTTTCAAATCCC-3´
PMC-QRT_fp5	61	21	5´-AGTGTGCTCTGATAAGACTGG-3´
PMC-QRT_rp3	61	20	5´-CTTCCTTTGTTCGCTAACGG-3´
FKS-QRT_fp5	61	20	5´-CACAATGTCAACAACGTCCT-3´
FKS-QRT_rp3	61	20	5´-GATTTCGTAAACCTGGCTCC-3´
PST-QRT_fp5	61	21	5´-TTGTCGTTGCTAACAACACTG-3´
PST-QRT_rp3	61	21	5´-GAGTCTAGGTTCAAGGAGGTG-3´
YLR194C-QRT_fp5	62	21	5´-GTAAGCACAACTTCAACTGCC-3´
YLR194C-QRT_rp3	62	21	5´-ATGTTGATGTCGTTGAGGAGG-3´
RFS-QRT_fp5	59	22	5´-GAGATAGCTGGTGGTGAGGCTG-3´
RFS-QRT_rp3	58	25	5´-CTGCATTAGTTTTGGCGACTTTGGC-3´
PNC-QRT_fp5	58	25	5´-CACCTTTAGGTTCCTTGACTGTTCC-3´
PNC-QRT_rp3	59	25	5´-GTGCCAGTCTCTATCAGCATCTTGC-3´

II.5.1 Fluorescence

The stress assay is performed as described in section **II.4.1**, except that cells were grown in 5ml culture and RNA was not isolated.

1. After 90 min take an aliquot (200µl)
2. For *FUS1* construct add FDG Molecular Probes (0,55 ml) after 90 min and incubate 10-90 min at 37°C

Preparation of FDG Solutions 1 & 2 (mix them in equal amount just prior to use):
Solution 1: 1mM FDG stock (10mM) in 25mM PIPES (pH 7,2)
Solution 2: 5% TritonX in 250mM PIPES (pH 7,2)

3. Spin 3000 rpm for 3 min
4. Resuspend in 1ml 1xPBS
5. Spin again 3000 rpm for 3 min
6. Resuspend pellet in 200µl of 3% paraform-aldehyde in PBS
7. Rotate tubes on room temperature for 30 min

8. Spin again and resuspend pellet in a drop (10µl) of a mounting medium on a glass slide
9. Cover with a slip and leave in 4°C to dry

Observe slides using contrast phase, different filters. Microscopy was performed on a Zeiss microscop with a oil immersion objective. Images were recorded on a Zeiss Axiocam The following filter sets were used:

CFP: ex:430/8, em:460/10
Citrine: ex:510/10, em: 520/10
MRFP: ex:590/7, em: 610/10
GFP: ex:485/5, em:510/10

II.5.2 Multilabel Counter (Victor^3)

The stress assay is performed as described in section **II.4.1**, except that cells were grown in 5ml culture and RNA was not isolated.

1. Dilute overnight culture in 5ml YPD to an OD_{600} of 0.2
2. Grow at 30°C to an OD_{600} = 1
3. Split into 2 cultures

For stress response assay
Stress: 250mM DTT, 25ng/ml caspofungin, 10-30mM KOH, 0,5M NaCl
Medium: YPD
Controls: YPD+WT (W303-1)

Time course of 2:30 hours, measurement every 5 minutes

Filters and overlength
CFP: ex:430/8, em:460/10
Citrine: ex:510/10, em: 520/10
MRFP: ex:590/7, em: 610/10
GFP: ex:485/5, em:510/10

II.6. Oligonuclotides, strains and plasmids used

II.6.1. Oligonucleotides

Name	Length bp	Tm	Sequence
Flo11-SacI-5	27	67	5´-CGAGCTCTCTCCACATACCAATCACTCG-3´
Flo11-SacII-3	27	64	5´-TCCCCGCGGCATAGTGTGCGTATATGG-3´
mRFP1-fl-5	46	76	5´-CCATATACGCACACTATGCCGCGGGGAATGGCCTCCTCCGAGGACG-3´
mRFP1-fl-3	42	79	5´-GCTTGCGGATCCGGAATTCCGGGGCCGCCACTCCACCGGCGC-3´
Pmc1-SacI-5	24	54	5´-CGAGCTCATTGCGTTAAATATTCG-3´
Pmc1-SacII-3	25	56	5´-TCCCCGCGGTATTTTTTTTGTTACG-3´
Citr-fl-5	45	66	5´-CGTAACAAAAAAAATACCGCGGGGAATGTCTAAAGGTGAAGAATTATTC-3´
Citr-fk-3	39	68	5´-GCTTGCGGATCCGGAATTCCGTTATTTGTACAATTCATCCATACCAT-3´
Fks2-SacI-5	27	64	5´-CGAGCTCATCATCGGCGCGTTCTTTCC-3´
Fks2-SacII-3	25	58	5´-TCCCCGCGGAACTATGACAGTTTAA-3´
yCFP-fl-5	24	67	5´-TTAAACTGTCATAGTTCCGCGGGGAATGTCTAAAGGTGAAGAATTATTC-3´
yCFP-fl-3	23	69	5´-GCTTGCGGATCCGGAATTCCGCCTTATTTGTACAATTCATCCATAC-3´
Ter-EcoRI-5	21	58	5´-CGGAATTCCGGATCCGCAAGC-3´
Ter-SalI-3	30	64	5´-ACGCGTCGACTGCATGCAGGGTATCAAAAC-3´
Glo1-SacI-5	28	64	5´-CGAGCTCCATTGTCAACCCTAGCTAAGCC-3´
Glo1-SacII-3	28	64	5´-TCCCCGCGGTGTTAGTTTGTAGCTGGTG-3´
GFP-SacII-5	25	61	5´-TCCCCGCGGATGTCTAAAGGTGAAG-3´
GFP-EcoRI-3	37	62	5´-CGGAATTCTTATTTGTACAATTCATCCATACCATGG-3´
SAT1-XhoI-5	24	61	5´-CCGCTCGAGCCGGGTTAATTAAGG-3´
SAT1-XhoI-3	25	61	5´-CCGCTCGAGGTTTAAACTGGATGGC-3´

II.6.2. Yeast strains

Name	Genotype	Mating type	Source/ Reference
W303-1A	ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63 ade2-101c can1-100	*MAT***a**	Rothstein, 1983
W303-1B	ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63 ade2-101c can1-100	*MAT*α	Rothstein, 1983
JJ-1A	arg1 thr1 rho^0	*MAT***a**	
JJ-1C	arg1 thr1 rho^0	*MAT*α	
YAK6	Isogenic to W303-1B; *FLO11*promoter-mRFP1	*MAT*α	This work
YAK4-1	Isogenic to W303-1A; *FKS2*promoter-yeCFP	*MAT***a**	This work
YAK4-2	Isogenic to W303-1B; *FKS2*promoter-yeCFP	*MAT*α	This work
YAK4-3	Isogenic to W303-1B; *FKS2*promoter-yeCFP	*MAT*α	This work
YAK4-4	Isogenic to W303-1B; *FKS2*promoter-yeCFP	*MAT*α	This work
YAK3-1	Isogenic to W303-1B; *PMC1*promoter-yeCitrine	*MAT*α	This work
YAK3-2	Isogenic to W303-1A; *PMC1*promoter-yeCitrine	*MAT***a**	This work
YAKFus-1	Isogenic to W303-1A; *FUS1*promoter-LacZ	*MAT***a**	This work
YAKFus-2	Isogenic to W303-1A; *FUS1*promoter-LacZ	*MAT***a**	This work
YAKFus-3	Isogenic to W303-1A; *FUS1*promoter-LacZ	*MAT***a**	This work

II.6.3 Plasmids

Name	Auxotrophic marker	DNA-Insert	Parental plasmid / Reference
pRS306	*URA3*	none	Sikorski et al., 1989
pRS304	*TRP1*	none	Sikorski et al., 1989
pRS303	*HIS3*	none	Sikorski et al., 1989
pMD25	*TRP1*	*FUS1* promoter_LacZ	This work
pAK6	*URA3*	*FLO11* promoter_mRFP1	This work
pAK4	*TRP1*	*FKS2* promoter_yeCFP	This work
pAK3	*HIS3*	*PMC1* promoter_yeCitrine	This work
pAK-SAT1	*SAT1*	*GLO1* promoter_yeGFP	This work

III. RESULTS

III.1. Analysis of gene expression induced by stress response

To quantify putative cross-signalling between different stress pathways in yeast, we looked for highly induced target genes upon various stress stimuli. Therefore, a real time PCR was performed following adaptation assays with sustained presence of stress.

Previous gene expression analysis in yeast has shown that a diverse number of genes is specifically modulated after environmental changes (Gasch et al. 2000). Hence, we looked at the chronological program of gene expression by real time PCR.

In these experiments, 250 ml aliquots at $O.D._{600}$=0.8 of *S. cerevisiae* culture (W303-1A) were submitted to various stresses: hyper-osmotic shock agent sorbitol, alkaline pH, reducing agent DTT, mating pheromone, and calcium over the course of 2 hours (Figure 8). One aliquot was left untreated as control. Samples were taken from each culture at certain time points to look at gene expression.

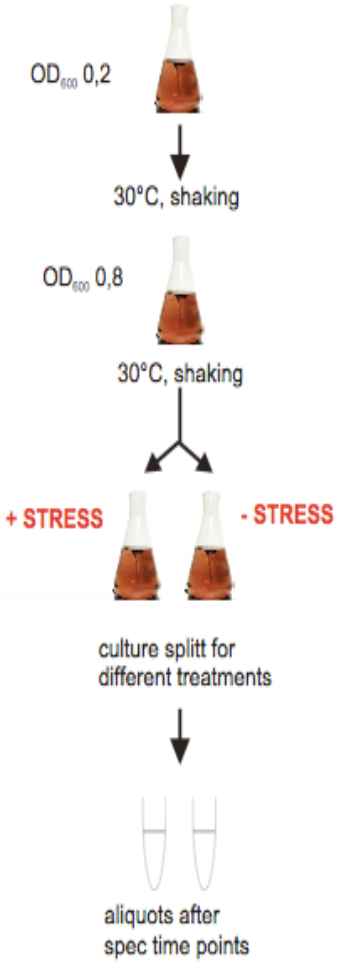


Figure 8: Stress Assay: Cell culture of W303-1A strain was grown over night in 50ml YPD medium. In the morning was diluted to 2L YPD medium at $O.D._{600}$=0.2 and allowed them to grow till $O.D._{600}$=0.8 so that all cells are in log phases of growth. At $O.D._{600}$=0.8 culture was split into 7 250ml portions and into each culture specific stress was added: 1.2M NaCl, 1M Sorbitol, 100mM KOH, 250mM DTT, 50nM αF1 factor and 200mM $CaCl_2$ at final concentration. At different time points an aliquot of 50ml of culture was taken from each culture.

After harvesting cells, cellular RNA extracted from stressed and unstressed yeast cells were reverse-transcribed, and subjected to real-time PCR analysis. Summary of all real time PCR results, done for each stress response, is presented in Figure 9. From the previous gene expression analysis (Gasch et al. 2000) we have chosen three characteristic genes for each pathway, which are highly induced upon certain stress but not under others (Figure 9).

For PKC pathway, expression of the glucan synthase catalytic subunits *FKS2,* the cell wall component *PST1* that contains a putative GPI-attachment site, and the structural constituent of the cell wall *YLR194C* attached to the plasma membrane by a GPI-anchor were characterized (www.yeastgenome.org). As shown in Figure 9A *YLR194C* mRNA was only slightly induced. Expression was increased about 2-fold in presence of 250mM DTT. The small induction in *PST1* mRNA levels could be explained since its regulation is coupled with a lack of *FKS1*. Remarkably, expression of *FKS2* was induced more than 6-fold in presence of DTT (Figure 9A). Thus, *FKS2* promoter region was chose to be fused with CFP reporter gene (Table1).

Genes of our choice involved in calcium stress adaptation to be tested were calcineurin regulator *RCN1,* calmodulin-dependent protein kinase *CMK1* and *PMC1,* vacuolar Ca^{2+}-ATPase involved in depleting the cytosol of Ca^{2+} ions. Addition of 200mM $CaCl_2$ to medium resulted in 20-fold increase of mRNA of *RCN1*, whereas induction of *CMK1* stayed very low. Furthermore, more then 80-fold induction was observed in mRNA levels of *PMC1* (Figure 9B) 90 minutes after stress stimulation.

These results lead us to use *PMC1* promoter region for activation of Citrine (yellow fluorescent protein) reporter gene by long-term calcium stress response.

In mating pathway, *FUS1* (Figure 9C) promoter region driving LacZ reporter was the best candidate, since *FUS1* is required for cell fusion and the most highly regulated gene in yeast.

In RIM pathway, *FLO11* was chosen for controlling expression of mRFP, since addition of 100 mM KOH triggered transcriptional induction of about 600-fold (Figure 9E). Flo11 is GPI-anchored cell surface glycoprotein (flocculin) required for diploid pseudohyphal formation and haploid invasive growth.

We also investigated the response to high-osmolarity glycerol (Figure 9D). Out of 200 target genes of HOG pathway (Gasch et al. 2000), we tested transcriptional profiles of three mostly HOG-responsive genes: *STL1*, glycerol proton symporter of plasma membrane that is strongly but transiently induced when cells are subjected to osmotic shock, Hsp12 plasma membrane protein protecting membranes from desiccation and *GLO1* monomeric glyoxalase I whose expression is regulated by methylglyoxal levels and osmotic stress. As shown in Figure 9D, addition of 1.2M NaCl impaired mRNA induction in all three genes. However, response of *HSP12* (5-fold) and *STL1* (2.5-fold) was lower that the one observed for *GLO1* (25-fold after 90 minutes). Therefore, we chose *GLO1* promoter region to drive GFP expression.

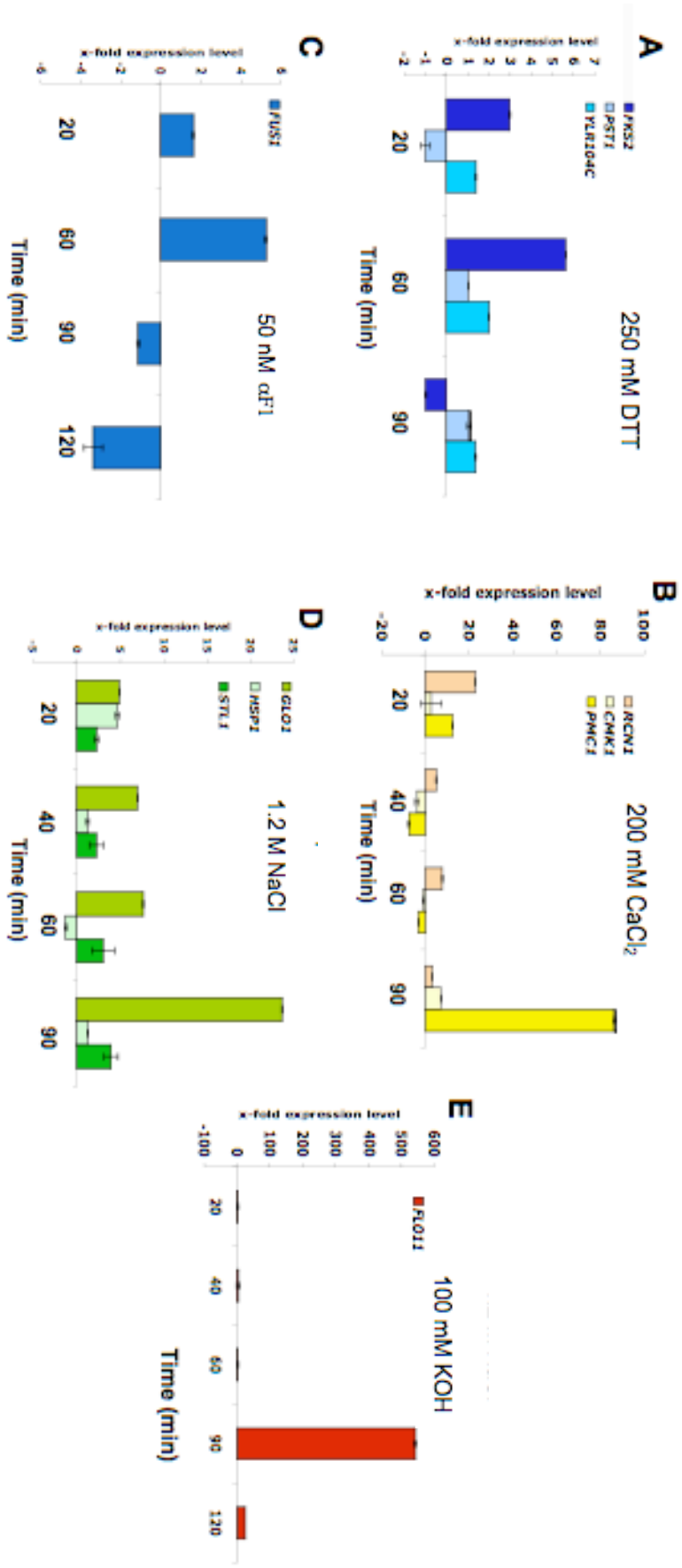


Figure 9: Different stress response mRNA synthesis induced in yeast.
Yeast cells were stressed with different stress stimuli: 250mM DTT with target genes for PKC pathway: *FKS2*, *PST1* and *YLR194C* (A), 200mM $CaCl_2$ with target genes for calcium pathway: *RCN1*, *CMK1* and *PMC1*(B), 50nM alpha Factor1 with *FUS1*gene(C), 1.2M NaCl with target genes for HOG pathway: *GLO1*, *HSP12* and *STL1*(D) or 100mM KOH for *FLO11* gene in RIM pathway. The incubation was performed as explained in **Fig 8.** After the indicated time points, total mRNA were extracted, reverse-transcribed, and analyzed by real-time PCR for specific genes expression.

To test the idea of the stress cross-talk in yeast, a *S. cerevesiae* multicolour strain was engineered by genomic integration of fluorescent reporters. If cross-signalling between different stress pathways exist, it should be possible to detect and quantify these events by measuring reporter gene expression (Fig 10).

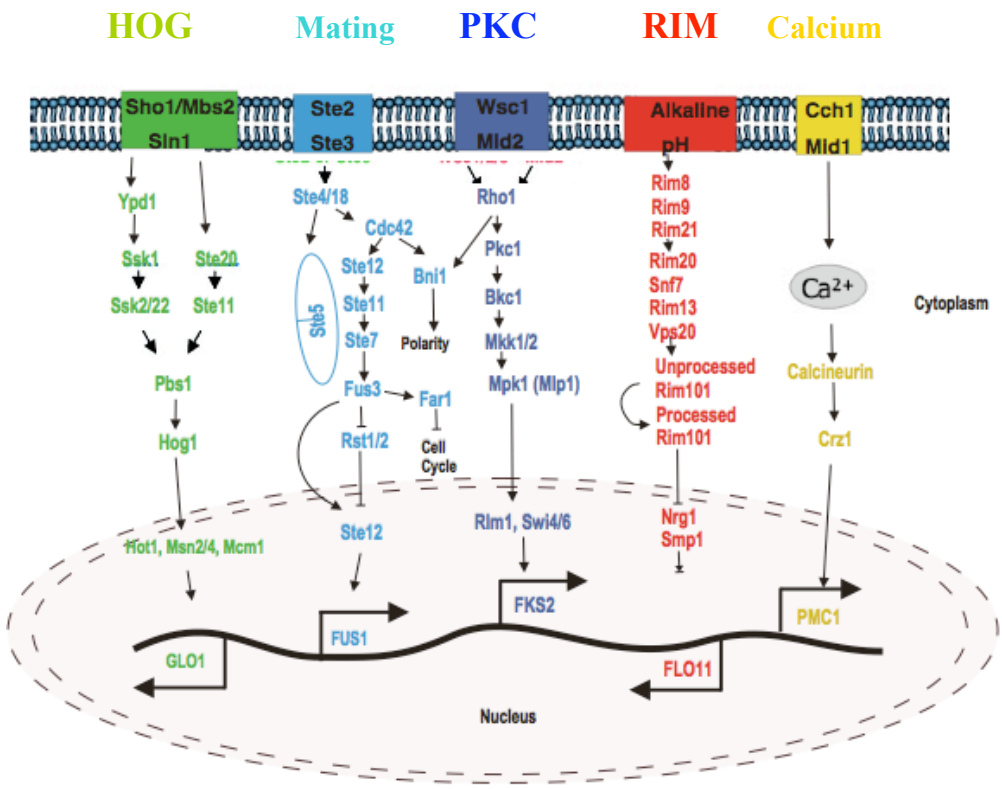


Figure 10: Putative cross signalling between different stress response pathways in *S.cerevisiae*

III.2. Construction of a multicolour fluorescent *S. cerevisiae* reporter strain

III.2.1. Construction of a promoter-reporter gene cassette

To generate a promoter-reporter cassette in *S. cerevisiae,* genomic *in vivo* recombination system was chosen. Promoter regions of *PMC1, FLO11, FKS2, FUS1 and GLO1* were inserted into a YIP (yeast integrative plasmid) vector upstream of a different reporter gene. The chosen reporter gene/promoter combinations are listed in Table 1. (Maps of all reporter plasmids can be seen in Supplement).

Reporter	Colour	Excitation (nm)	Emission (nm)	Promoter	Pathway	Overlapping
Citrine	yellow	516	529	*PMC1*	Calcium	LacZ
GFP	green	488	509	*GLO1*	HOG	LacZ
CFP	blue	433	475	*FKS2*	PKC	none
mRFP1	red	586	607	*FLO11*	RIM	none
LacZ	blue	485	530	*FUS1*	Mating	Citrine and GFP

Green fluorescent protein (GFP) has become an increasingly popular protein tag for determining protein localisation and abundance as well as dynamics. With the availability of GFP variants with altered fluorescence spectra, as well as GFP homologues from other organisms, multi-colour fluorescence with protein tags has become possible. We employed a set of yeast tagging vectors containing codon-optimized variants of GFP*,* CFP (cyan), YFP (yellow), and monomeric RFP (red) (Sheff and Thorn 2004), which is up to 15-fold more sensible than tags currently in use. These tags significantly improve the detection limits for live-cell fluorescence imaging in yeast, and provide sufficient distinguishable fluorophores for multi-colour

imaging. Each of the constructed YIP plasmids carries a different selection marker (*TRP1, HIS3, URA3, SAT1*). The promoter regions and terminators were integrated into YIP vectors via T4 ligation (Figure 11A). However, reporter genes were integrated into a YIP plasmid by *in vivo* recombination in *E.coli* due to homology of overlapping regions (red and green box, Figure 11B)

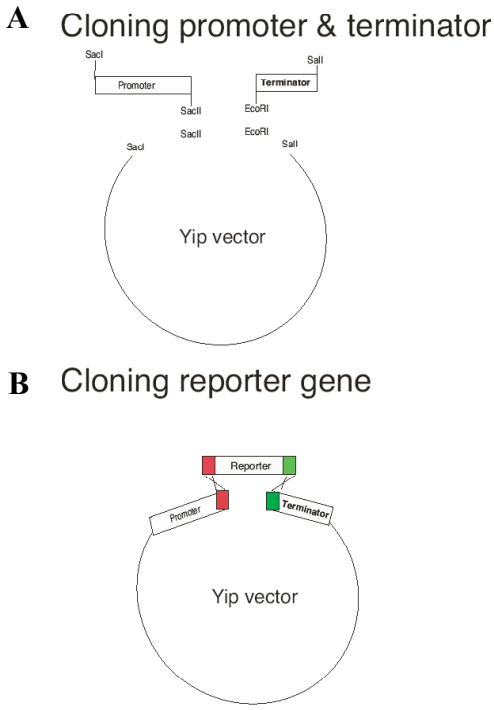


Figure 11: Cloning the fluorescent constructs

A. Chosen promoter region and terminator were integrated in YIP vector with T4 ligase.

B. Reporter gene was integrated additionally into a plasmid that already carries promoter and terminator by recombination in RecA+ *E.coli* strain TG1.

Vectors carrying cassettes *PMC1*-Citrine, *FKS2*-CFP, *FLO11*-mRFP, *GLO1*-GFP and *FUS1*-LacZ were named pAK3, pAK4, pAK6, pAK-SAT1 and pMD25, respectively.

III.2.2. Generating a single reporter gene strain

Vectors pAK3, pAK4, pAK6, pAK-SAT1 and pMD25 were linearized in the selective marker region and transformed into the wild type yeast strains W303-1A & W303-1B. The transformants were selected on corresponding selective plates for genomic integration of constructs and re-streaked as single colony. Each colony was then tested for the proper genomic integration of the cassette by colony PCR. After verifying the correct integration of constructs, we examined by fluorescence microscopy, the expression of each reporter gene under the specific stress condition in comparison with the wild type (Figure 12).

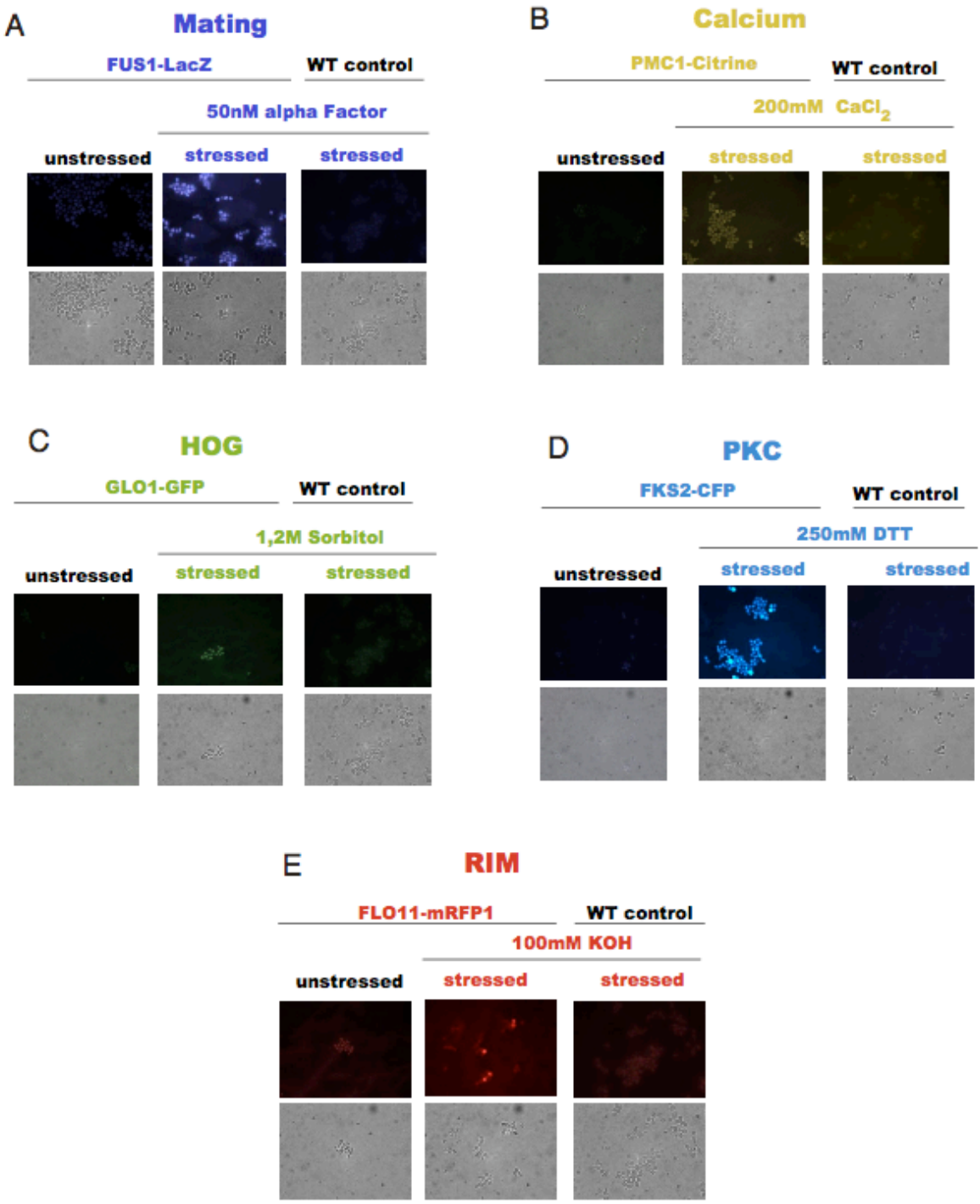


Figure 12: Single reporter strains under the fluorescent microscope upon activation.
S. cerevisiae cells were stressed with following stress conditions: A) 50nM αfactor1 to activate *FUS1* promoter driving LacZ; B) 200mM CaCl2 to activate *PMC1* promoter driving Citrine; C) 1.2M sorbitol to activate *GLO1* promoter controlling GFP; D) 250mM DTT to activate *FKS2* promoter controlling CFP; E) 100mM KOH to activate *FLO11* promoter driving the mRFP. After 90 min, fungal cells were checked under the microscope.

Figure 12 shows expression of all reporter genes during stress response adaptation. After 90 min of incubation unstressed strain that carry one construct (Figure 12, the first column in all pathways) shows basal level of fluorescence. However, stressed strain with one corresponding construct integrated into the genome shows full expression of the corresponding reporter gene (Figure 12, the second column in all pathways). Then again, the wild type strain (Figure 12, the last column in all pathways) expresses low or no fluorescence at all (basal level of fluorescence). The strain carrying the genomically integrated pAK3, pAK4, pAK6, pAK-SAT1 and pMD25 were named YAK3 (Calcium), YAK4 (PKC), YAK6 (RIM), YAKSAT1 (HOG) and YAKFUS (Mating), respectively (Table “Yeast Strains” in Material & Methods).

III.2.3. Making a two-reporter genes yeast strain

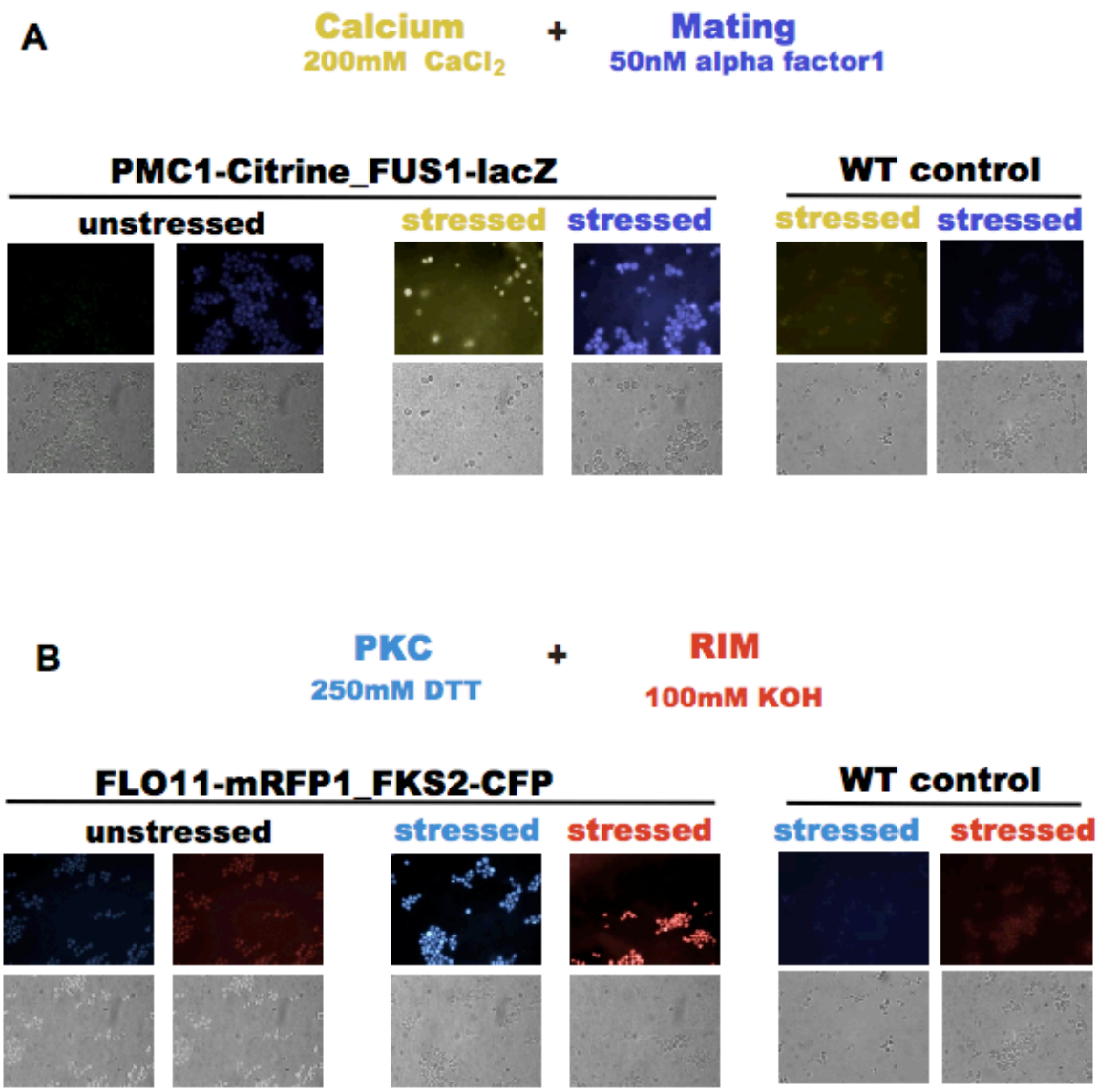
To obtain a two-reporters strain, the same procedure as described in III.3.2.2 was used. Vectors pAK3, pAK4, pAK6 and pMD25 were linearized in the selective marker region and transformed into the strains carrying already one reporter, YAK3, YAK4, YAK6 and YAKFUS respectively. Combination of all integration can be seen in Table 3.

2 colour strain, *MAT*a	**YAK3 (Calcium)**	**YAK4 (PKC)**	**YAK6 (RIM)**	**YAKFUS (Mating)**
pAK3 (Calcium)		YAK3-4	YAK3-6	YAK3-Fus,
pAK4 (PKC)	YAK3-4		YAK4-6	
pAK6 (RIM)	YAK3-6	YAK4-6		YAK6-Fus
pFus (Mating)	YAK3-Fus		YAK6-Fus	

2 colour strain, *MAT*α	**YAK3 (Calcium)**	**YAK4 (PKC)**	**YAK6 (RIM)**
pAK3 (Calcium)		YAK3-4	YAK3-6
pAK4 (PKC)	YAK3-4		YAK4-6
pAK6 (RIM)	YAK3-6	YAK4-6	

Table 3: Constructing a two-color yeast strain. Various combination of pAK integration into YAK strains used to obtain double reporter gene strain. First table shows reporter strains in *MAT***a** background and second table reporter strains in *MAT*α background.

After verification of the correct genomic integration by colony PCR, fluorescence microscopy was performed on stressed cells versus unstressed cells to monitor induction of the reporter gene (Figure 13).



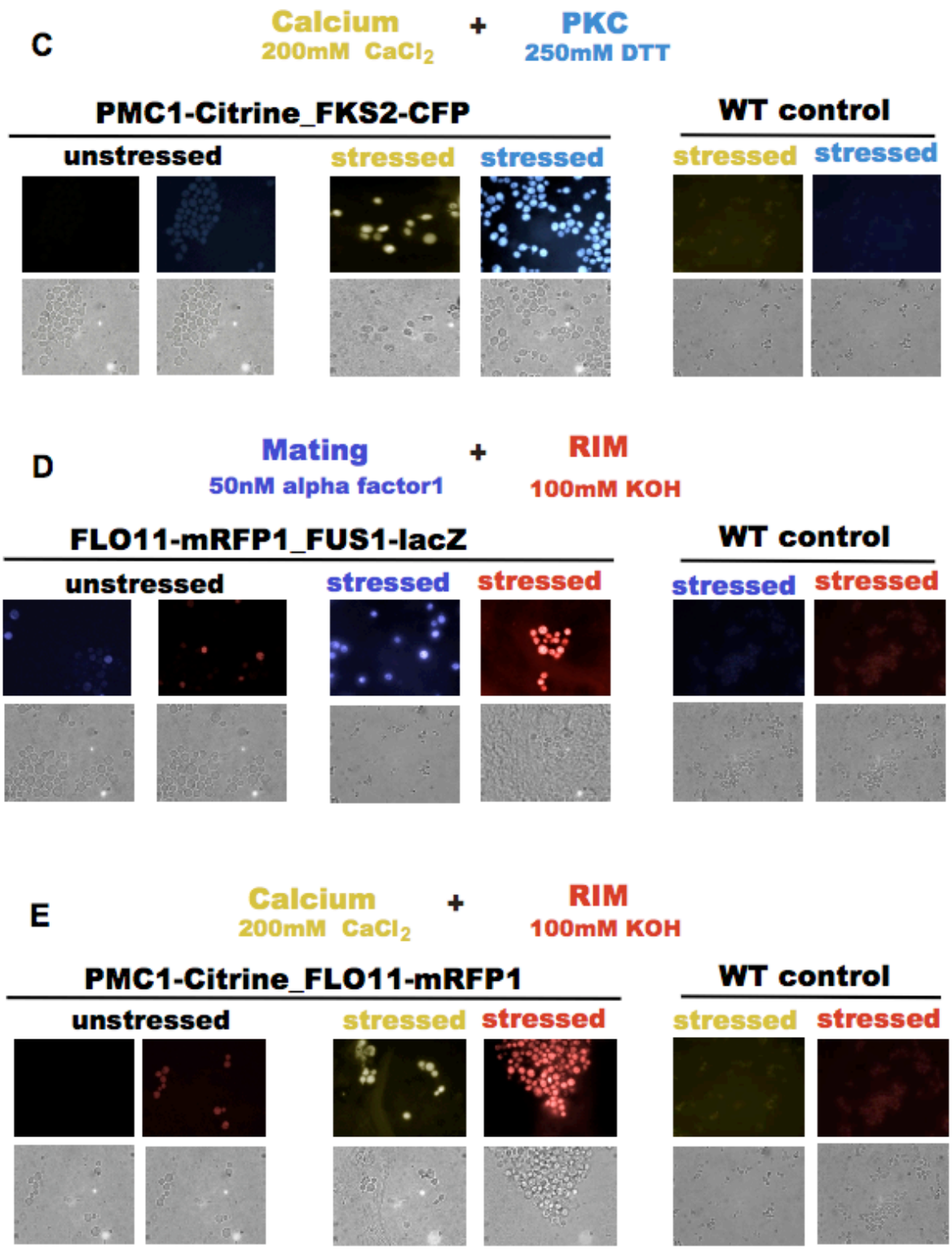


Figure 13: Microscopy of double-reporter strains under stressed vs. unstressed conditions. *S. cerevisiae* cells were stressed for 90min with the following stress conditions: A) 50nM αFactor1 to activate *FUS1* promoter driving *LacZ* and 200mM CaCl2 to activate *PMC1* promoter controlling Citrine; B) 250mM DTT to activate *FKS2* promoter driving *CFP* and 100mM KOH to activate *FLO11* promoter controlling *mRFP*; C) 250mM DTT to activate *FKS2* promoter driving *CFP* and 200mM CaCl2 to activate *PMC1* promoter controlling Citrine; D) 50nM αfactor1 to activate *FUS1* promoter driving *LacZ* and 100mM KOH to activate *FLO11* promoter controlling *mRFP*;

E) 100mM KOH to activate *FLO11* promoter driving *mRFP* and 200mM CaCl2 to activate *PMC1* promoter controlling *Citrine*.

Figure 13 shows the expression of a double-reporter strain during stress response adaptation. After 90 min of incubation unstressed strain carrying both corresponding constructs (Figure 13, the first and the second columns) showed basal level of fluorescence. Nevertheless, stressed strain with both corresponding constructs integrated into the genome showed full expression of the reporter gene (Figure 13, the third and the fourth column). The control wild type strains (Figure 13, the last two columns) displayed basal level of fluorescence. The strains carrying the combination of two genomically integrated corresponding vectors were named YAK3-FUS (Calcium, Mating) and YAK4-6 (PKC, RIM), respectively (Table “Yeast Strains” in Material & Methods).

III.2.4. *S. cerevisiae* strain with four-reporter genes

To construct a diploid strain carrying the four reporter genes in all combinations, yeast strains with mating type *MAT***a** and *MAT*α were crossed (Table 4).

4 Colour strain	**YAK3-FUS (Calcium, Mating), *MAT*a**	**YAK4-FUS (PKC, Mating), *MAT*a**	**YAK6-FUS (RIM, Mating), *MAT*a**
YAK3-4 (Calcium, PKC), *MAT*α			**YAK3-4-6-FUS**
YAK3-6 (Calcium, RIM), *MAT*α		**YAK3-4-6-FUS**	
YAK4-6 (PKC, RIM), *MAT*α	**YAK3-4-6-FUS**		

Table 4: Constructing a four-reporter yeast strain through crossing method.

Because mating type of some cells changes after transformation, both *MAT***a** and *MAT*α cells are present in each culture. Therefore, diploid cells were sporulated as described in “Materials and Methods”. Single spores were obtained, grown to colonies and their mating type was determined (Materials and Methods). The function of all four reporters was monitored by microscopy as described in III.3.2.2 (Figure 14).

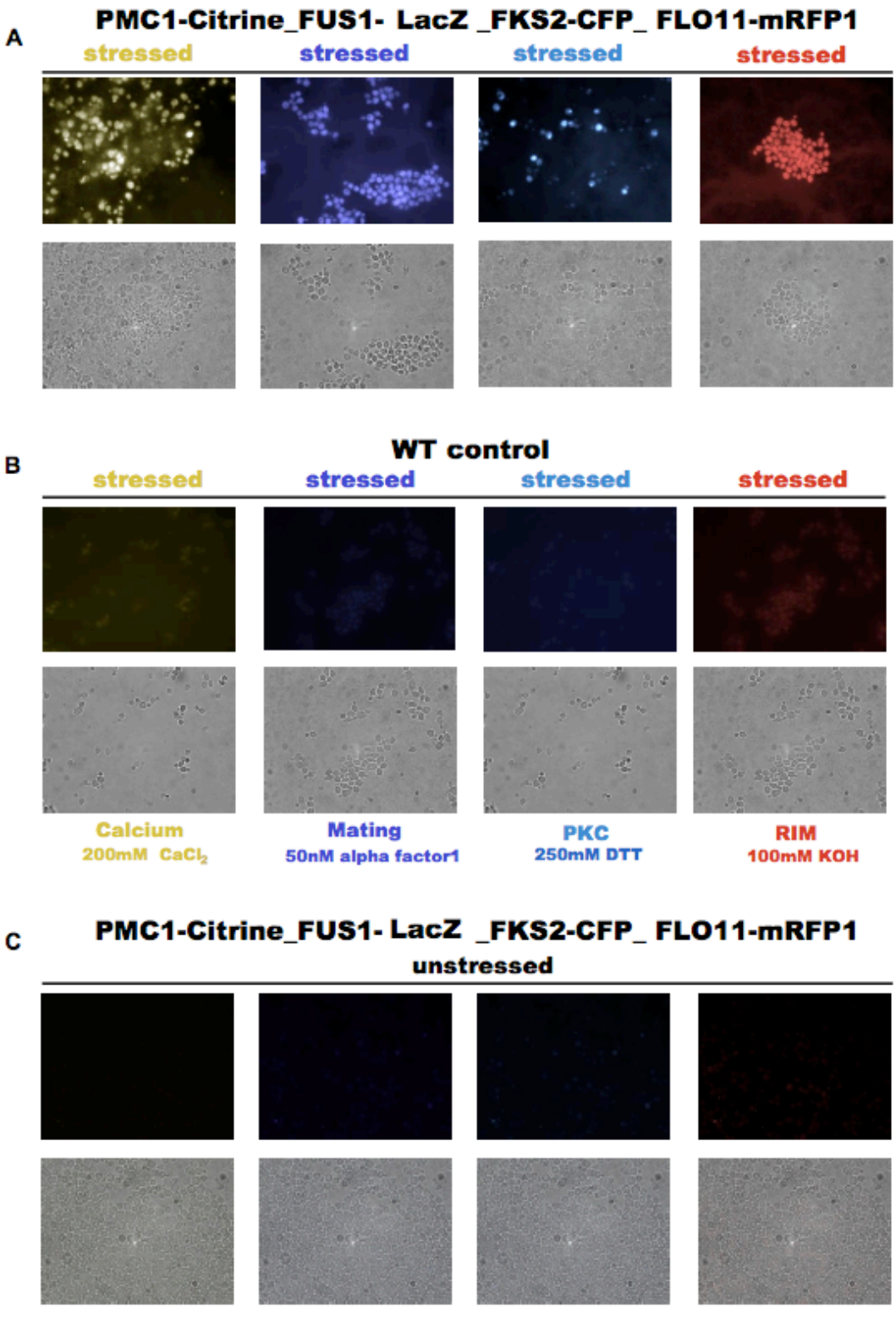


Figure 14: Microscopy of four-reporter gene strain. *S. cerevisiae* cells were stressed for 90 min with the following stress conditions: 50nM αFactor1 to stimulate *LacZ* expression under the control of *FUS1* promoter, 200mM CaCl2 to activate *PMC1* promoter driving *Citrine*, 250mM DTT to activate *FKS2* promoter controlling *CFP,* and 100mM KOH to stimulate *mRFP* expression under the control of *FLO11* promoter.

Figure 14 shows the expression of four reporter genes during various stress response adaptation. After 90 min of incubation, reporter strains showed full expression of respective reporter genes in response to a specific stress (Figure 14A). Only low or no fluorescence (basal level of fluorescence) was observed in the wild type control strain (Figure 14B). The same results were obtained with unstressed reporter strain (Figure 14C). The first columns in all rows corresponds to adaptation to calcium stress (yellow), the second columns (pink) to adaptation to mating stress, the third columns (blue) to cell wall damaging stress and the last (red) columns correspond alkaline pH stress.

All positive colonies were then further selected for their ability to mate with a tester strain. Those colonies that were able to mate with the tester strains JJ-1A (*MAT***a**) were selected as *MAT*α and vice versa. The resulting haploid *MAT***a** strain which is expressing all four reporter proteins was named YAK3-4-6-FUS (Calcium, PKC, RIM, Mating).

III.2.5. Engineering the final five-colour strain

To obtain a final, 5-colour strain, the 4-reporter strain was transformed with pAK-SAT1 vector according to III.3.2.2. Vector pAK-SAT1 was linearized in *GLO1* promoter region and transformed into the YAK3-4-6-FUS strain. The transformants were selected on selective plates containing nourseothricin (200µg/ml) to allow genomic integration of construct and re-streaked to single colonies. Single colonies were then tested for proper integration of the cassette by colony PCR. The correct genomic integration was verified from both ends of a construct. To show specificity, the same reactions were carried out with the wild type strain as a negative controls. After verifying the correct genomic integration of the construct, expression of the last reporter ye*GFP*, was monitored by fluorescence microscopy (Figure 15).

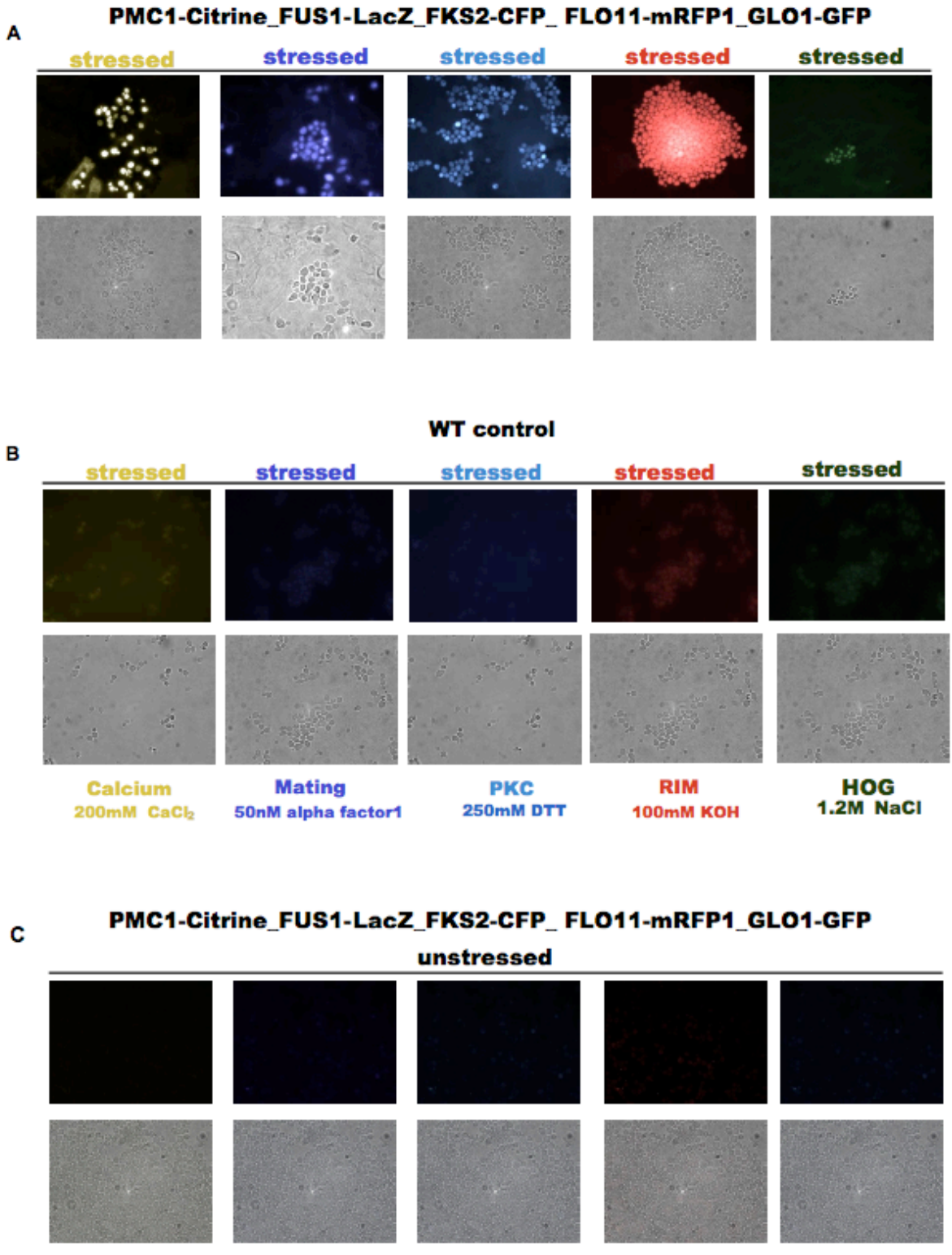


Figure 15: Microscopy of five-colour gene strain. *S. cerevisiae* cells were stressed for 90 min with the following stress conditions: 50nM αFactor1 to stimulate *LacZ* expression under the control of *FUS1* promoter, 200mM CaCl2 to activate *PMC1* promoter driving *Citrine*, 250mM DTT to activate *FKS2* promoter controlling CFP, 0.5M NaCl to stimulate *GFP* expression under the control of *GLO1* and 100mM KOH to stimulate *mRFP* expression under the control of *FLO11* promoter

Figure 15 shows the expression of all 5 reporters during adaptation to various stresses. After 90 min of incubation, all reporters showed full stimulation under the control of their respective promoters in response to specific stress (Figure 15A). The first columns in all rows present adaptation to calcium increase (yellow), the second columns (pink) to adaptation to mating stress, the third columns (blue) to cell wall damaging, the fourth (red) to alkaline pH environment, and the last (green) columns show high osmolarity response. No fluorescence signal was detected in the unstressed reporter strains (Figure 15C) as well as in the wild type strain (Figure 15B). Yeast strain carrying all five reporters was named as YAK5.

III.3 Cross-signalling between different stress-response pathways

III.3.1 Stress induction assay

To characterize and quantify cross signalling in *S. cerevisiae*, we followed the time course of stress induction in 96-well plate format and measured it in a Multilabel counter plate reader. For this purpose we used a YAK5 strain in which all five reporter genes (*GFP, Citrine, CFP, mRFP* and *LacZ*) are under control of corresponding yeast promotors (*GLO1, PMC1, FKS2, FLO11* and *FUS1,* respectively). During a time course of 160 minutes, we stressed the YAK5 strain with different stress stimuli followed by measurement after every 5 minutes. To distinguish the response of applied stress response to non-specific ones, wild type cells (strain without any reporter genes) were stressed in the same way and their

response was compared to response of the YAK5 strain. The following experiments present a typical sample experiment of displayed trends.

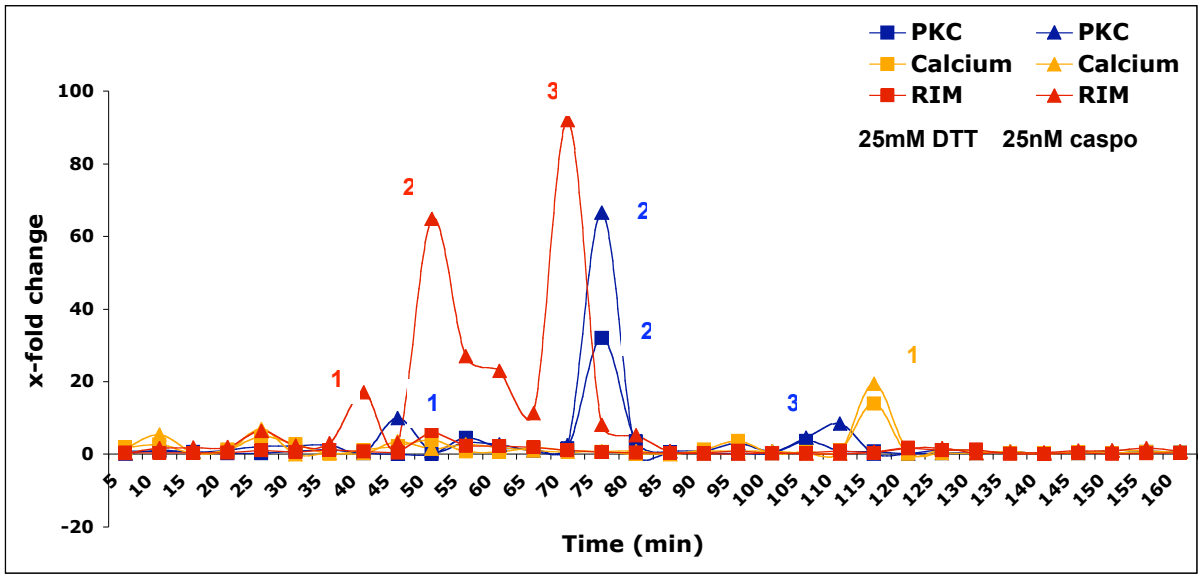


Figure 16: Stress response of the *S.cerevisiae* YAK5 strain to various cell wall damaging stimuli. The 5-reporter genes YAK5 strain was stressed with different stimuli: 25mM DTT (■) and 25ng/ml caspofungin (▲); Time course was performed for 160 minutes with fluorescence after every 5 minutes in a multilabel plate reader. Reporter genes expression were measured with corresponding filters: CFP (ex. 430/7nm, em. 460/15nm), YFP (ex. 510/10nm, em. 520/10nm), and mRFP (ex. 590/7, em. 610/10nm).

Figure 16 shows stress response of YAK5 yeast cells exposed to 250mM DTT or 25ng/ml caspofungin. Yeast response to DTT stress was to a certain extent different from these to antifungal drug caspofungin (Figure 16). Cell wall remodeling was active in both treatments, whereas activation of RIM pathway was shown only upon antifungal drug (Figure 16).

These results clearly show that PKC and RIM pathway act in parallel upon stimulation with antifungal agent (Figure 16). Activation of RIM pathway displayed three peaks increasing with time. The first response to caspofungin came 40 minutes upon stimulation within expression of mRFP and about 15-fold change (Figure 16, red triangle, first peak). Furthermore, 10 min later, another response of RIM pathway

to caspofungin treatment was observed with more then 60-fold change (Figure 16, red triangle, second peak), followed by the third peak of RIM pathway 70 minutes after caspofungin stimulation as 90-fold. Remarkably, response of PKC pathway to caspofungin showed a similar behavior, with three peaks, each following the previous one within delay of 30 minutes. The first and the third peak showed about 10-fold change, whereas the second peak showed a 60-fold increase. The second peak height of PKC pathway responding to caspofungin (Figure 16, blue triangle, about 60-fold) was also for more then 50% stronger compared to response to DTT (Figure 16, blue square, about 30-fold). However, Ca^{2+} influx in yeast (yellow) occurred much later, with low peak height and time delay of almost 2h after both, DTT and caspofungin stress stimuli.

The addition of different concentrations of KOH in YPD medium results in diverse pH changes (10mM, pH=6.5; 30mM, pH=8; 100mM, pH=10). PKC response to this pH stress occurs already 10 minutes after adding a stimulus (Figure 17), resulting probably in hydrolysis of the yeast cell wall (Figure 17, blue).

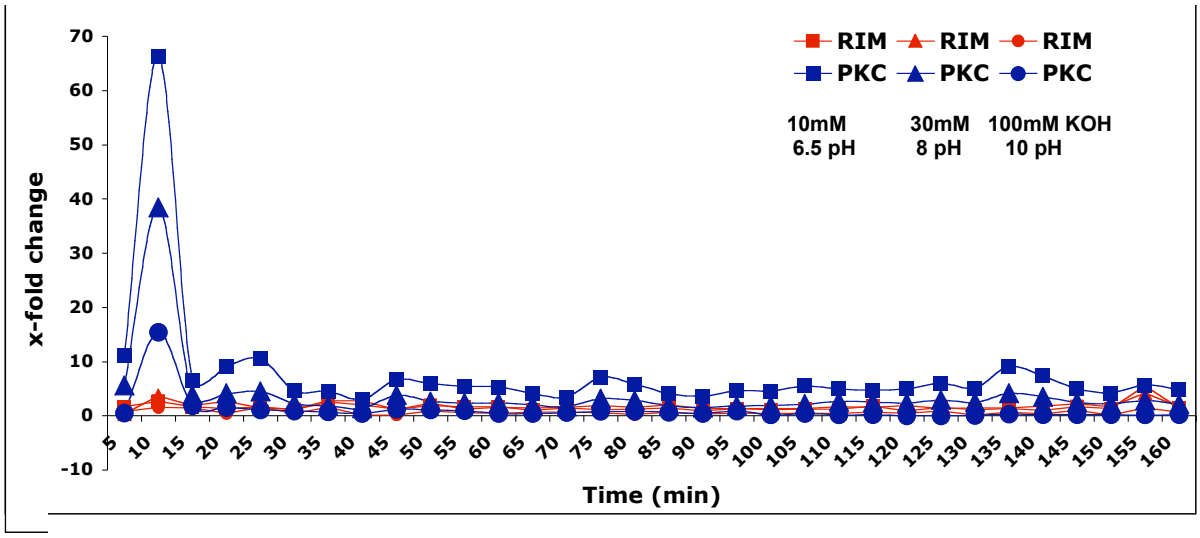


Figure 17: Stress response of the *S.cerevisiae* YAK5 strain to various pH. The 5-reporter genes YAK5 strain was stressed with different stimuli: 10mM (■), 30mM (▲) and 100mM KOH (●). Time course was performed for 160 minutes whereas fluorescence was measured after every 5 minutes in a multilabel plate reader. Reporter genes expression were measure with corresponding filters: CFP (ex. 430/7nm, em. 460/15nm), YFP (ex. 510/10nm, em. 520/10nm), and mRFP (ex. 590/7, em. 610/10nm).

Next, we examined the effect of Ca^{2+} in budding yeast. As shown in Figure 18, various $CaCl_2$ concentrations induce the Calcium pathway (yellow). Concentration of 0.2M $CaCl_2$ (Figure 18, yellow square) induced a similar response as that of 50mM (Figure 18, yellow round). Compared with 50mM and 0.2M $CaCl_2$-treated cells (Figure 18, yellow round and square), the pattern from the cells exposed to 0.1M $CaCl_2$ (yellow triangle) showed delay of 40 minutes. $CaCl_2$ had no stimulatory effect on cell wall remodelling (Figure 18, blue). Notably, response to $CaCl_2$ stimulated activation of RIM pathway (Figure 18, red). Unlike in PKC pathway (blue), two peaks were clearly observed after 60 minutes (Figure 18, red square) and 90 minutes (Figure 18, red round and triangle) time point.

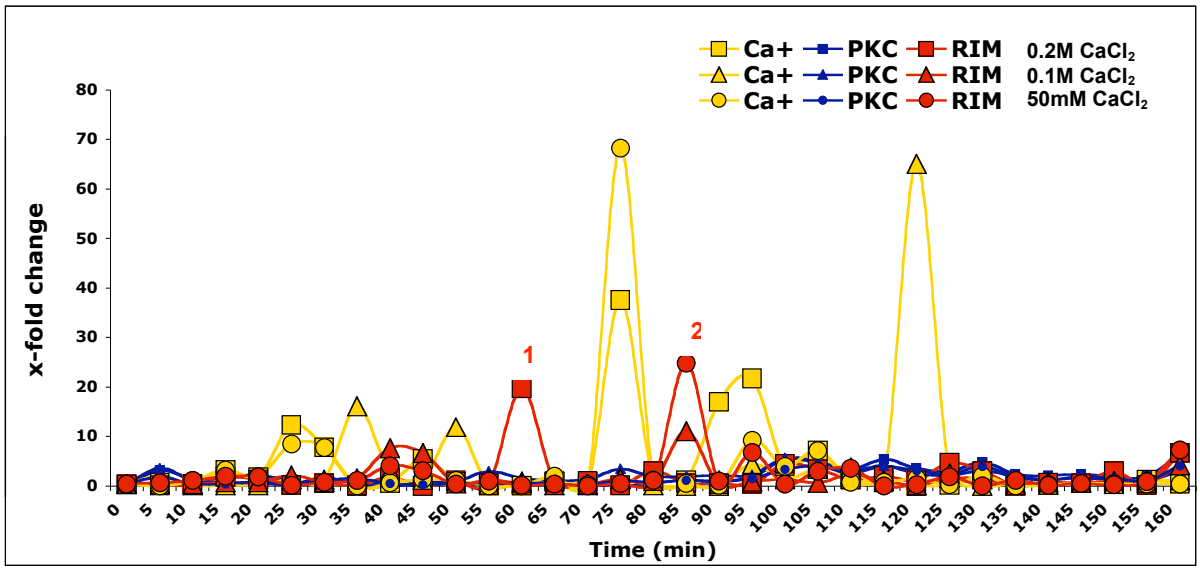


Figure 18: Stress response of the *S.cerevisiae* YAK5 strain to various calcium concentrations. The 5-reporter genes YAK5 strain was stressed with different stimuli: 0.2M (■), 0.1M (▲) and 50mM $CaCl_2$ (●). Time course was performed for 160 minutes whit fluorescence measurement after every 5 minutes in a multilabel plate reader. Reporter genes expression were measured with corresponding filters: CFP (ex. 430/7nm, em. 460/15nm), YFP (ex. 510/10nm, em. 520/10nm), and mRFP (ex. 590/7, em. 610/10nm).

IV. DISCUSSION

IV.1 Generating a multicolour yeast strain

Signal transduction enables cells to respond to changes in their environment, to survive and to maintain growth control. In the past, signal transduction was regarded explained as a linear connection between receptors stimulation and regulators of gene expression. However, signalling pathways are now being discovered to be a network of responses to environmental changes (Schwartz and Baron 1999). One of the best-described cascades, MAPK pathways, regulates diverse processes in cells (e.g. proliferation, differentiation and apoptosis). In budding yeast *Saccharomyces cerevisiae,* five signalling pathways involve MAPK cascades: the cell-wall integrity, the spore wall assembly, the filamentous/invasive growth, the pheromone response, and the high osmolarity glycerol pathway (Posas et al. 1998).

The aim of our study was to clarify how these different pathways, activated under different conditions of stress, cooperate or communicate with each other in order to regulate balance between survival and cell growth. This coordination may involve cross-talk between cascades due to the activation of kinases shared between these pathways (Gustin et al. 1998).

Previous studies have used strains carrying one or two chromosomal integrated reporter genes for investigating signalling pathway(s) of their interest: genes have been fused to GFP or luciferase (Deng et al. 2006, Leskinen et al. 2003) or tagged with various affinity tags such as TAP, His6 or GST (Gelperin et al. 2005, Ghaemmaghami et al. 2003, Huh et al. 2003, Martzen et al. 1999). To understand

the complex behaviour of signalling networks, we have generated a multi-colour fluorescent *S. cerevisiae* strain expressing five stress-induced reporter genes (ye*GFP*, ye*CFP*, ye*Citrine*, *mRFP* and *LacZ*) that are under control of corresponding yeast promotors (*GLO1, PMC1, FKS2, FLO11* and *FUS1,* respectively). To our knowledge, this is the first study on stress responses in yeast, where as many as five reporter genes were chromosomally integrated under control of five different promoters. This fact makes our YAK5 *S.cerevisiae* strain so unique as it allows the dynamic and simultaneous visualisation of different stress response pathways. To sense the activation status of each of the five stress pathways, the following promoters of highly regulated downstream genes were chosen:

- *LacZ* reporter is under *FUS1* control which gene products are required for proper cell fusion during mating.
- For expression of *mRFP* we used promoter region of *FLO11*, a GPI-anchored cell surface glycoprotein required for diploid pseudohyphal formation and haploid invasive growth (Kuchin et al. 2002).
- Recent study of endoplasmic reticulum stress in *S. cerevisiae* (Bonilla et al. 2002, Stathopoulos and Cyert 1997) showed an important link between calcium influx and cell wall integrity pathway. For that reason, we picked *FKS2*, an alternate subunit of glucan from PKC pathway and *PMC1*, a vacuolar Ca^{2+}-ATPase from calcium pathway (Hirayama et al. 2003) as genes of our interest. *FKS2* promoter drives LacZ reporter, whereas *PMC1* promoter controls Citrine (yellow fluorescent protein).
- GFP was under control of *GLO1,* monomeric glyoxalase I, whose expression is regulated by methylglyoxal levels and osmotic stress (Inoue et al. 1998).

IV.2. MAPK cross signalling in yeast

The results of exposing cells to caspofungin, an agent known to cause cell wall damage and surface stress (Bruno et al. 2006), clearly shows that it triggers several stress response pathways: strong activation of PKC and RIM pathway, as well as the activation of the calcium pathway at a low level (Figure 16). The first PKC response to caspofungin occurs very shortly after exposure. The second stronger PKC response occurs after 80 minutes following stimulation. Both PKC responses were followed with even stronger RIM response. These results clearly show that RIM and PKC pathways act in parallel in proper cell wall assembly as adaptation to antifungal drug. However, DTT was not able to trigger RIM pathway, but exclusively PKC and calcium. Calcium pathway activation appears as secondary response to cell wall remodelling as well as upon caspofungin treatment.

Possible explanation for this phenomena could be, that caspofungin mediate inhibition of glucan synthetase, might lead to leaks in the yeast cell wall. This cell wall permeability might in turn cause pH instability. Therefore, it is from high importance for yeast cell to survive two extremely inconvenience stress situations activating at the same time two totally independent and different stress response pathways, PKC and RIM. On the other hand, it seems that DTT attacks the cell wall but it allows cell wall permeability to stay intact.

In our study, we also show that alkaline pH exclusively triggers strong response in the PKC pathway (Figure 17). This would be explainable by the requirement of yeast to remodel their cell wall for growth at high pH. Our results are in part consistent with a model proposed by Castrejon (Castrejon et al. 2006) where

for the first time PKC pathway was triggered upon alkaline stress. However, why RIM pathway was not active by various extracellular pH changes is not clear. One possible explanation may be that high pH induce cell wall damage that in turn activate in a minute strong PKC pathway response (Figure 17) in order to manage cell wall remodelling and maintain its integrity.

A sustained adaptation to extracellular calcium was observed as long as cells were continuously exposed to low concentration as well as to high extracellular calcium (Figure 18). It is unlikely that low concentration of extracellular calcium directly activates calcineurin (Deng et al. 2006). There might exist a mechanism that senses an increase in extracellular calcium level and in turn causes a rise in intracellular calcium concentration, which then activates calcineurin to drive the continuous activation of the Pmc1 pump to maintain calcium homeostasis (Hirayama et al., 2003). Notably, increasing concentrations of extracellular calcium caused the increasingly activation of RIM as shown in Figure 18. This is the first report on the existence of activation of RIM pathway upon calcium stress (Figure 18). Nevertheless, mechanism of this interaction stays has to be discovered. PKC pathway stays inactive upon calcium influx in yeast cells (Figure 18).

IV.3 Future aspects of using multicolour fluorescent YAK5 strain

Five separate vectors were used to create the YAK strain carrying a stress-induced reporter explicit for one signalling pathway. Due to the existence of unique site of restriction enzymes (EcoRI, SacI, SacII, and SalI) in cassettes, it is possible to exchange the reporter gene or promoter region of these vectors, and therefore use them for additional reporter targets.

The *S.cerevisiae* Yak5 strain enable an easily monitoring of all five different stress pathways state, and therefore will also allow better modelling of these pathways and their interactions as a valuable tool for system biology approach.

Many features of cell wall construction and stress signalling are conserved between *Saccharomyces cerevisiae* and *Candida sp*. making *S. cerevisiae* a good model for the study of *Candida spp.* pathogenicity (Levin et al. 1990). *Candida species* are the major opportunistic human pathogens causing systemic fungal infections (Groll and Walsh 2001). Growing as harmless commensally colonizer in the gastrointestinal and genitourinary tracts and, on our skin *Candida sp.* can become an opportunistic pathogens, when the immune system of the host is weakened. Most host defences, are inducible after infection and rely mainly on the elimination of *Candida* by phagocytic cells of the innate immune system, especially through macrophages (Mansour and Levitz 2002). Since *Candida glabrata* codon table is similar enough to *S. cerevisiae*, our experiments can be used as a basis for generating a multicolour fluorescent *C. glabrata* strain as a useful tool in mouse experiments.

V. REFERENCES

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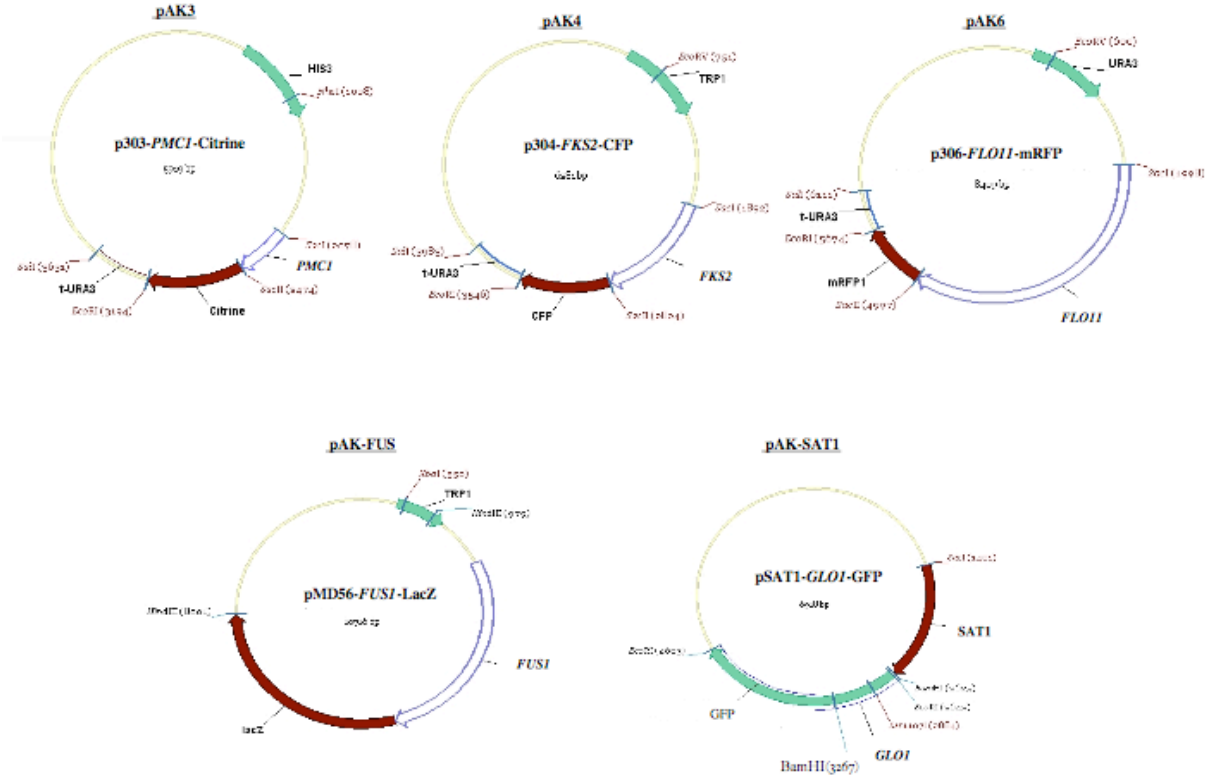
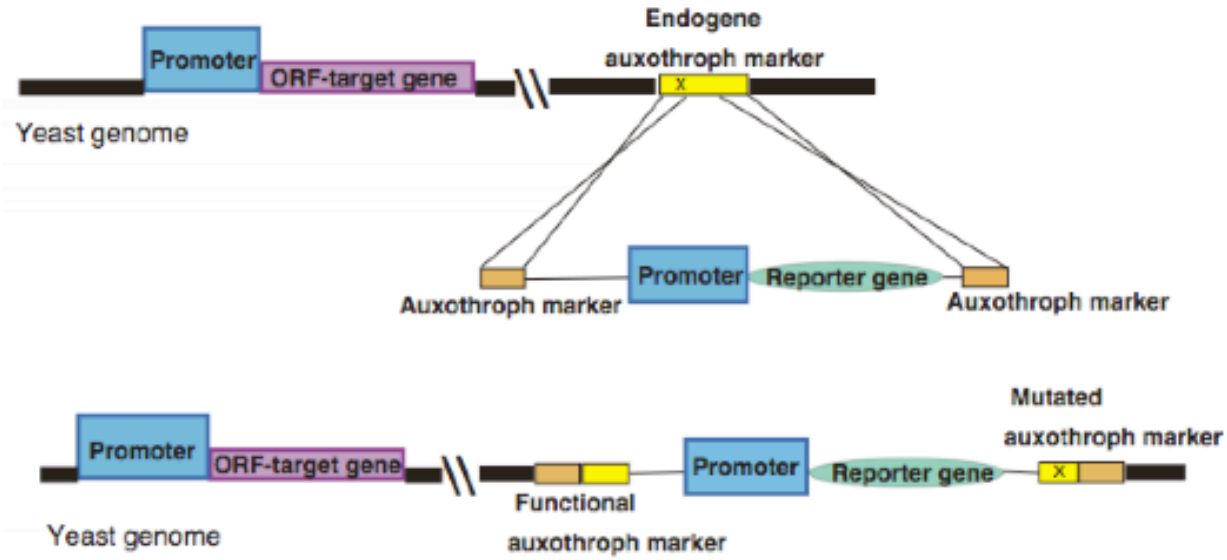
Supplement 1: Maps of used reporter gene plasmids, EUROSCARF, Sheff et al.(2002): yECitrine, yECFP and yEGFP

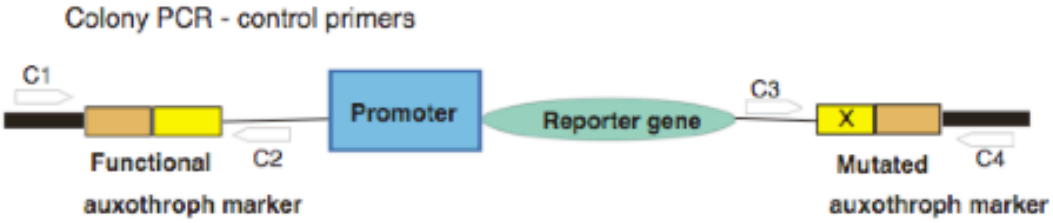
Supplement 2: Maps of pRSs (YIP) vectors used for transformation

Supplement 3: YIP fluorescent constructs



Supplement 4: Homologous Recombination Transformation & Colony PCR





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Curriculum Vita

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University Studies

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1998 – 2001, University of Belgrade, Serbia. Studied at the Faculty of Biology
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1986 – 1994, Elementary School, graduated with the maximal average mark.

Research & Professional Experience

Obligatory project:

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Group Leader: Karl Kuchler, Medical University of Vienna, Department of Molecular Genetics, Institute of Medical Biochemistry, Vienna, Austria

-

External project:

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- **August 2003**, "Development of intelligent vaccines"

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- **April – July 2003**, " RNA chaperone activity of large ribosomal subunit proteins of *E.coli*",

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- **July – September 2001**, " Imprinted status of the *Gallus Gallus* M6P-Igf2r gene", Group Leader: Denise P. Barlow, Institute of Molecular Biology, Austrian Academy of Sciences, Salzburg, Austria

External Courses:

- November 2004: "Medical Mycology" organized by Austrian Society for Medical Mycology at AKH, Vienna, Austria
- July 2003: Experimental Genetic "*In vitro* PCR-Mutagenesis" & " Gene disruption"

Experience in Management, Congress Organization

- **2004 – 2007**, Organizing Crew of the **1st**, **2nd FEBS Advanced Lecture Courses** on **Systems Biology (*"Systems Biology: From Molecules to Life"*)** with 200 participants in Gosau, Austria in March 2005 & 2007
- **2006 - 2008**, Organizing Crew of the **1st FEBS Special Meeting on ABC Proteins (*"ABC Proteins: From Multidrug Resistance to Genetic Disease"*)** with up to 350 participants in Innsbruck, Austria.
- **2006 - 2007**, Organizing Crew of the **32th FEBS Annual Congress (" *Molecular Machines*")** with up to 2000 participants in Vienna, Austria in July 2007
- **2006 - 2007**, **Organizing Committee** of the **7th FEBS Young Scientist Forum Meeting (" *Molecular Networks*")** with up to 104 participants from July 5-7, 2007 in Vienna, Austria

Poster Presentations

July 2007 at the 32nd FEBS Annual Congress "Molecular Machines" with the title "Cross-talk of MAP kinase pathways in *Saccharomyces cerevisiae*", Vienna, Austria

March 2008 at the 2nd FEBS Special Meating on ABC Proteins ("ABC Proteins: From Multidrug Resistance to Genetic Disease") with the title "Generating a multicolor *S. cerevisiae* strain", Innsbruck, Austria

Honors & Awards

1998, second price at the Serbian secondary schools-contest in Biology with a Project "Isolation of genomic DNA from mouse liver"

Languages

Serbian, Croatian, Bosnian: mother language level
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