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Functional Networks Involved in Cell Wall Biosynthesis and the Isoprenoid Pathway in the Yeast *Saccharomyces cerevisiae*

Ph.D. Thesis

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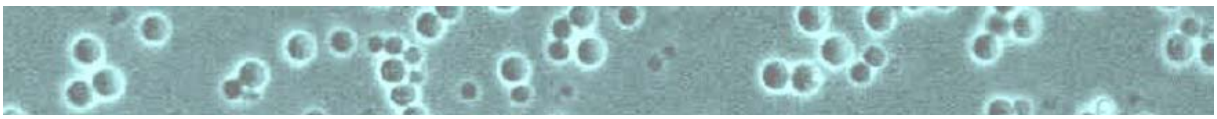
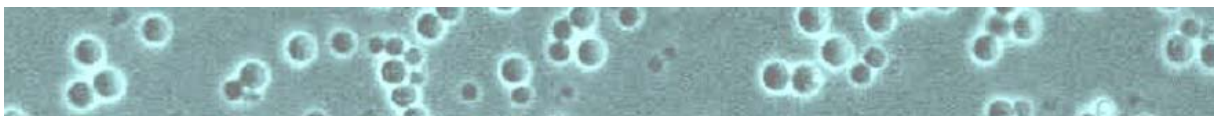


General Table of Contents

Detailed Table of Contents

INTRODUCTION



1 INTRODUCTION

Nowadays, we witness of impressive development of medical sciences that allow saving patients from life threatening diseases. However, at the same time the number of immunocompromised patients is growing. Patients of this group very often become infected with invasive or opportunistic fungi. Unfortunately, physicians have at their disposal only few antifungal drugs, which are at the same time harmful to the patients' health. Moreover, some fungal isolatesfrom the very specific environment of the hospital ward acquire resistance to the drugs used there. Therefore, an urgent demand exists for the discovery of novel antifungal drugs. For these reasons, many scientific teams are working hard studying fungal biology with the aim of revealing mechanisms which could be targeted by new antifungal drugs or which could explain the resistance to those already in use.

The majority of the presently available antifungal drugs (azoles, thiocarbamates and morpholines) target ergosterol biosynthesis, a branch of the isoprenoid pathway. Ergosterol in yeast is the equivalent of cholesterol synthesized in human, a critical health risk factor. Thus, the signal transduction involved in the regulation of both molecules, in yeast as well as in mammals, is of considerable interest. A second group of antifungal drugs target the fungal cell wall and are represented by echinochandins and papulacandins. For pathogenic fungi, the cell wall is the first point of interaction with the host. More importantly, it is essential for the fungus survival and morphogenesis while human cells do not possess this structure. For this reason, the fungal cell wall has been recognized as an attractive and safe target for antifungal drug development.

In this work, we affected, either pharmacologically or genetically, the cell wall integrity and the isoprenoid pathways, in an attempt to elucidate the mechanisms involved in the cell's response to stress conditions. For the experiments we chose the yeast *Saccharomyces cerevisiae* that is considered a good model to study fungal cell wall as well as the isoprenoid pathway. Most of the proteins and regulation features known so far are conserved between *S. cerevisiae* and pathogenic yeast, especially *Candida albicans*, one of the wide spread cause of fungal infections. Importantly, also yeast and mammalian cells are very similar in the arrangement and the regulation of the isoprenoid pathway.

Aim of the work

In this work, we focused our interest on the two pathways that are important for the survival of fungi treated with antifungal drugs, i.e. the cell wall assembly and isoprenoid biosynthesis. We examined the basic processes that are initiated while the cell struggles survival. Thus, the results obtained in this work may become useful in the future, in the attempts to design novel antifungal drugs.

In order to investigate the cellular response of yeast to an impairment of the cell wall, we disturbed the cell wall assembly using drugs that have distinct modes of action. Firstly, we treated cells with Papulacandin B, an inhibitor of the enzyme that synthesizes glucan which is one of the main components of the cell wall. Secondly, we treated cells with Congo red and Calcofluor white, compounds that do not inhibit enzymatic activities but bind directly to the fibrillar components of the cell wall, which causes weakening of the whole structure. Thirdly, we investigated caffeine, a supposed cell wall drug of unknown molecular function. Regarding the isoprenoid pathway, our goal was to investigate the response of the cells to chemical compounds that similarly to antifungal drugs inhibit this pathway.

The specific aims of the work were:

- Characterization of the yeast transcriptome response to the cell wall damage
- Investigation of the changes in the cell wall structure induced by the cell wall impairment and identification of the transcription factors involved
- Verification of caffeine action against the cell wall and identification of its cellular targets
- Characterization of the yeast transcriptome response to the isoprenoid pathway inhibitors that have opposing effects on the cellular availability of farnesyl diphosphate, a crucial molecule of this pathway
- Identification of genes that could potentially be regulated by changing the availability of farnesyl diphosphate
- Further investigation of the function of a representative gene belonging to the group of those potentially regulated by farnesyl diphosphate or its derivatives

1.1 YEAST CELL WALL

1.1.1 Characteristics of the fungal cell wall

The cell wall, despite its rigid appearance, is a highly dynamic structure adapting to changing environmental conditions. The yeast cell during its life increases in size and buds, thus changing its shape constantly. Moreover, during growth, the cell wall can adapt to a limited space, which was elegantly shown by obtaining cubic yeast cells (Suzuki *et al*., 2004a) (Figure 1). Modification of the cell wall shape can also occur more rapidly due to changes in the osmotic pressure of the environment. The yeast cell can shrink by up to 50% of its volume and this change of the shape is reversible, which illustrates the great elasticity of the cell wall. This elasticity could also explain why the cell wall of live cells is permeable for molecules larger than 760 Da, the mass that was established during studies on the isolated cell wall (Scherrer *et al*., 1974). Remarkably, it has been shown that the yeast cell wall exhibits local periodic nanomechanical motion depending on the temperature and ATP production (Pelling *et al*., 2004). However, the reason for the occurrence of these motions remains completely unexplained. Importantly, the cell wall not only changes its shape but also its composition and the present work is focused on these changes that occur in response to cell wall damaging drugs.

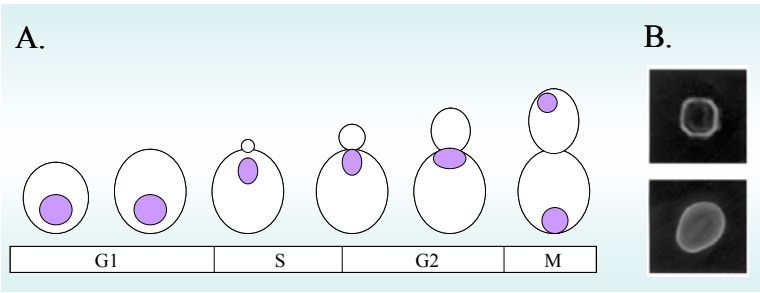


Figure 1: The shape of the cell wall is constantly changing during growth.

A. Main stages of the yeast cell cycle illustrating changes of the cell shape. **B**. Upper panel shows a yeast cell with altered shape, which was placed in a cubic hole and grown for 4 h. The lower panel shows an elliptical cell grown on the surface of a silicon wafer for 4 h (Suzuki *et al*., 2004a).

Electron microscopy shows that the cell wall is a layered structure. As indicated in Figure 2, this structure consists of an electron-transparent inner layer mainly built of β-1,3-glucan, β-1,6-glucan and chitin, and an electron-dense outer layer built of heavily glycosylated mannoproteins. While the inner layer gives the wall mechanical strength, the

outer layer is involved in cell-cell recognition and limits the permeability; together, the two layers provide physical protection and osmotic support to the cell (Smith *et al*., 2000).

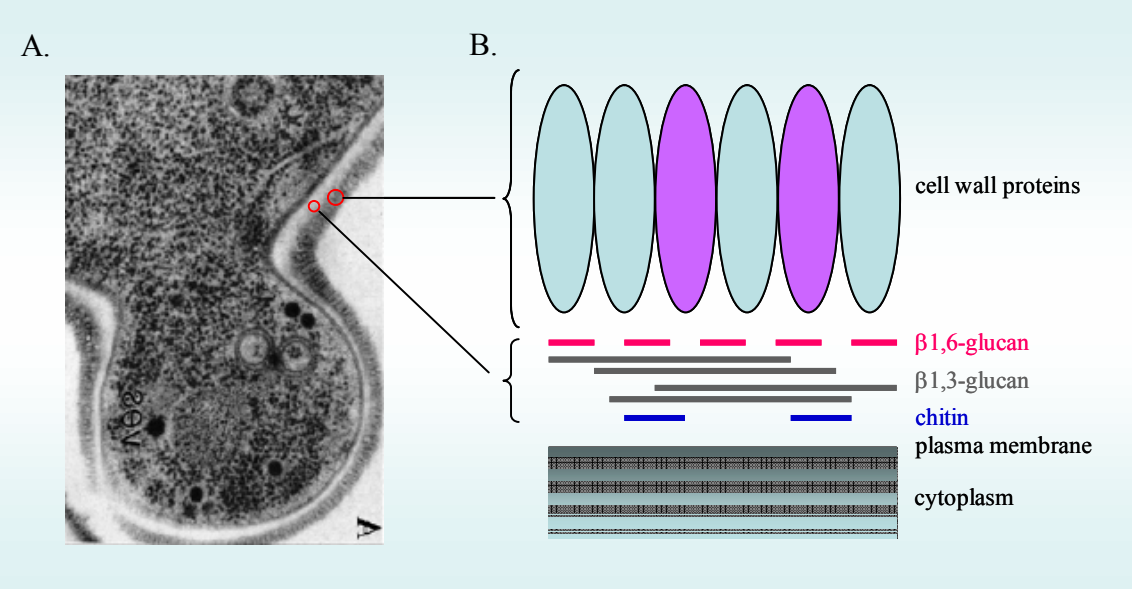


Figure 2: Outline of the yeast cell wall structure.

A. Budding cell of *S. cerevisiae*. Electron-transparent inner layer and electron-dense outer layer are visible (from (De Groot *et al*., 2005)). **B**. Model of the cell wall structure (adapted from (Smits *et al*., 1999)). The outer layer of proteins and the inner layer containing a network of carbohydrate polymers: β1,6-glucan, β1,3-glucan and chitin are indicated.

Characteristics of the cell wall constituents are summarized in Table 1 below. The mechanical strength of the cell wall and the above mentioned elasticity are mainly due to **β-1,3-glucan** consisting of approximately 1500 glucose monomers and representing approximately 50-55% of the dry weight of the cell wall. β-1,3-glucan chain forms a helix which can exist in different states of extension, explaining the elasticity of the structure formed. β-1,3-glucan is synthesized as a linear chain but the mature form contains 3-4% of β-1,6-linked glucose residues. This fact suggests the existence of additional processing enzymes rearranging the newly synthesized chains and integrating them into the existing wall. Proteins like Gas1p, Bgl2p and Crh1p are likely involved in these processes but their function *in vivo* is not well understood. Fraction of β-1,3-glucan is partially crystalline but the moderate branching of the mature chain prevents extensive crystallization.

Approximately 5 to 10% of the cell wall consists of a highly branched water-soluble polymer, **β-1,6-glucan**, built of about 130 glucose monomers. Although several genes have been identified to be involved in the biosynthesis of β-1,6-glucan (for a review see (Shahinian

and Bussey, 2000)), the mechanism of its synthesis remains largely unknown. The main function of this polymer *in vivo* is to enable connection of GPI- dependent cell wall proteins to the β-1,3-glucan network but it may also serve as an acceptor site for chitin.

Another constituent of the cell wall accounting only for 1-2% of the wall dry mass is **chitin** concentrated mainly in achitin ring and in the bud scars. In some exceptional situations, when the cell wall is weakened, synthesis of chitin is activated and then it can reach even 20% of the wall dry mass (Molano *et al*., 1980). This linear polymer is built of about 190 *N*-acetylglucosamine monomers and in *S. cerevisiae* it is synthesized by three tightly regulated chitin synthases (Cabib *et al*., 2001).

Table 1: Cell wall composition in the yeast *Saccharomyces cerevisiae* (from (Klis *et al*., 2002))

Macromolecule	Wall dry weight (%)	Site of synthesis	Branching
Mannoproteins	35-40	Secretory pathway	Highly branched
β-1,6-glucan	5-10	(Plasma membrane)	Highly branched
β-1,3-glucan	50-55	Plasma membrane	Moderately branched
Chitin	1-2	Plasma membrane	Linear

The outer protein layer accounts for about 40% of the wall dry mass. Proteins are covalently linked directly to the β-1,3-glucan-chitin network or indirectly through the β-1,6-glucan moieties. **Cell wall proteins** (CWPs) are so heavily glycosylated that their carbohydrate fraction can amount to over 90% of their mass. Usually, highly branched carbohydrate side chains are linked to asparagine residues, whereas short oligomannosyl chains are linked to the residues of serine and threonine. While the general function of the CWPs is limiting the cell wall permeability, the functions of particular proteins are often unknown. In general, CWPs are involved in processes such as mating, flocculation, biofilm formation, invasive growth, sporulation and the stationary phase of growth, response to low temperature and anaerobic growth (for a review see (Klis *et al*., 2002)).

Depending on the attachment to the cell wall constituents, CWPs can be divided into two groups, i.e. GPI-CWPs and Pir-CWPs. The first group of proteins are modified in the endoplasmic reticulum (ER) by addition of a glycosylphosphatidylinositol (GPI) anchor. This modification is required for trafficking of the protein to the plasma membrane. Then, depending on the sequence of the protein it will remain in the plasma membrane or will be liberated and linked to β-1,6-glucan through a remnant of the GPI anchor. In yeast, the

existence of approximately 60 GPI-CWPs has been predicted (Caro *et al*., 1997). The second group contains Pir proteins (Pir-CWPs) that are probably directly linked to β-1,3-glucan through an alkali-sensitive linkage. In *S. cerevisiae* four such proteins have been found, Pir1p, Pir2p/Hsp150p, Pir3p and Pir4p/Cis3p being involved in wall strengthening (Toh-e A *et al*., 1993). Additionally, some cell wall proteins like sexual agglutinin Aga2p or protease Bar1p might retain their cell wall localization through disulfide bonds with other proteins.

1.1.2 Cell wall biosynthesis and organization

1.1.2.1 Basic regulation of cell-wall polymer biosynthesis

In general, expression of CWP-encoding genes increases during bud formation, separation of the daughter cell and while mating projections are being formed, i.e. when the cell wall has to change its shape or structure (for a review see (Smits *et al*., 1999)). Consequently, expression of the cell wall genes is regulated by the cell cycle, nutrient availability and environmental conditions. Transcript levels of some genes encoding CWPs change upon respiratory growth or in the stationary phase. Some genes like *DAN1*, *TIP1* and *TIR1* are specifically induced under hypoxic conditions. Some others like *FLO11* and *PIR2* increase transcription during pseudohyphal growth.

From studies of genetic cell wall defects, it was concluded that lowered level of β-1,3-glucan caused an increase of chitin content in lateral walls and increased the amount of GPI-CWPs bound through β-1,6-glucan to chitin (Dallies *et al*., 1998; Garcia-Rodriguez *et al*., 2000b; Ram *et al*., 1998). Reduced level of β-1,6-glucan resulted in an increased amount of Pir-CWPs, β-1,3-glucan and chitin in the cell wall but also in the release of the GPI-CWPs into the medium (Kapteyn *et al*., 1999; Lu *et al*., 1995). Furthermore, defects in glycosylation caused an increase in chitin level (Kapteyn *et al*., 1999).

As we mentioned before, the major filamentous component of the cell wall, responsible for its rigidity, is β-1,3-glucan synthesized by **β-1,3-glucan synthase**. This enzyme is localized to the plasma membrane together with cortical actin patches allowing its translocation directly to the sites of the cell wall remodeling (Utsugi *et al*., 2002). There are two alternative catalytic subunits of glucan synthase, which are encoded by the *FKS1* and

FKS2/GSC2 genes and one regulatory subunit, Rho1p. The small GTPase Rho1p plays an essential role in the control of the cell wall synthesis, as in its active GTP-bound form it binds and activates Fks1p (Drgonova *et al*., 1996).

The expression of *FKS1* predominates in the optimal growth conditions and is cell-cycle regulated (Mazur *et al*., 1995; Ram *et al*., 1995) while *FKS2* is induced by mating pheromone, high extracellular Ca^{2+} concentration, in the *fks1*Δ mutant or in the stationary phase of growth.

Regarding β-1,6-glucan, which is the crucial component of the cell wall interconnecting all other components (Kollar *et al*., 1997), a gene coding for a putative **β-1,6-glucan synthase** has not been found. However, several genes were identified to be involved in the biosynthesis of this component, since their deletion caused a decrease in β-1,6-glucan and subsequent resistance to K1 killer toxin (for a review see (Bussey *et al*., 1990)). These genes were named *KRE* for killer toxin resistant (for a review see (Shahinian and Bussey, 2000)).

In *S. cerevisiae*, three **chitin synthases** are known Chs1p, Chs2p and Chs3p. They have the same polymerizing activity but deposit chitin at different times during the cell cycle and at different locations. Chs1p acts at the end of the cytokinesis to deposit chitin to the birth scar on the daughter cell. Chs2p synthesizes chitin in the primary septum. The majority of chitin is synthesized by Chs3p, which is localized to the ring at the base of the emerging bud and also to a lesser extent in the lateral walls (for a review see (Henar *et al*., 1999)). Expression of *CHS3* is not regulated by the cell cycle (Choi *et al*., 1994). In the steady state, Chs3p is localized to the internal stores called chitosomes, but under stress conditions, it is shifted to the plasma membrane where the biosynthesis occurs (Valdivia and Schekman, 2003). This redistribution is very rapid and requires the G protein Rho1p and protein kinase Pkc1p. The enzymatic activity and trafficking through the ER to the plasma membrane is regulated by several proteins – Chs4p/Skt5p, Chs5p, Chs6p and Chs7p. Chs4p is crucial for Chs3p binding to the septin ring in the bud-neck region. Chs5p and Chs6p are involved in the transport of chitosomes to the plasma membrane (Santos *et al*., 1997; Ziman *et al*., 1998). Chs7p plays a role in the exit of Chs3p from the ER (Trilla *et al*., 1999).

1.1.2.2 Guardians of the cell wall integrity

1.1.2.2.1 Cell wall integrity (CWI) pathway

In this chapter we describe the defensive mechanisms of the yeast cell allowing reinforcement of the cell wall. Indisputably, the cell wall integrity pathway (CWI) is so far the best characterized pathway permitting changes of the cell wall structure in response to a vast spectrum of events that a cell might encounter during its life. These events include high temperature (Kamada *et al*., 1995), hypotonic shock (Davenport *et al*., 1995), impairment of the cell wall synthesis (Ketela *et al*., 1999; Lagorce *et al*., 2003), exposure to cell wall binding agents such as Calcofluor white and Congo red (Garcia *et al*., 2004; Martin *et al*., 2000) as well as a response to rapamycin, an antibiotic that inhibits the TOR pathway (Torres *et al*., 2002). Depending on the source and the type of the stimulus, the CWI pathway can be activated at the 'top', through cell surface sensors, or laterally by activating one of the kinases eventually activating a mitogen activated protein (MAP) kinase, Mpk1p (Harrison *et al*., 2004).

As shown in Figure 3 below, the main activators of this pathway (Wsc1p, Wsc2p, Wsc3p and Mid2p) are integral plasma membrane proteins with heavily O-mannosylated extracellular domains (Ketela *et al*., 1999; Lodder *et al*., 1999). These proteins, upon stress of the cell wall, are able to activate a MAP kinase cascade (Pkc1p-Mpk1p) through Rom2p (Philip and Levin, 2001) and Rho1p, a small G-protein. Rho1p is active in its GTP-bound form and inactive in the GDP-bound form. Thus, both GTPase activating proteins (GAPs) and guanosine nucleotide exchange factors (GEFs) can regulate its action. Nevertheless, the main regulator of Rho1p, regarding response to the cell wall stress, is the Rom2 GEF (Ozaki *et al*., 1996). Rom2p can bind phosphatydylinositol-4,5-bisphosphate which allows its proper localization to the plasma membrane, where it can interact with the Wsc1 and Mid2 proteins can the bind inactive form of Rho1p in order to activate it. Four known GAPs, Bem2p, Sac7p, Bag7p and Lrg1p, seem to inactivate Rho1p in a target-specific manner. Thus, Lrg1p is involved in controlling Rho1p function concerning glucan synthase activity, Bem2p and Sac7p affect the MAP kinase cascade, while Bag7p is rather devoted to the control of the actin cytoskeleton.

When active, Rho1p activates in turn a linear MAP kinase cascade comprised of Pkc1p, Bck1p, two redundant Mkk1p and Mkk2p, and Mpk1/Slt2p. Loss of function of any of

the kinases below Pkc1p causes cell lysis at an elevated growth temperature. This effect can be prevented by osmotic protection, e.g. supplementation with 1 M sorbitol. Loss of the Pkc1p function causes lysis at any temperature and this effect is also osmoremedial. A few other phenotypes like sensitivity to Congo red, Calcofluor white or caffeine are typically associated with the MAP kinase cascade mutants (for an extensive review on CWI see (Levin, 2005)).

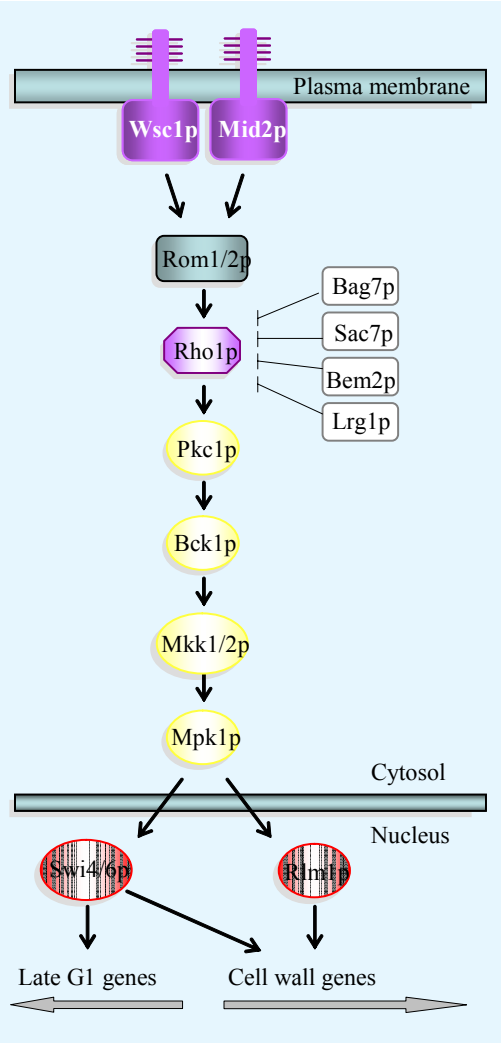


Figure 3: CWI in *Saccharomyces cerevisiae*.

The sensors of the cell wall damage located in the plasma membrane (Wsc1-3p, Mid2p) pass the signal through the Rom1/2 proteins to Rho1p, which in turn activates the Pkc1p-dependent cascade of MAP kinases (Bck1p, Mkk1/2p). The Mpk1 kinase activates its main targets, the Swi4p and Rlm1p transcription factors that activate expression of the cell wall genes. Arrows indicate activation of signaling, and bars indicate repression (adapted from (Levin, 2005)).

The main known output of the MAP kinase cascade is the activation of the SBF (Swi4/Swi6p) and Rlm1p transcription factors, which control, respectively, the expression of cell-cycle regulated genes at the G1/S transition (Madden *et al.*, 1997) and the cell wall-related genes (Jung and Levin, 1999). However, SBF has also been reported to control expression of several cell wall genes. This transcription factor is comprised of two proteins, Swi6p and Swi4p, of which the latter is activated by Mpk1 kinase in order to induce expression of the cell wall genes (Andrews and Herskowitz, 1989; Madden *et al.*, 1997). These genes are involved in different aspects of the cell wall biosynthesis that is coordinated with the cell cycle (β-1,3-glucan: *FKS1*, *GAS1*; β-1,6-glucan: *KRE6*; mannoproteins: *MNN1*, *VAN2*; and chitin: *CHS3*) (Igual *et al.*, 1996). In addition, Swi4p was proposed to activate the *GSC2* gene during a cell wall stress (Levin, 2005). Consistently, it was reported that cells deleted for *SWI4* were hypersensitive to Calcofluor white and sodium dodecyl sulfate, which suggests weakened cell wall.

1.1.2.2.2 An involvement of the TOR pathway in the cell wall organization

The TOR pathway is the major controller of growth and proliferation in eukaryotic cells. TOR kinases were discovered in yeast in a search of molecular targets of the immunosuppressive and anticancer drug rapamycin (TOR- target of rapamycin) (Heitman *et al*., 1991). This drug forms a complex with a conserved immunophilin, FKBP12 (*FPR1*-encoded in *S. cerevisiae*), which binds and inhibits Tor kinase. Yeast, having two homologous genes, *TOR1* and *TOR2*, are an exception among eukaryotes which have only one *TOR* gene. However, all eukaryotic organisms have two protein complexes TORC1 and TORC2 which are composed of distinct protein partners, and this organization is conserved also in yeast (TORC1: Tor1p or Tor2p, Lst8p, Kog1p and Tco89p; TORC2: Tor2p, Lst8p, Avo1/3p and Bit61p (Reinke *et al*., 2004)). As shown in Figure 4, the two yeast TOR proteins have a redundant function in the 'temporal' control of cell growth by regulating translation, transcription, ribosome biogenesis, nutrient transport and autophagy in response to nutrient availability (for a review see (Martin and Hall, 2005)). In addition, *TOR2* uniquely has a rapamycin-insensitive function in the 'spatial' control of cell growth by regulating the cell-cycle-dependent polarization of the actin cytoskeleton. This 'spatial' control of the cell growth by TORC2 is mediated by Rho1 GTPase and Pkc1 kinase (Helliwell *et al*., 1998b). Thus, Tor2p is able to perform all essential functions of Tor1p but Tor1p cannot perform all essential functions of Tor2p. This is reflected also in the fact that only deletion of *TOR2* is lethal.

Although TOR kinases are members of the protein family containing phosphatidylinositol 3-kinase (PI3K) related kinases (other yeast members: Tel1p, Mec1p and Rad53p), their lipid kinase activity has never been shown. On the other hand, both mammalian and yeast TOR kinases posses a Ser/Thr protein kinase activity. The role of the yeast TOR kinases has been extensively studied (for a review see (Crespo and Hall, 2002)) and it was reported to control the cell growth in response to nutrient signals. Under favorable nutrient conditions, TOR maintains some starvation-specific transcription factors (Gat1p, Gln3p, Msn2/4p, Rtg1/3p) in the cytoplasm where they are inactive. Upon starvation TOR is inactivated and the transcription factors can enter the nucleus where they induce transcription of specific genes. For instance, when TOR is inhibited by rapamycin or by nitrogen starvation, a heterodimeric transcription factor composed of Rtg1p and Rtg3p is immediately accumulated in the nucleus and activates transcription of TCA cycle and glyoxylate cycle genes (Komeili *et al*., 2000). Further, the expression of nitrogen responsive genes is regulated

by two GATA transcription factors, Gat1p and Gln3p. When TOR is active, Gln3p is in phosphorylated form and is retained in the cytosol by Ure2p. Probably, Ure2p also regulates Gat1p but a direct interaction has not been shown (Beck and Hall, 1999). The other proteins regulated by TOR are two redundant Zn^{2+} finger transcription factors, Msn2p and Msn4p. Active TOR promotes formation of the complex of Msn2p and Msn4p with cytoplasmic 14-3-3 proteins Bmh1p and Bmh2p preventing their entrance into the nucleus (Beck and Hall, 1999; Bertram *et al*., 1998). The stress response controlled by TOR includes also regulation of tRNA, rRNA and r-protein genes (Zaragoza *et al*., 1998). Moreover, it has been shown that repression of polymerase III transcription upon depletion of TOR kinases occurs via Maf1p (Pluta *et al*., 2001; Upadhya *et al*., 2002).

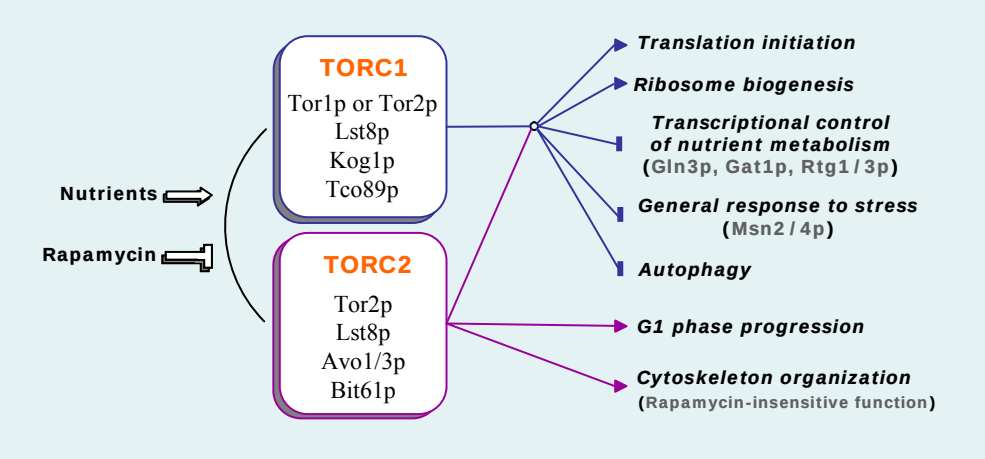


Figure 4: The TOR pathway in *Saccharomyces cerevisiae*.

In the presence of nutrients, the TORC1 and TORC2 complexes promote cell growth and proliferation. In the figure, functions shared by two complexes are shown and TORC2-specific functions such as G1 phase progression and cytoskeleton organization. The functions of TORC1 as well as TORC2 are inhibited by rapamycin except for cytoskeleton organization. Protein components of TORC1 or TORC2 are listed.

Recently, the TOR pathway has been implicated also in the regulation of the cell wall integrity, in particular the Pkc1p-Mpk1p signaling cascade. Torres and co-workers showed that Mpk1p was rapidly activated when TOR was inactivated by rapamycin or when cells entered stationary phase (Torres *et al*., 2002). Those authors proposed that TOR function could maintain the signal(s) that prevent the cell integrity pathway from being activated when cells grow under favorable nutrient conditions. Interestingly, another study showed that rapamycin-induced depletion of TOR caused remodeling of the cell wall that was dependent on Pkc1p but independent of Mpk1p (Krause and Gray, 2002). Further, some groups reported cell wall defects associated with particular TOR complexes. Regarding TORC2, it has been shown that a temperature-sensitive ts of *AVO3* exhibited cell wall defects and caffeine

sensitivity (Ho *et al*., 2005). In another report, a connection between Tor2p and Rom2p has been established. However, this link concerned only regulation of the actin cytoskeleton through the components of the CWI pathway, Rom2p and Rho1p, while effects on the cell wall integrity were not tested in that case. Recently, Powers and co-workers described cell wall-related phenotypes of the *tor1*Δ and *tco89*Δ mutants (Reinke *et al*., 2004). Additionally, they identified an *SSD1*-mediated cell integrity pathway as partially alleviating these phenotypes. Importantly, the observed cell integrity phenotypes were not rescued by additional copies of *PKC1* or *MPK1*. Overall, depletion of TOR causes remodeling of the cell wall but the exact way of signaling from TOR to the cell wall remains largely uncharacterized.

1.1.2.2.3 An involvement of the cAMP signaling cascade in cell wall organization

The Ras/cAMP pathway, similarly to the TOR pathway, is responsible for the control of growth in response to nutrients and mediates global stress response. As shown in Figure 5, an increased level of cAMP in the cell activates protein kinase A (PKA), which regulates transcription of numerous genes. PKA holoenzyme is a heterotetramer composed of two regulatory and two catalytic subunits (Toda *et al*., 1987a; Toda *et al*., 1987b). Binding of cAMP to the regulatory subunits of PKA results in the releasing of the catalytic subunits and in their activation. cAMP is biosynthesized by *CYR1*-encoded adenylate cyclase. There are two major ways to activate this enzyme (for a review see (Thevelein and de Winde, 1999)). The first is mediated through Ras2 GTPase. To make this activation possible, Ras2p has to be modified and translocated to the plasma membrane with the help of Erf2p. The second way to activate adenylate cyclase goes via Gpr1p receptor and Gpa2 G-protein that activates adenylate cyclase in response to high glucose or low nitrogen. Another protein, Plc1p, enables a physical interaction between these two proteins (Ansari *et al*., 1999). Recently, it has been proposed that the Ras/cAMP pathway is also under control of TOR kinases. The subcellular localization of PKA catalytic subunit, Tpk1p, depends on TOR kinases (Schmelzle *et al*., 2004). When TOR and/or PKA are inactivated, cells exhibit similar effects like the G_1 cell cycle arrest, accumulation of storage carbohydrates (trehalose, glycogen) and enter the stationary phase of growth, which is accompanied by reinforcement of the cell wall structure. The entrance into the stationary phase of growth requires Rim15p. In the presence of

nutrients, Rim15p is inhibited by PKA-mediated phosphorylation and, additionally, TOR prevents its nuclear localization (Pedruzzi *et al*., 2003). However, Rim15p is not the only protein known to be regulated by both Ras/cAMP and TOR pathways, since it is also the case of the Msn2/4p transcription factor (Beck and Hall, 1999; Boy-Marcotte *et al*., 1998; Gorner *et al*., 1998). The exact mechanism of Msn2/4p repression by PKA is not known, although these proteins link functionally the Ras/cAMP signaling to the cell wall integrity. Lagorce and co-workers have shown that Msn2/4p contributes to the response of yeast cells to the cell wall impairment (Lagorce *et al*., 2003). In that study, a cluster comprising 79 genes that were induced in the cell wall mutants was identified. Fifty-seven percent of those genes contained in their promoter regions the STRE element that is a binding site for Msn2p and Msn4p transcription factors.

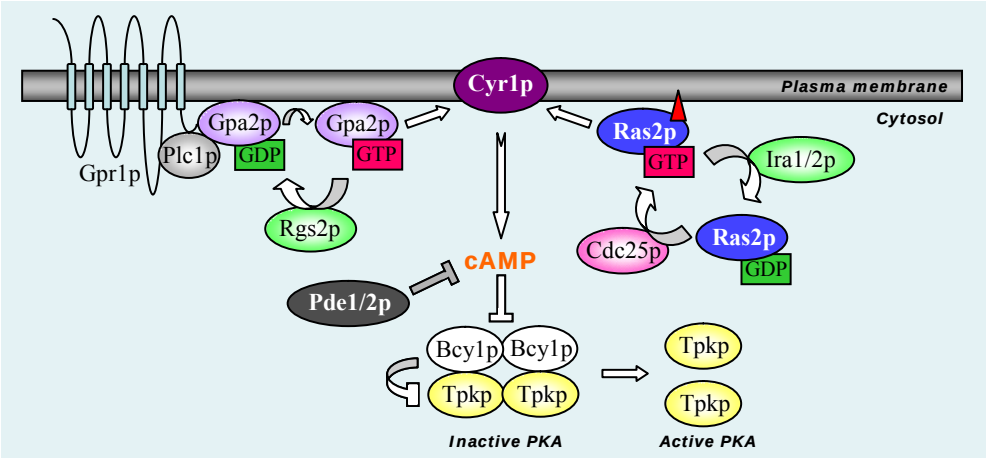


Figure 5: Activation of the cAMP signaling cascade.

The common action of Gpr1p receptor, Plc1p and Gpa2 G protein activates adenylate cyclase (Cyr1p). Gpa2p can be inactivated by GAP Rgs2p. Another way to induce cAMP biosynthesis goes through the Ras signaling. Ras2p is anchored to the plasma membrane via palmitoyl moiety (red triangle). GEF Cdc25p promotes an active state of Ras2p while GAPs, Ira1p and Ira2p, inactivate this protein. The intracellular concentration of cAMP can be reduced by the action of the Pde1 and Pde2 cAMP phosphodiesterases. A complex of protein kinase A (PKA) comprises two regulatory subunits (Bcy1p) and two catalytic subunits (Tpk1p, Tpk2p or Tpk3p). cAMP binds PKA regulatory subunits which leads to dissociation of the complex and activation of the catalytic subunits.

Another link between the Ras/cAMP pathway and the cell wall integrity is the fact that PKA is a negative regulator of Crz1p transcription factor (Kafadar and Cyert, 2004). Crz1p is required for the induction of the *GSC2/FKS2*-encoded glucan synthase's expression but only in some particular conditions like response to mating pheromone, high concentration of $CaCl_2$ or loss of *FKS1* function (Zhao *et al*., 1998). While PKA phosphorylates Crz1p and negatively regulates its import to the nucleus, calcineurin, a protein phosphatases, promotes nuclear localization of Crz1p where it is active. Calcineurin is activated by an increased level

of Ca^{2+} in the cell. Probably, when the cell wall structure is weakened, an increased plasma membrane tension activates mechanosensitive channels and intracellular concentration of Ca^{2+} is elevated.

To summarize, the cell wall integrity can be affected also by Msn2/4p and Crz1p transcription factors, which are both regulated by the Ras/cAMP pathway.

1.1.3 Cell wall drugs - Papulacandin B, Congo red, Calcofluor white and Caffeine

In order to study cell wall defects, two approaches -'genetic' and 'chemical'- are often in use. The genetic approach is based on studies of cell wall defects in mutants impaired in the cell wall functions. This method is limited in the sense that weakening of the cell wall caused by deletion of a given gene results in various reinforcing reactions which at the same time depend on the particular defect. In other words, for instance, many mutations can cause a decrease in β-1,3-glucan level but the response of the cell will depend not only on the fact that the level of β-1,3-glucan is decreased but also on the particular mutation which permits or not a proper cellular response. The chemical approach uses various chemicals to disturb the cell wall structure. An obvious limitation of this method is the fact that usually the exact mechanism of action of a given agent is not known and we cannot exclude activation of some additional cellular processes that are not implicated in cell wall biosynthesis. In this work, we investigated the action of four drugs affecting the cell wall, however, in some cases, we observed transcriptional effects which could not be simply explained by impairement of the cell wall structure.

1.1.3.1 Papulacandin B

In this study, we focused our interest on Papulacandin B, a lipophilic antifungal antibiotic synthesized in nature by the fungus *Papularia spaerosperma*. Structurally, Papulacandin B is a glycolipid, which consists of a modified disaccharide linked to two fatty-acyl chains (see structure in Figure A 1, pg 157) (Traxler *et al*., 1977). This compound is known mainly for its inhibitory effect on β-1,3-glucan synthase (Baguley *et al*., 1979; Kopecka, 1984b) but it was shown to inhibit also other plasma membrane enzymes like ATP

synthase, adenylate cyclase and 3',5'-cyclic phosphodiesterase (Surarit and Shepherd, 1987). Papulacandin B in yeast causes a transition of the polar growth into spherical one and the cell walls become apparently fragile and amorphous. Dividing cells fail to separate properly and at a higher concentration of the antibiotic (8-32 μg/ml) perforations in the cell wall occur, leading to cell lysis. These perforations are the most frequent at the basis of the bud or at its apex and eventually the cell proliferation is stopped (Komiyama *et al*., 2002; Kopecka, 1984a). Mutations in 22 genes caused hypersensitivity to Papulacandin B but, surprisingly, no correlation was found with changed levels of glucose, mannose or glucosamine (Lussier *et al*., 1997; Ram *et al*., 1994) leading to the conclusion that Papulacandin B- hypersensitivity was not specific to the cell wall mutants. In order to learn the molecular mechanisms which drive the cellular effects mentioned above we decided to analyze the transcriptional response of yeast cells to Papulacandin B.

1.1.3.2 Congo red and Calcofluor white

Here, we describe two compounds, Congo red and Calcofluor white, which exert their effects through a similar mechanism leading to the loss of the cell wall integrity. Both compounds induce phosphorylation of Mpk1p, an effect that is mediated by the Mid2p sensor, and can be diminished by 1 M sorbitol. Remodeling of the cell wall in response to these agents is dependent on Mpk1 kinase (de Nobel *et al*., 2000).

Congo red (CR) is a dye known to interfere with the glucan network, which results in the loss of the cell wall rigidity (see structure in Figure A 1, pg 157) (Kopecka and Gabriel, 1992). Nevertheless, it was also suggested that it could bind chitin (Raclavsky *et al*., 1999). A model was proposed in which CR binds the helical chains of β-1,3-glucan leading to the loss of interaction between the helices. A second model, which is not exclusive with the first one, proposes that CR could interfere with the action of enzymes responsible for the assembly of the cell wall components (for a review see (Klis *et al*., 2006)). Whatever the reason, protoplasts regenerating the cell wall in the presence of CR cannot form thick fibrils of glucan assembled into broad ribbons, which are present in the walls of cells growing without the presence of the dye (Kopecka and Gabriel, 1992). In addition, cells treated with CR were shown to deposit more chitin in the lateral walls and septa (Vannini *et al*., 1987) and they formed chains of cells, as they were unable to disconnect (Kopecka and Gabriel, 1992).

Calcofluor white (CFW) is an antifungal drug that interacts specifically with chitin in yeast (Roncero *et al*., 1988a; Roncero and Duran, 1985) and with cellulose in other organisms (see structure in Figure A 1, pg 157). Yeast cells in the presence of this drug in the medium increase the biosynthesis of chitin, but the biosynthesized polymer has an abnormal structure and does not provide an appropriate support for the cell. Several mutants have been isolated in which resistance to CFW was accompanied by a decreased chitin content (Roncero *et al*., 1988b; Trilla *et al*., 1999). Although all these data suggested that chitin was a primary target of CFW, later other mutants were discovered which had apparently functional chitin biosynthesis (Lussier *et al*., 1997). Additionally, it has been shown that the sensitivity to CFW is also dependent on the high-osmolarity glycerol response (HOG) pathway. This pathway is normally required in yeast for growth in a high-osmolarity environment, like medium supplemented with 0.9 M NaCl or 1.5 M sorbitol. However, an involvement of this pathway in the CFW sensitivity is not clear. While deletion of genes coding for particular components of the HOG pathway increased resistance to CFW, activation of Hog1p was not detected in wild-type cells grown in the presence of CFW. Nevertheless, the intracellular level of glycerol was increased upon CFW treatment (Garcia-Rodriguez *et al*., 2000a).

1.1.3.3 Caffeine

Caffeine (1,3,7-trimethylxanthine) is an analogue of purine bases that has been implicated to affect a variety of functions in different organisms including antagonistic effects on adenosine receptors or stimulation of muscle contraction (see structure of caffeine in Figure A 1, pg 157). There is also increasing evidence that caffeine inhibits formation of some tumors and induces apoptosis in already existing tumors (Hashimoto *et al*., 2004). However, effects of caffeine at the cellular level are not clear (exemplary effects are reported in Table 2). Original studies on the caffeine action have shown that it could affect the level of intracellular cAMP, hence modifying the cAMP-dependent protein kinase (PKA) pathway (description of the PKA pathway is given in chapter 1.1.2.2.3, pg 13). However, the effects of caffeine on cAMP level and PKA pathway are very controversial. Some authors reported an increase of cAMP due to the inhibition of the enzyme cyclic 3',5' nucleotide phosphodiesterase (Butcher and Potter, 1972; Liao and Thorner, 1981; Tsuzuki and Newburgh, 1975), others observed a blockage of a glucose-induced cAMP increase in the

presence of caffeine (Tortora *et al*., 1982); {J. François, unpublished data} or an absence of any effects of this drug on cAMP (Tsuboi and Yanagishima, 1973).

Another reported effect of caffeine was an exacerbation of chromosomal breakages induced by UV irradiation, alkylating agents or maleic hydrazide in plants and animals (Kihlman *et al*., 1973; Lehmann and Kirk-Bell, 1974). The recent finding that caffeine can inhibit *in vitro* phosphoinositide 3-kinase (PI3K)- related protein kinases ATM and ATR (Blasina *et al*., 1999; Hall-Jackson *et al*., 1999), whose major role is to control DNA damage checkpoints during the cell cycle (Kuruvilla and Schreiber, 1999), may give some clues regarding the mechanism by which caffeine elicits the hypersensitivity to the DNA-damaging agents. Yeast homologues of those proteins, Tel1p and Mec1/Rad3p, are regulators of telomere length, and their inhibition causes reduction of telomere length, which could account for the lethal actions of caffeine in yeast (Saiardi *et al*., 2005).

In fungi, caffeine is currently used as a phenotypic criterion to evaluate the performance of the cell wall integrity (CWI) pathway, since mutants defective in the components of this pathway are usually caffeine-sensitive (Costigan *et al*., 1992; Jacoby *et al*., 1998; Martin *et al*., 2000; Martinez-Pastor *et al*., 1996; Park *et al*., 2005; Watanabe *et al*., 1997). The sensitivity to caffeine was also correlated with the cell wall related mutants (Lussier *et al*., 1997). So far, the best-studied effect of caffeine is an increase in the cellular amount of phosphorylated Mpk1 kinase, which is diminished by addition of sorbitol (Martin *et al*., 2000).

It was thought that the cell wall integrity pathway mutants were hypersensitive to caffeine due to their weakened cell wall. However, for several reasons it is unlikely that caffeine acts on the cell wall directly; rather, it exerts its effect only after entering the cell. First, caffeine was suggested to be actively imported into the yeast cells by an unknown adenine permease (Bard *et al*., 1980) and addition of adenine to the yeast culture alleviated caffeine effects. Another reason is the fact that overexpression of *SNQ2*, a gene coding for a plasma membrane transporter involved in multidrug resistance, was able to suppress the caffeine sensitivity of the *mpk1*Δ mutant (Martin *et al*., 2000). This result suggested that *SNQ2* could be involved in pumping caffeine out of the cells. Altogether, these reports imply that by blocking the entrance into the cell or by expulsing caffeine out of the cell, we can eliminate its detrimental effects.

Table 2 : Exemplary cellular effects of caffeine observed in various organisms

Caffeine effect:	Organism/Cells	Reference
Increase of cAMP	- Day 7 and day 15 guinea pig endometrium cells in culture	(Naderali and Poyser, 1997)
	- Alveolar macrophages from rat lung	(Jafari and Rabbani, 2000)
	- ADPKD (Autosomal dominant polycystic kidney disease) cells	(Belibi *et al*., 2002)
	- Porcine oocyte	(Kren *et al*., 2004)
	- *Trichoderma harzianum*	(Silva *et al*., 2004)
	- *Schizophyllum commune*	(Kinoshita *et al*., 2002)
Decrease of cAMP	- HKC (Human Kidney Cortex) cells	(Belibi *et al*., 2002)
	- *Saccharomyces cerevisiae*	(Tortora *et al*., 1982)
Induction of apoptosis	- Human neuroblastoma cells	(Jang *et al*., 2002)
	- Human pancreatic adenocarcinoma cells	(Gururajanna *et al*., 1999)
	- Human A549 lung carcinoma cells	(Qi *et al*., 2002)
	- Mouse epidermal JB6 Cl 41	(He *et al*., 2003)
Repression of cell proliferation	- Human lung adenocarcinoma	(Qi *et al*., 2002)
Induction of cell prolirefation	- Human fibroblast	(Taylor *et al*., 1993)
	- Murine T-lymphoma cells	(Palayoor *et al*., 1995)
	- Chinese hamster V 79 cells	(Labanowska *et al*., 1988)
Exacerbation of chromosomal breakage	- plant and animal cells	(Kihlman *et al*., 1973)
	- mammalian cells	(Lehmann and Kirk-Bell, 1974)
Inhibition of PI3K-related kinases	- breast cancer cells	(McMahon *et al*., 2005)
	- HeLa cells	(Blasina *et al*., 1999)
	- HEK293 cells	(Hall-Jackson *et al*., 1999)
Induction of Mpk1 kinase phosphorylation	*Saccharomyces cerevisiae*	(Rajavel *et al*., 1999) (Martin *et al*., 2000)

1.2 ISOPRENOIDS in yeast

1.2.1 Isoprenoid biosynthetic pathway

In nature, the biosynthesis of isoprenoids occurs through two distinct pathways, using two distinct precursors, i.e. deoxyxylulose 5-phosphate in eubacteria and higher plants (Lange and Croteau, 1999), or mevalonate characteristic for archebacteria and eukaryotes. In eukaryotic cells, production of isoprenoids starts with the biosynthesis of acetoacetyl coenzyme A catalyzed by *ERG10*-encoded thiolase (Figure 6). This and subsequent reactions lead to the biosynthesis of mevalonate which in the following steps is transformed into farnesyldiphosphate (FPP), a compound serving in the cell as a precursor of numerous essential metabolites like sterols, dolichols, ubiquinons and as a substrate for protein prenylation. In human, this pathway produces also such critical end products like steroid hormones, cholesterol and bile acids and for this reason it has been extensively studied in mammals, while its equivalent in yeast gained considerably less interest. In yeast, the two last reactions leading to the synthesis of FPP are catalyzed by FPP synthase (FPPS) encoded by the *ERG20* gene. In the first step of this reaction, FPPS condenses isopentenyl diphosphate (IPP) and dimethylalyl diphosphate (DMAPP) to form geranyl diphosphate (GPP), in order to add another molecule of IPP in the second step (Anderson *et al*., 1989b). Prior to the synthesis of FPP, a molecule of DMAPP is created by *IDI1*-encoded IPP isomerase (Anderson *et al*., 1989a). Interestingly, FPPS competes for the limited pool of DMAPP with Mod5p responsible for the synthesis of isopentenylated (i^6A) tRNA, thus disrupting the balance between both enzymes is going to affect not only FPP synthesis but also translation (Benko *et al*., 2000).

Once FPP is synthesized, various enzymes consume it. However, the cell requirement for sterols is greater than for the non-sterol products of the IP pathway. The first enzyme consuming FPP and committed to the branch of **sterol biosynthesis** is squalene synthase (SQS). In yeast, SQS is known to be regulated at the transcriptional level by heme-activating protein transcription factors, namely Hap1/2/3/4p, yeast activator protein transcription factor yAP-1 and the phospholipids transcription factor complex Ino2/4p (Kennedy *et al*., 1999). Further, in a sequence of 15 enzymatic reactions, squalene is converted to the final sterol product, ergosterol that is accumulated in yeast. This final product is integrated into

membranes and secretory vesicles (Bagnat *et al*., 2000) and it plays a role in endocytosis (Heese-Peck *et al*., 2002) and oxygen sensing (Hughes *et al*., 2005).

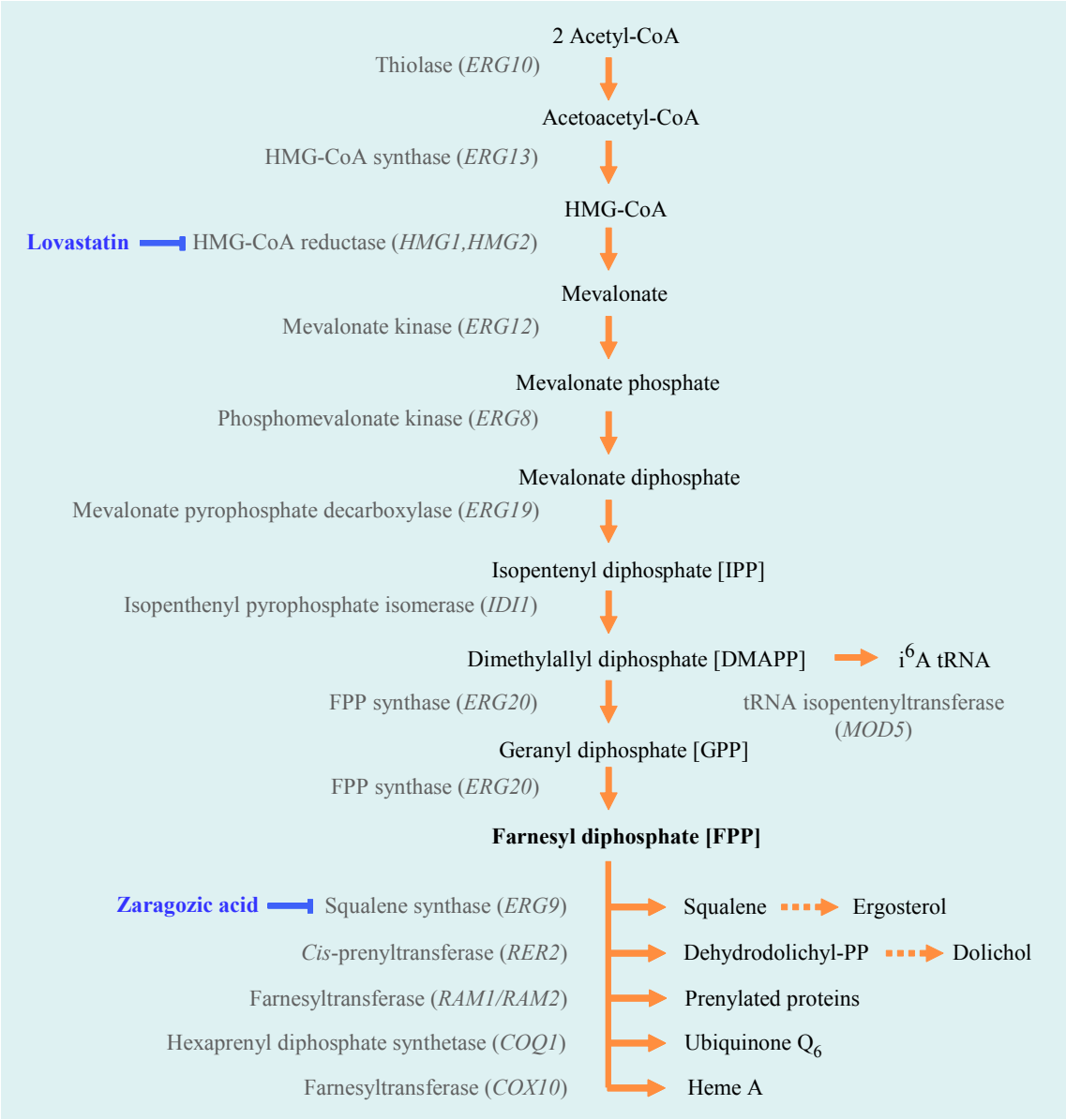


Figure 6: Isoprenoid (IP) pathway in the yeast *Saccharomyces cerevisiae*.

The sequence of reactions is shown with the enzymes catalyzing them. Names of the genes coding for the enzymes are given in parentheses. Enzymes affected by lovastatin and zaragozic acid, inhibitors of the IP pathway, are indicated. For some compounds abbreviations of the names, which are used later in the text, are given in the brackets. The dashed lines indicate the multi-component pathways (adapted from (Grabinska and Palamarczyk, 2002)).

The next branch of the IP pathway depending on FPP is the **biosynthesis of dolichol**. The first enzyme, exclusively dedicated to this task, is *cis*-prenyltransferase encoded by the *RER2* gene. Rer2p is a peripheral ER protein and as revealed by immunofluorescence microscopy, it is present in the form of dots in the ER membrane (Sato *et al*., 2001). Cis-

prenyltansferase uses FPP as a starter and catalyzes subsequent additions of IPP molecules to form a long-chain (12-24 isoprene units) polyprenyl diphosphate (Adair, Jr. and Cafmeyer, 1987). Polyprenyl diphosphate is immediately dephosphorylated by isoprenoid phosphatases and by the action of unknown α–saturase dolichol is formed (for a review see (Grabinska and Palamarczyk, 2002)). In addition, the *SRT1* gene has been found to encode a protein with enzymatic activity of *cis*-prenyltransferase, however, *SRT1* is expressed only under particular conditions of growth and synthesizes longer polyprenols then does the main *cis*-prenyltransferase ((Sato *et al*., 2001; Szkopinska *et al*., 1997). The number of isoprene residues in the dolichol molecule is specific for each species and varies from 14 to 17 for *Saccharomyces cerevisiae* (Quellhorst, Jr. *et al*., 1998) to 19 to 22 in mammals (Rip *et al*., 1985). The function of dolichol is generally associated with the biosynthesis of glycoproteins, but in fact, it is dolichol phosphate, localized mainly to the ER membranes, that is involved in this process. Free dolichol in the cells is present mainly in the lysosomes and peroxisomes and has been shown to reside in the center of the membrane bilayer, parallel to its plane. Up to date the precise function of free dolichol remains unclear. Based on membrane studies, it has been proposed that dolichol can inhibit the leak of protons (H^+, Na^+ and K^+) through biological membranes (for a review see (Haines, 2001)) which could at least partially explain its high amount in the membrane of organelles with extreme pH, like lysosomes or peroxisomes. Interestingly, dolichol accounts for 14% of the mass of neuromelanin pigment in dopaminergic neurons of the human *substantia nigra* and it was proposed to play there an antioxidant role (Bergamini *et al*., 2004).

FPP or GPP can be also used for **protein prenylation**, a post-translational modification of proteins that are usually implicated in signal transduction pathways. Generally, this modification is regarded to be important in the attachment of proteins to the cellular membranes but also in protein-protein interactions including those that facilitate protein trafficking and subcellular localization. In yeast, four proteins are involved in this kind of protein modification, i.e. type II (RAB-protein) geranylgeranyltransferases Bet2p and Bet4p, and both farnesyltransferases and geranylgeranyltransferases Ram2p and Ram1p. Ram1p and Ram2p are both required for membrane localization of Ras proteins and α-factor but only Ram2p is an essential protein (He *et al*., 1991).

Ubiquinone Q is another product of the isoprenoid pathway requiring FPP for its biosynthesis. This lipid, a soluble antioxidant, participates as an electron carrier in the mitochondrial respiratory chain, oxidation of sulfide and also regulates the physicochemical properties of membranes. All the genes identified in yeast (*COQ1-8*) that are involved in the

biosynthesis of ubiquinone Q have a mitochondrial targeting signal, nevertheless, ubiquinone Q is probably actively transported as it is found in all the cellular compartments (for a review see (Turunen *et al*., 2004)).

FPP is also used in the biosynthesis of **heme A**, a prosthetic group of cytochrome c oxidase. This process begins with farnesylation of heme B (protoheme) by *COX10*-encoded farnesyltransferase (Tzagoloff *et al*., 1993). In human, a *COX10* ortholog is associated with mitochondrial disorders.

Altogether, products of the IP pathway take part in various cellular processes, therefore it is important to precisely regulate the flux of intermediates through the pathway to satisfy the needs of the cell. Briefly, we can say that transcription of the genes encoding enzymes of the IP pathway is regulated by the levels of the cellular sterols as well as the non-sterol derivatives of FPP, and by FPP itself. Aspects of this regulation are discussed in the next chapter.

1.2.1.1 Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase.

So far, the best-studied enzyme of the early steps of this pathway is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). This enzyme is responsible for the overall control of the synthesis of the non-sterol and sterol products, which is of particular importance in human, where HMGR controls the biosynthesis of cholesterol. Currently, statins, a class of HMGR inhibitors are used to treat hypercholesterolemia (described in the next chapter 1.2.2). There is a high degree of similarity between mammalian and yeast HMGR. Unlike the prokaryotic enzyme, the eukaryotic one is not freely dispersed in the cytoplasm but instead is tethered to the ER membrane through an additional domain (Hampton *et al*., 1996). Yeast have two HMGR isozymes encoded, respectively, by the *HMG1* gene that provides 83% of the total cellular activity and the *HMG2* gene (Basson *et al*., 1986). These two isozymes are differently regulated, which is probably a consequence of different destination of their products. The *HMG1*-encoded HMGR is the dominant enzyme in the logarithmic phase of growth in aerobic conditions while the *HMG2*-encoded enzyme becomes more important in anaerobiosis or in the stationary phase of growth. Therefore, it was proposed that Hmg1p would rather supply isoprenoids for processes requiring oxygen, such as heme biosynthesis, cyclization of squalene and demethylation of lanosterol while the need for Hmg2p activation would occur due to the accumulation of isoprenoids during anaerobiosis (Casey *et al*., 1992;

Thorsness *et al*., 1989). Interestingly, a very recent study which investigated sterol-regulated gene expression in *Schizosaccharomyces pombe* (fission yeast) discovered that regulation of gene expression by sterol responsive element was a part of the oxygen-sensing mechanism (Hughes *et al*., 2005). It is likely that this pathway is used for the same purpose in other yeast species, especially that sterol synthesis requires oxygen.

In addition, overexpression of the *HMG1* and *HMG2* genes brings divergent results for the cell morphology. Both genes when overexpressed cause proliferation of smooth ER membranes that nevertheless have different protein composition and different cellular localization. The Hmg1p-induced membranes (karmellae) are tightly associated with the nucleus while the Hmg2p-induced ones can be located in the cytoplasm or associated with the nucleus or plasma membrane (Hampton *et al*., 1996).

The regulation of HMGR occurs at multiple levels including transcription, translation, and protein stability controlled by the IP pathway products (for a review see (Panda and Devi, 2004)). While Hmg1p seems to be very stable, with a half-life in excess of 8 h, Hmg2p half-life is regulated by rapid degradation depending on signals derived from mevalonate. The physiological role of this degradation is not clear, since it is Hmg1p that serves as the main source of the HMGR activity and not Hmg2p. However, since Hmg2p has been a model protein for studies of protein degradation we have now a clear picture of the feedback regulation of the mevalonate pathway acting via Hmg2 p but we lack the information about Hmg1p regulation. Hmg2p in response to signals derived from mevalonate is ubiquitinated and targeted to proteasome 26 S for its degradation. The signals for degradation can originate from the different steps of the mevalonate pathway:

i) Inhibition of HMGR with lovastatin leads to a decrease of Hmg2p ubiquitination and degradation. Conversely, inhibition of squalene synthase with zaragozic acid leads to a rapid degradation of Hmg2p. This was consistent with the regulation through FPP level and it was further confirmed by genetic experiments showing that overexpression of a gene encoding squalene synthase had a stabilizing effect on Hmg2p while downregulation of this gene facilitated protein degradation (Gardner and Hampton, 1999; Hampton and Bhakta, 1997). Thanks to a recent work led by Hampton (Shearer and Hampton, 2005) it became clear that it was not FPP itself but the FPP-derived farnesol that was responsible by changing the Hmg2p structure into a less folded state more sensitive for degradation.

ii) Degradation of Hmg2p is enhanced upon oxysterol accumulation in the cells. Oxysterol can be created via cyclization of 2,(3S),(22S),23-dioxidosqualene. However, unlike

in mammalian cells oxysterols alone are not a sufficient signal to cause the Hmg2p degradation in yeast (Gardner *et al*., 2001).

Information concerning Hmg1p regulation is more restricted. It was shown that Hmg1p was not ubiquitinated but high level of ergosterol strongly inhibited *HMG1*-dependent squalene synthesis when this process was barely affected in a strain expressing only *HMG2*. In mammals, regulation of gene expression by sterols is possible due to the presence of sterol regulatory element (SRE-3) in their promoter sequence which is recognized and positively regulated by SRE-3-binding protein 1 (SREBP1) (Spear *et al*., 1994). Whether this kind of regulation exists in yeast remains an open question.

1.2.2 Isoprenoid pathway inhibitors.

In this work, we investigated the effects of two inhibitors of the IP pathway, lovastatin and zaragozic acid (see structures in Figure A 1, pg 157). The exact sites of their action are presented in Figure 6 (pg. 21).

Lovastatin (Lo) belongs to a larger group of statins which are known to inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) (Alberts *et al*., 1980) In human, HMGR catalyzes the rate-limiting step in cholesterol biosynthesis and due to this fact statins are extensively used in medical practice. They bind HMGR at nanomolar concentrations leading to competitive displacement of the natural substrate, HMG-CoA, which binds at micromolar concentrations (Istvan and Deisenhofer, 2001). However, the medical use of statins suffers major setbacks, as their effects in the cell are broader than those that could be explained just by inhibition of HMGR. For example, lovastatin has been shown to cause cytoskeletal actin fragmentation in cardiomyocytes (Kong and Rabkin, 2004) and to affect immune response in the mouse (Sun and Fernandes, 2003). Lovastatin-induced apoptosis was proposed to be a mechanism of its suppressing effects on tumor genesis and metastasis described in various rodent models (Otsuki, 2004). In yeast, lovastatin (40-50 µg/ml) has been shown to inhibit the biosynthesis of squalene, oxysterol and ergosterol and to increase the cellular amount of Hmg1p (Dimster-Denk *et al*., 1994; Gardner *et al*., 2001). Moreover, the inhibition of Hmg2p ubiquitination was observed upon lovastatin treatment (25 µg/ml, 30 min incubation) (Gardner and Hampton, 1999).

Zaragozic acid (ZA) was discovered in a fungal culture isolated from a water sample. The name of this compound comes from the place of the origin of this sample, the city of Zaragoza in Spain. The established function of this naturally derived compound is inhibition of squalene synthase (SQS). The general structure of zaragozic acid is analogous to that of presqualene diphosphate, an intermediate in squalene synthesis, thus it was proposed to mimic its binding to the enzyme (Bergstrom *et al*., 1993). Inhibition of SQS causes an increase of FPP utilization in other branching pathways of isoprenoid metabolism. In mammalian cells, inhibition of squalene synthase by zaragozic acid was accompanied by a decrease of cholesterol levels and an increase in IPP (5-fold), FPP (20-fold), farnesol and organic acids levels. Additionally, the levels of dolichol and dolichyl phosphate increased (3-fold) as well as the amount of ubiquinone (90%) (Bergstrom *et al*., 1993; Keller, 1996). Most likely the observed increase in the amount of organic acids is caused by conversion of farnesol into farnesoic acid and consequently in a dicarboxylic acid (Vaidya *et al*., 1998). Farnesol is produced from FPP by farnesyl pyrophosphatase (Bansal and Vaidya, 1994) and it exhibits various biological effects like cell growth inhibition or induction of HMG-CoA reductase degradation (Bradfute and Simoni, 1994; Meigs *et al*., 1996). In yeast, zaragozic acid (10 µg/ml) has been shown to inhibit the biosynthesis of squalene, oxysterol and ergosterol without affecting other modes of acetate metabolism, like fatty acids biosynthesis (Dimster-Denk *et al*., 1994; Gardner *et al*., 2001). Effects of zaragozic acid have been shown on the both Hmg1 and Hmg2 proteins. Treatment of yeast cells with this inhibitor caused a decrease of *HMG1*-lacZ activity and induced Hmg2p ubiquitination (Dimster-Denk *et al*., 1994; Gardner and Hampton, 1999). It has also been shown that zaragozic acid caused an increase in expression of *ERG9*, a gene coding for SQS (Kennedy *et al*., 1999).

To summarize, both inhibitors have a repressing effect on the sterol branch of the IP pathway. However, their major action differs in the effects on the part of the pathway at the level of FPP biosynthesis. While lovastatin would decrease the flux of the substrates also through this section, zaragozic acid, by inhibiting the main FPP-consuming enzyme, would increase FPP availability in the cell. Since FPP and FPP-derived farnesol are positive signal for the Hmg2p ubiquitination (Gardner and Hampton, 1999; Hampton and Bhakta, 1997) in many studies lovastatin and zaragozic acid were used to alter inversely the availability of these compounds in the cell and to examine details of Hmg2p degradation (e.g. (Cronin *et al*., 2000; Gardner *et al*., 2001; Shearer and Hampton, 2005)). In the present work, we used this model to study response of yeast cells to the impairment of the isoprenoid pathway.

1.2.3 General description of Yta7 protein.

Here, we present a characteristic of Yta7p based on the work of other groups and our previous unpublished results that are not included in the present study.

YTA7 is not an essential gene and its deletion does not cause any severe phenotype except the slight sensitivity to 6% ethanol (Agostoni Carbone *et al*., 1998) and the decreased sensitivity to killer toxin 1 (K1) (Page *et al*., 2003) that binds the cell wall β-glucan receptor subsequently forming lethal pores in the plasma membrane. It was also proposed that Yta7p could be one of the targets for Cdk1/Cdc28 kinase (Ubersax *et al*., 2003), suggesting that its activity could be dependent on the progression of the cell cycle. Our group found Yta7p to interact in a two-hybrid screen with Erg20p (FPP synthase). Moreover, it was shown, using gas liquid chromatography, that the amount of squalene increased 2-fold in the *yta7*Δ mutant {Grabińska, unpublished}.

The *YTA7* gene has been sequenced and the resulting protein is attributed to the family of ATP-ases Associated with diverse cellular Activities (AAA) as it contains two ATP-binding sites (Figure 7) (Agostoni Carbone *et al*., 1998). However, with increasing knowledge about this family it becomes clear that the 'diverse cellular activities' are in fact mediated by structural remodeling, i.e. unfolding and disassembly of proteins and protein complexes (for a review see (Lupas and Martin, 2002)). The AAA domain is wide-spread in nature and represented in all kingdoms. In *S. cerevisiae* alone 22 proteins of the "triple-A" family have been found. Yta7p contains also a degenerated bromodomain. Bromodomains have been shown to be acetyllysine-binding modules (Hudson *et al*., 2000) and have been suggested to bind acetylated histones and other chromatin-associated proteins. However, Yta7p lacks the critical conserved tyrosine residue required for acetyllysine recognition by proteins involved in restriction of silencing (Dhalluin *et al*., 1999) and this position is taken by serine and threonine. Yta7p orthologues, in the related species of *S. paradoxus*, *S. mikatae*, *S. bayanus* and the less related *Debaromyces hansenii*, contain the same serine-threonine substitution in their bromodomains. Similar substitution exists in mouse and human Tif1 b (Jambunathan *et al*., 2005).

Then, a more precise database search allowed the identification of three additional domains in Yta7p sequence (Figure 7) {M. Grynberg, unpublished data}. The N-terminal part (8-312aa) of Yta7p shows high similarity to an internal repeat fragment of the rat

microtubule-associated protein MAP1A (1336-1630), a structural protein involved in the filamentous cross-bridging between microtubules and other skeletal elements (Langkopf *et al*., 1992). The C-terminal part of Yta7p (1114-1329) was homologous to the N-terminal domain of syntaxin 1A, a protein required for membrane fusion (Bennett *et al*., 1993). At both the N- and C-termini of Yta7p, ER membrane retention signals were discovered (Teasdale and Jackson, 1996).

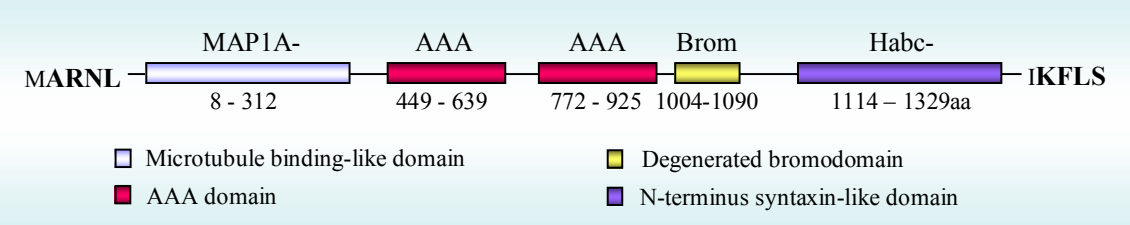


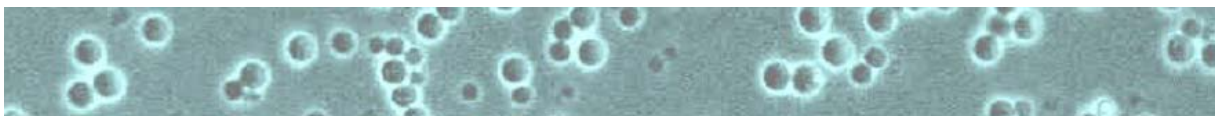
Figure 7: Model of domain structure of Yta7p.

The numbers below the structure depict the boundaries of each domain. The names of domains are given above. The Bromodomain name was shortened for space purposes to Brom. The bolded amino acid sequences shown at the termini are the putative ER membrane retention signals {M. Grynberg, unpublished data}.

While this work was in progress Tackett and coworkers (Tackett *et al*., 2005) proposed that Yta7p was a component of a chromatin-associated complex of proteins that helps to define and preserve boundaries separating silent and active chromatin. In yeast, the majority of chromatin remains active and the remaining ~10% is concentrated mainly near the telomere at the rRNA-encoding locus. The best-studied example of this epigenetic regulation are the silent mating type loci, HML and HMR. In this work, Yta7p was copurified with Top2, Rpb2, Spt16, Sas3, Ylr455 and histones H3, H2B, H2A, Htz1 and H4. Moreover, deletion of the *YTA7* gene caused an increased silencing of the *URA3* reporter gene placed near a boundary region which suggested an involvement of Yta7p in maintaining active transcription near the silent regions of chromatin. A function of Yta7p in this process was further supported by another work of Jambunathan and coworkers (Jambunathan *et al*., 2005). In their study *YTA7* was identified as a gene which, when mutated, allowed inappropriate spreading of silencing from HMR. Moreover, the degenerated bromodomain of Yta7p has been shown to be capable of binding histones *in vitro*.

Altogether, these data supported mainly an involvement of Yta7p in silencing but other features of this protein suggested that it could be additionally involved in other cellular processes.

RESULTS and DISCUSSION



2 RESULTS and DISCUSSION

2.1 YEAST RESPONSE TO CELL WALL DAMAGE

2.1.1 Response to an inhibitor of β-1,3-glucan synthase, Papulacandin B

In order to examine the response of yeast cells to the inhibition of glucan synthesis, we performed genome-wide expression analysis in wild-type cells treated with various concentrations of Papulacandin B. After heaving determined the minimal inhibitory concentration (MIC) as 2.5 µg/ml {A. Lagorce, unpublished data}, we decided to treat cells with 0.1, 1 and 10×MIC value for 1h. MIC was the smallest concentration of the drug that caused total inhibition of the growth measured after 24 h of incubation with the drug. As a result, we obtained three transcription profiles where sub-inhibitory (0.1 MIC) and inhibitory (1 MIC) concentration of Papulacandin B affected similar number of genes (48 and 59, respectively), and the highest concentration used affected roughly 4 times as many genes (207) (Figure 8A).

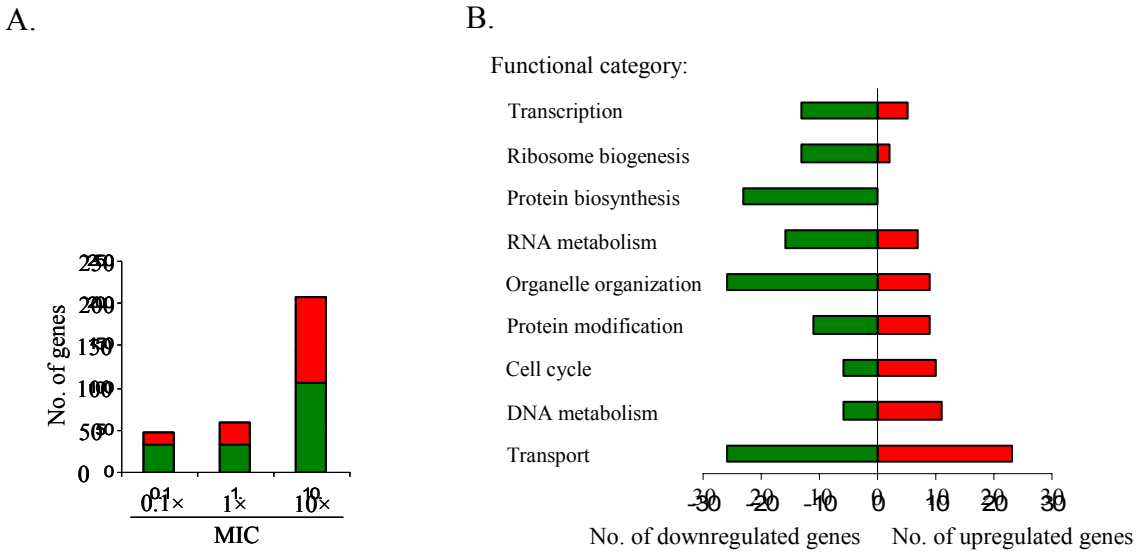


Figure 8: Transcriptional response of yeast cells to Papulacandin B.

A. Number of genes whose expression significantly changed due to 1h treatment with indicated concentrations of Papaulacandin B. Overexpressed genes are represented in red and underexpressed genes in green. **B**. Distribution of differentially expressed genes among functional categories according to Slim Mapper.

To have an overview of the general changes of gene expression in response to Papulacandin B we pooled the genes affected by all concentrations tested and distributed them among the functional categories according to Slim Mapper (Figure 8B, above) (method in chapter 3.1.4.3.2, pg 122). It could be seen that the majority of these genes fell into broad categories such as transport, organization of organelles and RNA metabolism. Interestingly, we observed a strong downregulation of genes involved in biogenesis of ribosomes and the biosynthesis of proteins, which was consistent with the observed inhibition of growth.

Next, to assess the contribution of the expression of genes from specific gene classes to the total gene expression, we grouped the genes according to gene ontology nomenclature (1389 groups), using T-Profiler. This software uses an unpaired *t* test which gives a measure of significance to the difference between the mean of a specific class of genes and the mean of the remaining genes of the total gene expression profile (see method in chapter 3.1.4.3.2, pg 122). As expected, Papulacandin B affected mainly genes encoding proteins localized to the cell wall and in particular some cell wall structural constituents, which was in agreement with its action against β-1,3-glucan synthase (Table 3, below). Moreover, we again detected downregulation of ribosome biogenesis and protein biosynthesis. However, since this effect was elicited only by the highest concentration of this drug, we can suppose that downregulation of these categories was probably not associated with the specific action of Papulacandin B but it was a result of strong growth inhibition. Analysis of the data sets with T-Profiler also allows the identification of regulatory motifs in the promoters of responsive genes. It is important to note that T-Profiler does not take into account how often a particular motif occurs in a promoter region. Using this tool, we detected significant (*t* value = 4.21) upregulation of genes containing in their promoters the Rlm1p-binding motif (TAWWWWTAGM). This result suggested that Papulacandin B induces changes in the cell wall structure through the Rlm1p transcription factor, the major target of the CWI pathway.

Table 3: Functional categories affected by Papulacandin B treatment [a]

Functional category (no. of ORFs)	*t*-value at MIC:		
	0.1	1	10
Sructural constituent of the cell wall (9)	4.57	4.01	-
Cell wall component (91)	-	5.18	4.44
Protein biosynthesis (440)	-	-	-6.35
Component of ribosome (248)	-	-	-7.39

[a] The number of ORFs in each category is shown in parentheses. The selected categories have a number of ORFs ≤ 500 and a *t* value ≥ 4.
'-' indicates that a given category was not significantly changed (P value < 0.05).

Further, we used the GeneSpring software that allows clustering of similarly expressed genes (see method in chapter 3.1.4.3, pg 120). Using this method, we clustered all the genes whose expression was significantly changed and obtained a sub-cluster comprising 29 genes that were overexpressed at all concentrations of Papulacandin B tested (Figure 9, below). Among these genes, we found *MLP1*, a gene coding for Mpk1-like protein kinase that has been proposed to activate the Rlm1p transcription factor independently of Mpk1p (Watanabe *et al*., 1997). This result implied that this kind of regulation might occur in response to Papulacandin B, which was further supported by the fact that upregulation of the *MPK1* gene was not detected in any of the tested conditions. On the other hand, the expression of *BCK1*, encoding an upstream component of the Pkc1p-Mpk1p signaling cascade, was induced. A genetic interaction between *MLP1* and *BCK1* has already been reported: overexpression of *MLP1* rescued a caffeine-sensitive phenotype of cells depleted in the *BCK1* gene (Watanabe *et al*., 1997). Therefore, we can envisage that Papulacandin B, by impairing cell wall biosynthesis, activates the CWI pathway but the signal to the Rlm1p transcription factor is relayed by Mlp1p instead of the Mpk1 kinase. Verifying this hypothesis needs further investigation.

Additionaly, in the cluster of upregulated genes, we found some typical cell wall genes like *CWP1*, *PIR3*, *FIT3*, *FLO11*/*MUC1* and *SED1*. *SED1* is a gene coding for the major structural constituent of the cell wall that is normally expressed during the stationary phase of growth (Shimoi *et al*., 1998). We also found a protein of unknown function, encoded by *YDR134C*, which was nevertheless localized to the cell wall. Another upregulated gene was *PRM5* that is known to be regulated by the Rlm1p transcription factor (Jung and Levin, 1999). Interestingly, this cluster contained also *RHK1*/*ALG3*. Alg3p is an alpha 1,3-mannosyltransferase that catalyzes the addition of the sixth mannose moiety to the lipid-linked oligosaccharide required for N-glycosylation of proteins. This protein was shown previously to be involved in cell wall mannoprotein biosynthesis, thus its presence in this cluster is also justified (Kimura *et al*., 1997).

ORF	Gene	Description
YKL161C	*MLP1*	Mpk1-like protein kinase
YKL096W	*CWP1*	Cell wall mannoprotein
YML100WA		Uncharacterized
YKL147C		Uncharacterized
YKL141W	*SDH3*	Succinate dehydrogenase
YOR383C	*FIT3*	Cell wall protein involved in iron transport
YPR044C		Uncharacterised
YDR077W	*SED1*	Cell surface glycoprotein
YDL122W	*UBP1*	Ubiquitin-specific protease
YKL163W	*PIR3*	Cell wall protein
YDR134C		Protein localized to the cell wall
YML083C		Uncharacterized
YBR267W		Uncharacterized
YBL082C	*RHK1*	Dol-P-Man dependent α1,3-mannosyltransferase
YDR543C		Uncharacterized
YIL102C		Uncharacterized
YDR197W	*CBS2*	Cytochrome b translational activator
YGL157W		Dihydrokaempferol 4-reductase
YKR046C		Protein involved in aerobic respiration
YDL197C	*ASF2*	Anti-silencing protein
YGL023C	*PIB2*	Phosphatidylinositol 3-phosphate binding protein
YLR378C	*SEC61*	ER protein of translocation apparatus
YNL017C		Uncharacterized
YKL214C	*YRA2*	Yeast RNA Annealing protein
YDR093W	*DNF2*	Potential aminophospholipid translocase
YNR071C		Uncharacterized
YIR019C	*FLO11*	Cell surface flocculin
YIL117C	*PRM5*	Pheromone-Regulated Membrane protein
YNR077C		Uncharacterized

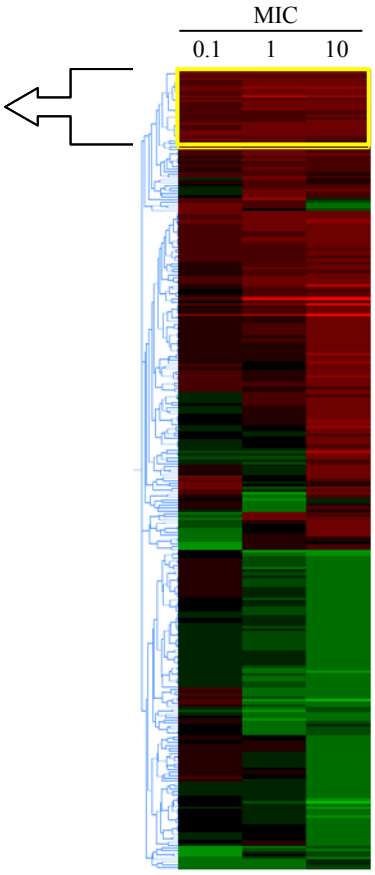


Figure 9: Hierarchical clustering of genes responding to Papulacandin B treatment.

Figure represents cluster of 287 genes that significantly changed expression in response to Papulacandin B. The list of genes represents a sub-cluster comprising 29 genes that were overexpressed at all concentrations of the drug (0.1, 1 and 10 MIC) tested. For clustering, standard correlation was used as a similarity measure.

The next step of the analysis was to identify more specific and narrow categories of genes affected by Papulacandin B stress. To this end, we used Term Finder, a statistical tool provided at the SGD database, which estimates significance of an involvement of a particular biological process in the response elicited by the conditions tested. The number of genes representing a given process in the transcription profile was compared with the number of genes representing this process in the whole genome, without taking into account their ratio of expression (see method in chapter 3.1.4.3.2, pg 122).

This analysis revealed upregulation of genes involved in the invasive growth and cell adhesion as the processes significantly affected by Papulacandin B (Table 4, below). Importantly, this group of genes comprised *FLO11*, a hallmark of the invasive growth. This result brings an important insight into the mechanism of defence of yeast against antifungal drug as it suggests that the presence of the drug could in fact stimulate biofilm formation. Biofilm is a tight structure of normal yeast cells mixed with elongated pseudohyphal forms,

which are attached to solid surfaces. Formations of this kind often colonize various medical devices and cause serious problems at intensive care units, since biofilm is much more resistant to antifungal drugs than cells growing separately. The ability of cells to grow invasively and to increase their adhesion is essential for biofilm formation. Thus, it would be interesting to verify whether treatment of yeast with a non-inhibitory concentration of Papulacandin B could result in a change of the yeast growth to pseudohyphal.

In addition, it turned out that the tested drug elicited induction of genes involved in ATP biosynthesis, namely *OLI1*/*ATP9*, *ATP8* and *ATP6* encoding integral membrane proteins and components of the mitochondrial ATP synthase (for a review see (Devenish *et al*., 2000)). *OLI1*- and *ATP6*-encoded subunits are components of the proton channel, while *ATP8*-encoded subunit remains unassigned to any particular function. Remarkably, *OLI1* was the gene that reached the highest rate of induction in the whole dataset (10.6-fold induction). This result suggested that Papulacandin B could inhibit ATP biosynthesis. This explanation could be supported by one of the original reports showing that Papulacandin B inhibited ATP-ase, localized to the plasma membrane in the yeast *Candida albicans* (Surarit and Shepherd, 1987).

Moreover, Term Finder pointed out to the repression of polyamine biosynthesis as strongly associated with the Papulacandin B stress (Table 4, below). This most significantly changed category (P value 7.13^{-05}) contained just three genes *SPE1*, *SPE2* and *SPE3*, although the whole process involves only four genes. Spe1p, ornithine decarboxylase, catalyzes the first step in polyamine biosynthesis. Spe2p, S-adenosylmethionine decarboxylase, produces S-adenosylmethioninamine that serves in subsequent reaction, catalyzed by Spe3p, for the synthesis of spermidine. The explanation of this result is not easy, since the biological function of polyamines in yeast is not clear. For instance, it is known that spermidine is required for growth of wild-type cells. The probable function of polyamines is stabilization of DNA by association of their amino groups with the phosphate residues of DNA. They are especially abundant in quickly dividing cells (Tabor and Tabor, 1985).

Table 4: Processes associated with Papulacandin stress according to Term Finder

Process	Probability	Gene	Ratio	Description
Invasive growth (activated)	0.00070	*RIM21*	1.54	Protein that affects invasiveness of haploid strains
		FLO11	1.66	GPI-anchored cell surface glycoprotein required for diploid pseudohyphal formation and haploid invasive growth
		STE20	1.67	Signal transducing kinase of the PAK (p21-activated kinase) family, involved in pheromone response and pseudohyphal/invasive growth

		DIA1	1,57	Protein of unknown function, involved in invasive and pseudohyphal growth
		STE5	1.57	Scaffold protein that, in response to pheromone, shuttles from the nucleus to the plasma membrane and assembles kinases Ste11p, Ste7p, and Fus3p into a specific signaling complex
Cell adhesion (activated)	0.00610	*MUC1*	1.66	GPI-anchored cell surface glycoprotein required for diploid pseudohyphal formation and haploid invasive growth
		HSP12	1.83	Plasma membrane localized protein that protects membranes from desiccation; induced by heat shock, oxidative stress, osmostress, stationary phase entry, glucose depletion, oleate and alcohol
		SAG1	2.13	Alpha-agglutinin of alpha-cells, binds to Aga1p during agglutination
ATP synthesis coupled to proton transport (activated)	0.00812	*OLI1*	10.66	F0-ATP synthase subunit 9 (ATPase-associated proteolipid)
		ATP6	5.0	Mitochondrially encoded subunit 6 of the F0 sector of mitochondrial F1F0 ATP synthase
		ATP8	2.37	Subunit 8 of the F0 sector of mitochondrial inner membrane F1-F0 ATP synthase
Polyamine biosynthesis (repressed)	7.13^{-05}	*SPE1*	0.47	Ornithine decarboxylase
		SPE2	0.58	S-adenosylmethionine decarboxylase
		SPE3	0.66	Putrescine aminopropyltransferase

2.1.1.1 Discussion

Papulacandin B is an antifungal drug that exerts its main action by inhibiting β-1,3-glucan synthase (Kopecka, 1984b). However, this enzyme is not the only cellular target of this antibiotic, since it has detrimental a effect also on protoplasts (cells of which the cell walls were removed by treatment with cell-wall lytic enzymes) (Komiyama *et al*., 2002). The genome-wide expression analysis of cells treated with various concentrations of Papulacandin B performed in this work has shown that an additional target of this drug could be the mitochondrial complex of ATP synthase. Since this antibiotic has a lipophilic character it could possibly interact with this transmembrane complex. This hypothesis is likely because Papulacandin B has already been shown to be capable of inhibiting plasma membrane proton pumping ATP-ase activity in a related fungus, *Candida albicans*. However, it was not the mitochondrial ATP synthase but a synthase localized to the plasma membrane.

As we expected, genes coding for the cell surface proteins were the most affected. We observed a significant upregulation of the cell wall genes as well as a significant upregulation of genes containing an Rlm1p-binding motif in their promoter regions. This result implies that in the tested conditions transcription of the cell wall genes could be activated through the Rlm1p transcription factor, which is the case in other kinds of the cell wall stress (Garcia *et al*., 2004; Jung *et al*., 2002; Jung and Levin, 1999). Interestingly, one of the genes identified

by this study to be strongly associated with Papulacandin B was *MLP1*, a gene implicated in the regulation of the Rlm1 transcripton factor (Watanabe *et al*., 1997). One of the perspectives of this work could be investigation of the Mlp1p function using Papulacandin B as an inducer of its transcription.

Another feature of Papulacandin B transcription profile was induction of genes involved in the invasive growth and cell adhesion. These genes are required for biofilm formation. Hence, it would be interesting to verify whether small amounts of Papulacandins or other antibiotics introduced in the environment of the fungus could stimulate formation of the biofilm and whether biofilm formation could be in fact a mechanism of defense against harmful conditions and not only starvation.

Actually, since all suggestions mentioned above are based exclusively on the transcriptome data they should be treated cautiously until confirmed by biochemical experiments.

2.1.2 Response to drugs destabilizing the cell wall structure, Congo red and Calcofluor white

2.1.2.1 Response of yeast cells to Congo red

2.1.2.1.1 Response to Congo red as deduced from transcriptome profiling

In order to study the early transcriptomic response of yeast cells to Congo red (CR), we analyzed changes in gene expression caused by 20 µg/ml and 100 µg/ml CR. These two concentrations exert an effect smaller than 10% of that caused the IC_{50} value and twice that of the IC_{50} value and were termed a 'non-inhibitory' concentration and an 'inhibitory' concentration, respectively (Kuranda *et al*., 2006). Interestingly, the 'non-inhibitory' and 'inhibitory' concentrations caused similar numbers of genes to respond and this number was increasing when measured after 5, 15 and 90 minutes of growth (Figure 10A; method in chapter 3.1.4, pg 117). The induction of the observed transcriptional changes was relatively slow, which was in agreement with the known fact that Congo red does not enter the cell but acts directly on the cell wall. Therefore, the transcriptional changes occur only after the cell will have sensed a perturbation of its outer layer or the plasma membrane.

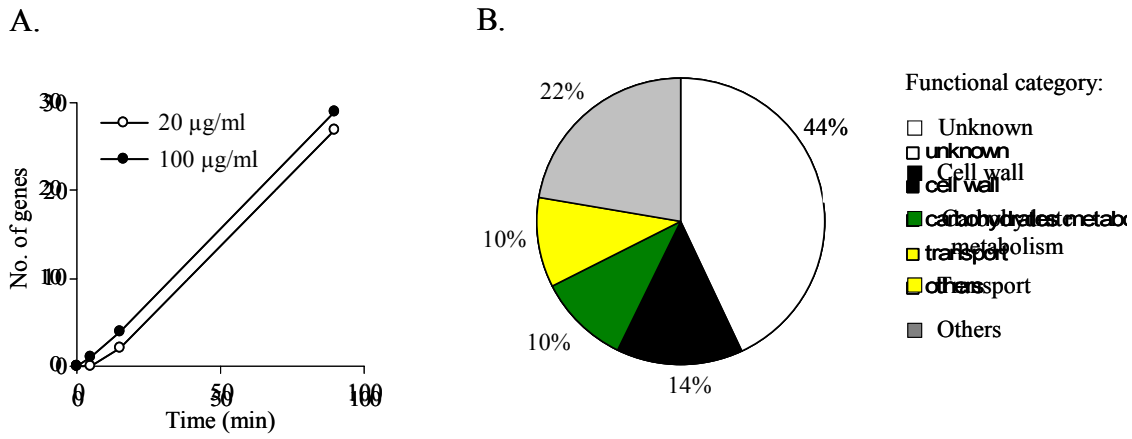


Figure 10: Transcriptomic response of yeast cells to Congo red.

A. The number of genes whose expression changed due to CR treatment. Wild-type cells were treated with indicated concentrations of CR, and the number of differentially expressed genes was scored after 5, 15 and 90 minutes of growth. **B**. The main functional categories of the genes affected by CR treatment according to Slim Mapper.

Genes whose expression changed in response to CR could be assigned mainly to two functional categories, i.e. cell wall organization and biogenesis (14.3% of genes) and carbohydrate metabolism (10.2% of genes) (Figure 10B, above). In the first category, we can find representatives of both cell wall protein families: *CWP1*, encoding glycosylphosphatidylinositol (GPI)-dependent cell wall protein, and genes encoding PIR proteins (*PIR1*, *PIR3*). Also *KRE2*, coding for α-1,2-mannosyltransferase, involved in mannosylation of the cell wall proteins was overexpressed. Additionally, we detected an overexpression of the *CHS7* gene, involved in chitin biosynthesis by regulating chitin synthase III (Chs3p) export, and the *SKT5* gene, which activates chitin synthase III during sporulation. The up-regulation of these genes is commonly regarded as a compensation for the cell wall damage in order to maintain the cell wall integrity. In general, the same cell wall genes were induced by the high and low concentrations of CR (see Table A 1, pg 155).

The group of genes implicated in carbohydrate metabolism contained *ENO1*, coding for enolase catalyzing the first common step of glycolysis and gluconeogenesis; *ZWF1*, encoding glucose-6-phosphate dehydrogenase, and *GAL1*, involved in galactose catabolism.

Overexpression of the *GAL1* gene is quite interesting as some groups have speculated that the cell wall could contain minor amounts of galactose. It has been shown that β1,6-glucan extracted from the cell walls by hot acetic acid contains traces of galactose (Manners *et al*., 1973), which in some genetic backgrounds could be a part of the GPI-anchor remnant of GPI-CWPs (Muller *et al*., 1992). The overexpression of genes involved in carbohydrate metabolism could be explained by possibly higher energy demands of the cell remodeling its cell wall.

Forty four percent of the genes responding to CR treatment had unknown functions. However, among these genes we found interesting ones like *YAL053W*, *YHR097C* and *YHR209W* whose involvement in the cell wall organization is still not proved but is supported by our and other author's findings (see Table A 1, pg 155). All of these genes were previously found to be overexpressed due to CR treatment in an independent study (Garcia *et al*., 2004). Moreover, *YAL053W* has been shown to have synthetic interactions with *GAS1* and *KRE1*, both genes involved in the cell wall assembly. Additionally, both *YAL053W* and *YHR097C* were overexpressed in several strains bearing cell wall mutations (Lagorce *et al*., 2003).

Comparing our data with those obtained by Garcia *et al*. (Garcia *et al*., 2004) who focused their analysis on later time-points (2, 4 and 6 h) of the culture with CR, we found similar changes in the expression of 9 genes: *SRL3*, *CWP1*, *YPS3*, *PIR1*, *PIR3*, *PRM10*, *YHR097C*, *YHR209W* and *YAL053W*.

To conclude, regarding the transcripomic response to Congo red treatment, activation of genes involved in the cell wall remodeling prevailed. Remarkably, at this early moment of the cell wall stress, we did not detect changes in the expression of genes encoding components of the Pkc1p-Mpk1p signaling cascade.

2.1.2.1.2 Induction of Mpk1p phosphorylation in response to Congo red

In order to understand better the transcriptomic response of yeast cells to CR, we studied the state of the CWI pathway by observing Mpk1 kinase phosphorylation (method in chapter 3.1.8.1, pg 128). To this end, we measured the level of Mpk1p phosphorylation in the same conditions and at the same time-points at which we analyzed changes of gene transcription. In addition, to examine whether the state of phosphorylation lasted longer, we also took samples after 20 h of CR stress. An increase of the Mpk1p phosphorylated form was observed after 1.5 h of growth in the presence of the 'inhibitory' (100 μg/ml) concentration of the drug (Figure 11, results after 0, 5 and 15 min are not shown). Thus, the increase of phosphorylated Mpk1p was simultaneous with the previously observed overexpression of the cell wall genes. This result suggested that consistently with our knowledge phosphorylated Mpk1p activated its main downstream target, the Rlm1p transcription factor, which in turn could activate expression of the cell wall genes. This relatively slow response (1.5 h) could be explained by the gradually increasing damage of the cell wall. CR does not destroy the already existing glucan network but interferes with polymerization of new chains. Therefore, as the cell wall is slowly but constantly renewed at the presence of CR, the cell wall will gradually loose its integrity finally reaching the threshold of the activation of the CWI pathway. As we did not detect Mpk1p phosphorylation after 20 h of incubation with CR, we could conclude that it was a transient event.

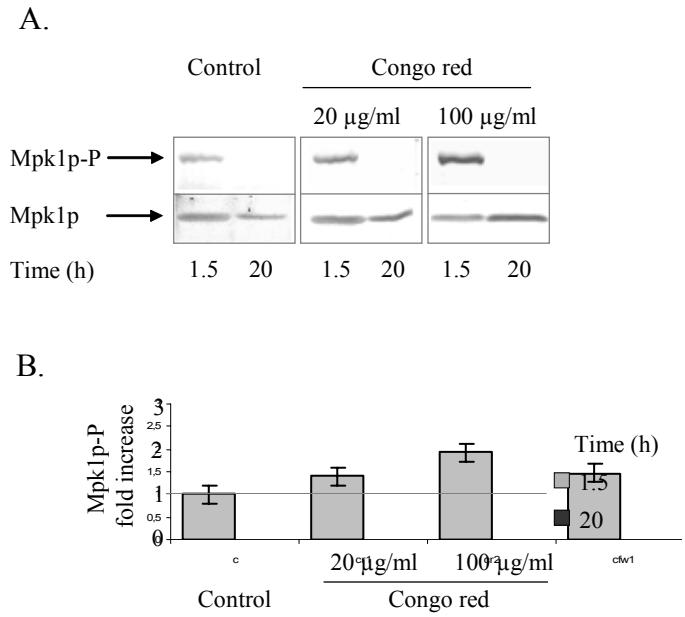


Figure 11: Induction of Mpk1p phosphorylation in response to CR.

A. Early exponential cultures of BY4742 strain were exposed to the 'non-inhibitory' and the 'inhibitory' concentrations of CR. Samples were taken, at the indicated times, and assayed for the phosphorylation levels of Mpk1p. Anti-phospho-Mpk1p antibodies and anti-Mpk1p antibodies were used to quantify phosphorylated Mpk1p (Mpk1p-P) and total amount of Mpk1p (Mpk1p). Data shown represent one typical experiment that was performed at least twice with consistent results.

B. The ratio between phosphorylated Mpk1p and total Mpk1p was calculated for all Western blots, and this ratio was normalized to 1 at time 0 (not shown) for each of the tested conditions.

2.1.2.1.3 Effect of Congo red on the yeast cell wall composition

In order to verify whether the CR-induced expression of the cell wall genes was followed by actual changes in the cell wall structure, we assayed cells preincubated with CR for sensitivity to Zymolyase, a cocktail of cell wall lytic enzymes. In general, an increased resistance of cells to the lysis caused by Zymolyase indicates strengthening of the cell wall structure (Boorsma *et al*., 2004). Yeast were cultivated for 90 minutes with the 'inhibitory' or 'non-inhibitory' concentrations of CR or without any additions (control). Then, the cells were collected, treated with Zymolyase and cell lysis was followed as a decrease in OD_{600} (Figure 12A; method in chapter 3.1.5.1, pg 123). During 90 min, the 'non-inhibitory' concentration of CR did not change the Zymolyase sensitivity, which was in agreement with the negligible activation of Mpk1 kinase in these conditions. Nevertheless, prolonged incubation (5 h) of the cells with the low CR concentration resulted in a decrease of their sensitivity to Zymolyase (result not shown). An 'inhibitory' dose of CR caused a decrease in the sensitivity to Zymolyase already after 90 minutes. This result supported our transcriptomic data by showing that activation of the cell wall genes resulted in apparent changes of the cell wall composition. Both effects were simultaneous with the activation of Mpk1p.

Further, to examine the character of the changes in the cell wall structure caused by CR stress, we measured levels of three main carbohydrate components of the cell wall (Figure

12B/C). Wild-type cells were grown in the presence of the 'inhibitory' or 'non-inhibitory' concentrations of CR, or without any additions, then the cell wall fraction was isolated and assayed for its main components (method in chapter 3.1.5.2, pg 124). The most significant changes caused by CR concerned mannans and chitin content. Cell walls of yeast growing at the 'inhibitory' concentration of CR contained 54% more mannans and 77% more chitin compared to non-treated cells. The 'non-inhibitory' concentration of CR also caused an increase in the mannose (43%) and glucosamine (42%) content but these changes were less pronounced.

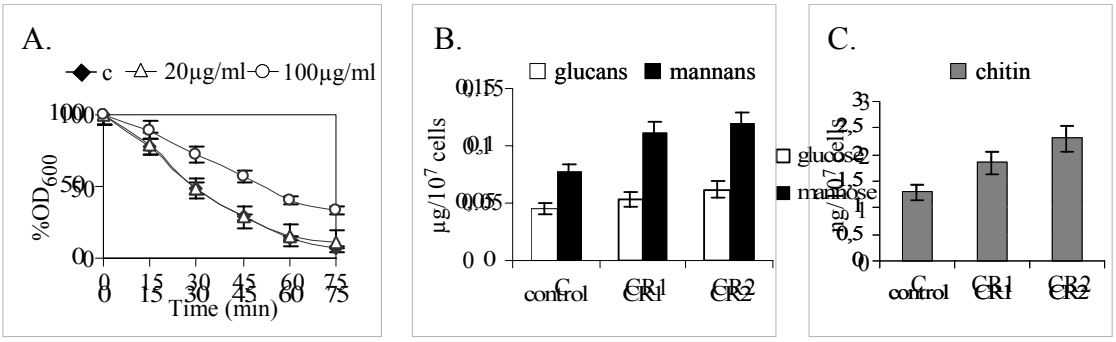


Figure 12: CR-induced cell wall remodeling

A. Zymolyase sensitivity. Yeast cells (BY4742) were challenged for 90 minutes with CR prior to Zymolyase assay. The cell lysis is expressed as a decrease of OD at 600 nm (percentage of the initial value). **B**. Changes in the main cell wall components caused by CR. After 20 h of growth of the wild type strain BY4742 in the absence (C) or presence of Congo red (CR1 20 µg/ml; CR2 100 µg/ml), yeast cell wall was purified and glucan, mannan, and glucosamine were determined by HPAIE. Results are averages of two independent experiments.

2.1.2.1.4 Involvement of particular transcription factors in the CR-induced cell wall remodeling

The group led by J. Arroyo has shown that the transcriptional response to long-term (2-6 h) CR stress involves induction of genes potentially regulated by the Rlm1p, Crz1p, Swi4p or Msn2/4p transcription factors (Garcia *et al*., 2004). To investigate which of those transcription factors is necessary for the strengthening of the cell wall during short-term treatment with CR, the Zymolyase sensitivity assays were performed in the respective mutant strains (method in chapter 3.1.5.1, pg 123). The increase of the Zymolyase resistance in wild-type cells grown in the presence of CR was taken as 100% of resistance and compared to the increase of resistance observed in the *rlm1*Δ, *crz1*Δ, *swi4*Δ or the *msn2*Δ*msn4*Δ mutants. It turned out that Rlm1p played the major role in the remodeling of the cell wall caused by CR. However, when the *RLM1* gene was deleted the strengthening of the cell wall still took place

(50% of the wt level) suggesting a contribution of another transcription factor(s), most likely Crz1p, as the loss of this protein function also caused a slight (to 75% of the wt level) decrease in the resistance to Zymolyase. In contrast to the proposition of Arroyo's group, we did not observe dependence of CR-induced cell wall remodeling on Swi4p and Msn2/4p. This result can be explained by different duration of the CR stress.

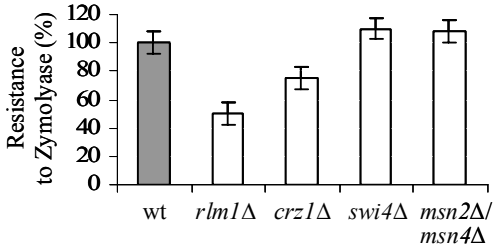


Figure 13: Involvement of chosen transcription factors in the CR-induced cell wall remodeling.

Cells were treated for 90min with 100 μg/ml CR, washed and treated with Zymolyase. Resistance was measured as the OD_{600} of cells remaining after lysis provoked by digestion of the cell wall with Zymolyase during 90 minutes. The increase of resistance after treatment of yeast with CR in the wild type strain was taken as 100% and the resistance of the mutant strains was compared to this value.

In Table 5, genes overexpressed due to CR treatment and containing in their promoters binding sites for the transcription factors tested are listed. In agreement with the previous result, the majority of those genes contained a binding site for the Rlm1p transcription factor in their promoter regions.

Table 5: Genes containing in their promoter sequence binding sites for Rlm1p, Crz1p, Swi4p or Msn2/4p, whose expression increased due to Congo red treatment

Regulated genes	Regulatory protein				Description
	Rlm1	Crz1	Swi4	Msn2/4	
CWP1	*		*	p	Cell wall mannoprotein, involved in cell wall organization
PIR1	*			p	O-glycosylated protein required for cell wall stability
PIR3	*			p	O-glycosylated cell wall protein required for cell wall stability
PRY2	*		*		Uncharacterized
SEC59	*				Dolichol kinase required for protein N-glycosylation
SRL3	*	p		p	Uncharacterized
YAL053W	*	*		p	Uncharacterized
YHR209W	*			p	Uncharacterized
YOL159C	*				Uncharacterized
YPS3	*		*	p	Aspartic protease, attached to the plasma membrane via a GPI anchor
BAG7	p	*		p	Rho GTPase activating protein (RhoGAP)
KHA1		*		*	Putative K+/H+ antiporter
YHR097C		*	p	p	Uncharacterized
GAL1				*	Galactokinase

* -documented binding site; p -potential binding site

2.1.2.2 Response of yeast cells to Calcofluor white

2.1.2.2.1 Response to Calcofluor white as deduced from transcriptome profiling

Calcofluor white (CFW) is a fluorescent dye binding to chitin and disturbing the cell wall assembly (Elorza *et al*., 1983). To study short-term response of yeast cells to this compound, as for Congo red, we chose two concentrations; 'non-inhibitory' equal to 20 µg/ml and 'inhibitory' equal to 100 µg/ml CFW (Kuranda *et al*., 2006). Similarly to CR, CFW caused changes in gene transcription mainly after 90 minutes but these changes concerned about twice as many genes as in the case CR (Figure 14A; method in chapter 3.1.4, pg 117). There was no significant difference between the numbers of genes affected by the low and high doses of the drug, which was also similar to the effect of CR.

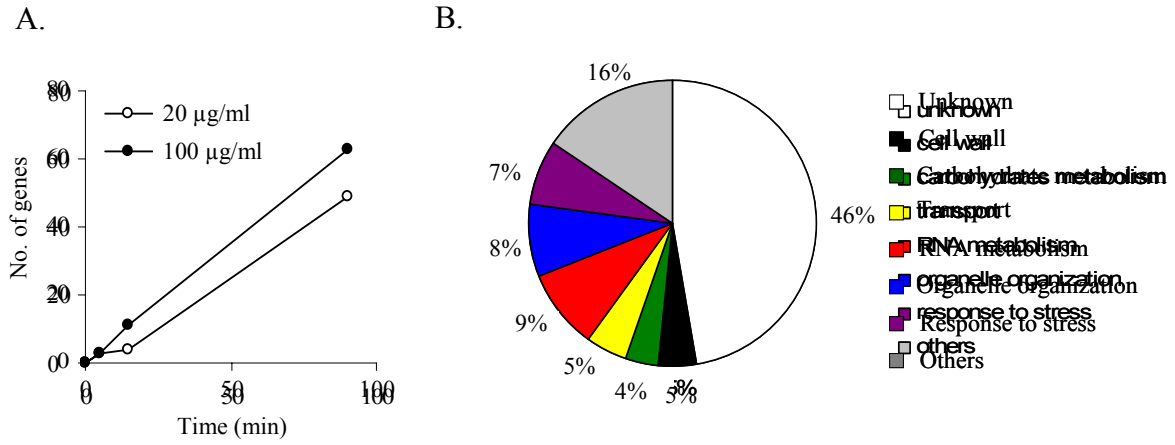


Figure 14: Transcriptomic response of yeast cells to CFW.

A. The number of genes whose expression changed due to CFW treatment. Wild-type cells were treated with indicated concentrations of CFW, and the number of differentially expressed genes was scored after 5, 15 and 90 minutes of growth. **B**. The main functional categories of genes affected by CFW treatment according to Slim Mapper.

Genes whose expression changed significantly in response to CFW were distributed among the functional categories according to Slim Mapper (Figure 14B). Genes of unknown function represented 46% of all affected genes, which makes interpretation of these data rather incomplete. However, two among these genes, *TIR4* and *YLR194C*, have been localized to the cell wall implying a cell-wall related function. According to our data, CFW caused changes mainly in five functional categories: RNA metabolism, ribosome biogenesis and

protein biosynthesis (presented in Table 6), response to stress (*PER1*, *MSB2*, *HSP12*, *HSP30*, *BNI1*, *PEP4*, *SIP5*, *LSP1*) and organelle biogenesis (*MSB2*, *LSB6*, *SFK1*, *PTC1*, *CTF19*, *BNI1*, *VIK1*, *MDM30*, *PAC11*). The latter category contained mainly genes involved in cytoskeleton organization. Two of these genes were down-regulated, *BNI1* encoding formin which nucleates the formation of linear actin filaments and *LSB6* encoding a protein involved in actin patch assembly and actin polymerization. Other genes like *CTF19* (required for attachment of spindle microtubules to the kinetochore), *SFK1* (suppressor of *STT4*), *VIK1* (required for sister chromatid cohesion) and *PAC11* (dynein intermediate chain) were overexpressed. Their presence in this analysis is justified since it is known that properly working cytoskeleton is vital for the cell wall assembly (Utsugi *et al*., 2002).

Table 6: List of genes whose expression changed due to Calcofluor white treatment, involved in RNA metabolism, ribosome biogenesis and protein synthesis

ORF	Gene*	Description
		RNA metabolism
YPR182W	***SMX3***	Sm or Sm-like snRNP protein, nuclear mRNA splicing via spliceosome
YGR006W	***PRP18***	pre-mRNA splicing factor
YDL006W	***PTC1***	type 2C protein phosphatase (PP2C), involved in precursor RNA splicing
YHR085W	***IPI1***	Protein that may be involved in rRNA processing
YKL208W	*CBT1*	Subunit of complex involved in processing of cytochrome b pre-mRNA
YPL097W	*MSY1*	Mitochondrial tyrosyl-tRNA synthetase
YOR168W	*GLN4*	Glutamine tRNA synthetase
YHR148W	*IMP3*	Component of the SSU processome, which is required for pre-18S rRNA processing
YBR084W	*MIS1*	Mitochondrial C1-tetrahydroflate synthase
YOR004W		Protein involved in processing of 20 S pre-rRNA
		Ribosome biogenesis and assembly
YPR102C	***RPL11A***	Protein component of the large (60S) ribosomal subunit
YKL021C	*MAK11*	Protein involved in ribosomal large subunit biogenesis
YIL148W	*RPL40A*	Ribosomal protein L40A also encodes a ubiquitin protein
YNR053C	*NOG2*	Part of a pre-60S complex
YDR101C	*ARX1*	Protein involved in ribosomal large subunit biogenesis
		Protein biosynthesis
YDR515W	***SLF1***	Protein that associates with translating ribosomes
YNL284C	*MRPL10*	Mitochondrial ribosomal protein of the large subunit
YHR038W	*RRF1*	Mitochondrial ribosome recycling factor

*Genes represented in bold were overexpressed, other genes were underexpressed.

The *CWP1*, *PIR1*, *PIR2*, *PIR3* and *CSR2* genes represented the cell wall organization and biogenesis category. All of them, except *CSR2*, which is a multicopy suppressor of the temperature-sensitive phenotype of the *chs5*Δ*spa2*Δ double mutant (Santos and Snyder, 2000), were overexpressed by the 'inhibitory' and 'non-inhibitory' concentrations of CFW.

However, contrary to our expectations, known cell-wall related genes amounted only to 5% of the total number of genes affected by CFW. This result suggests that CFW could interfere with other processes in the cell than the cell wall assembly, for instance with RNA metabolism. Genes representing this latter category constituted 9% of all responsive genes and they are listed in Table 6 (above).

As it was previously suggested by Garcia-Rodriguez and co-workers (Garcia-Rodriguez *et al*., 2000a), the HOG (High-Osmolarity Glycerol) pathway is involved in the cellular response to CFW in yeast. Consistently, we found an up-regulation of two genes implicated in this pathway: *MSB2*, encoding an osmosensor protein, which can activate Hog1p, and *PTC1*, coding for type 2C protein phosphatase (PP2C) which inactivates Hog1p.

Finaly, we compared our data with those obtained by Boorsma *et al*. (Boorsma *et al*., 2004), where 51 genes were shown to be overexpressed due to 10 µg/ml CFW after 2 hours of exposure. Twelve of those genes (*CWP1*, *SRL3*, *PIR1*, *PIR2*, *PIR3*, *HSP12*, *DDR2*, *YAL053W*, *YLR194C*, *YBR071W*, *YMR315W* and *YLR414C*) were also induced in our analysis (see Table A 1, pg 155), suggesting that they were a constant trait of transcriptomic response to CFW.

2.1.2.2.2 Induction of Mpk1p phosphorylation in response to Calcofluor white

Since the previous analysis of the genome-wide expression did not detect genes known to be involved in the CWI pathway, we sought to verify if this pathway was active in the tested conditions. To answer this question we measured the level of phosphorylated Mpk1p in cells treated with the 'inhibitory' and 'non-inhibitory' concentrations of CFW (Figure 15). This experiment showed that the 'inhibitory' concentration of CFW caused a significant induction of Mpk1p phosphorylation, which indicated the activation of the CWI pathway at the protein level. The 'non-inhibitory' concentration also caused an increase in the abundance of phosphorylated Mpk1p but the effect was less pronounced (method in chapter 3.1.8.1, pg 128). Similarly to the effect of CR, the CFW-induced activation of the cell wall integrity pathway was transient, as we did not detect phosphorylated Mpk1p after 20 h of incubation with CFW.

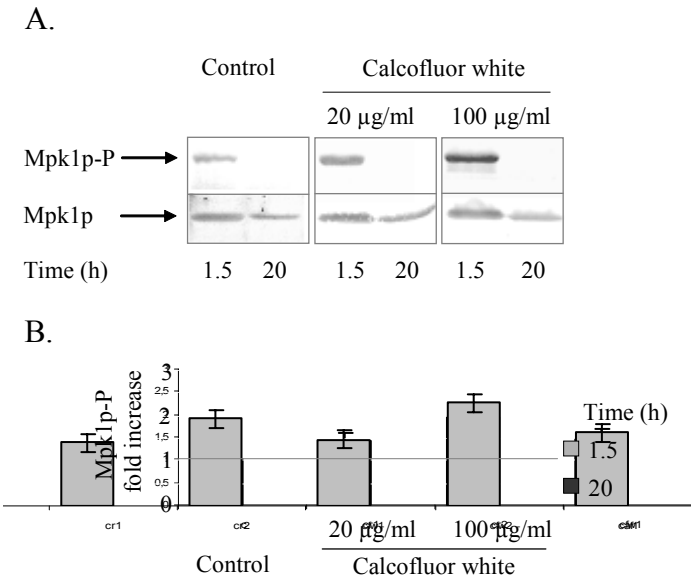


Figure 15: Induction of Mpk1p phosphorylation in response to CFW.

A. Early exponential cultures of BY4742 strain were exposed to the 'non-inhibitory' and 'inhibitory' concentrations of CFW. Samples were taken, at the indicated times, and assayed for the phosphorylation level of Mpk1p. Anti-phospho- Mpk1p antibodies and anti-Mpk1p antibodies were used to quantify phosphorylated Mpk1p (Mpk1p-P) and total amount of Mpk1p (Mpk1p). Data shown represent one typical experiment that was performed at least twice with consistent results.

B. The ratio between phosphorylated Mpk1p and total Mpk1p was calculated for all Western blots, and this ratio was normalized to 1 at time 0 (not shown) for each of the tested conditions.

2.1.2.2.3 Effects of Calcofluor white on the yeast cell wall composition

The next step in the study of the yeast response to CFW was verifying whether this drug could actually elicit changes in the cell wall composition after a short exposure and whether these changes were tuned with the observed Mpk1p phosphorylation. To this end, we used the standard procedure testing the sensitivity of the cells to Zymolyase treatment (method in chapter 3.1.5.1, pg 123). It turned out that only the 'inhibitory' concentration was able to elicit changes in the cell wall structure and these changes were detectable after 90 min of treatment (Figure 16A; measurements after 30 and 60 min are not shown). This result was in agreement with the induction of the cell wall genes and the increase of the amount of phosphorylated Mpk1p that were observed at the same time.

To determine the character of the changes in the cell wall composition we measured carbohydrate compounds in the cell walls isolated from yeast treated with Calcofluor white (method in chapter 3.1.5.2, pg 124). The 'inhibitory' concentration of CFW did not cause significant changes in glucan content but levels of mannans and chitin increased by 44% and 61% comparing to the wild type level, respectively (Figure 16B,C). The 'non-inhibitory' concentration caused a significant change only in chitin level (by 53%) confirming the fact that cells treated with CFW deposit additional chitin in the cell wall.

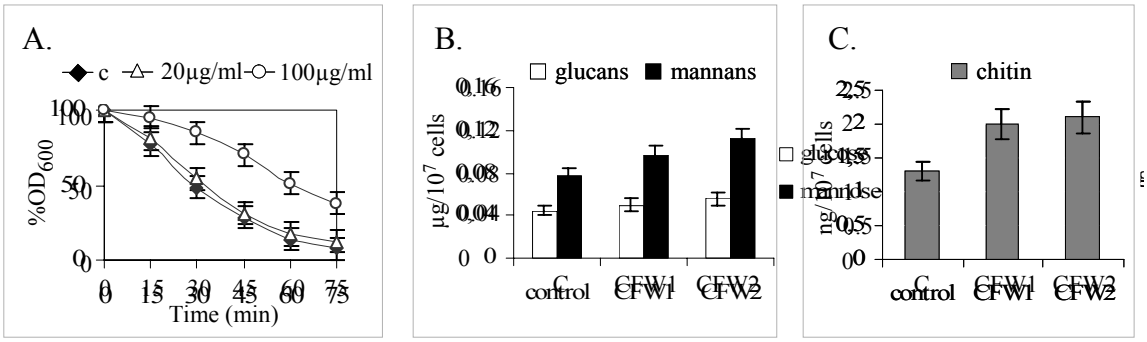


Figure 16: CFW-induced cell wall remodeling.

A.Yeast cells (BY4742) were challenged for 90 minutes with CFW prior to Zymolyase assay. The cell lysis is expressed as a decrease of OD at 600 nm (percentage of the initial value). **B**. Changes in the main cell wall components caused by CFW. After 20 h of growth of the wild type strain BY4742 in the absence (C) or presence of Calcofluor white (CFW1 20 µg/ml; CFW2 100 µg/ml), yeast cell wall was purified and glucan, mannan, and glucosamine were determined by HPAIE. Results are averages of two independent experiments.

2.1.2.2.4 Involvement of particular transcription factors in the Calcofluor white-induced cell wall remodeling

When we verified the occurrence of changes in the cell wall structure due to treatment with the 'inhibitory' concentration of Calcofluor white, after 90 min, we asked which transcription factor was necessary for these early changes. Previously, it has been proposed that the Rlm1p transcription factor is involved in response to CFW stress, nevertheless this proposition was based only on transcriptional data (Boorsma *et al*., 2004). To answer this question, we tested the Zymolyase sensitivity of mutant cells depleted in some transcription factors, which could be potentially involved in the cell wall remodeling (Figure 17) (Jung and Levin, 1999; Lagorce *et al*., 2003; Zhao *et al*., 1998). This experiment showed that the strengthening of the cell wall was exclusively dependent on the Rlm1p transcription factor, as the Zymolyase resistance in the *rlm1*Δ mutant was almost completely abolished. Changes in the resistance to Zymolyase in other mutants tested (*crz1*Δ, *swi4*Δ, *msn2*Δ*msn4*Δ) were not significant. This result suggests that during the response to CFW, other proteins that could activate expression of the cell wall genes cannot replace Rlm1p.

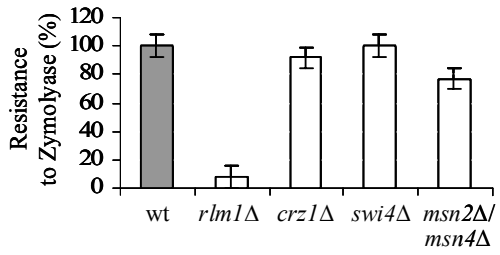


Figure 17: Involvement of chosen transcription factors in the CFW-induced cell wall remodeling.

Cells were treated for 90min with 100 µg/ml CFW, washed and treated with Zymolyase. Resistance was measured as the OD_{600} of cells remaining after lysis provoked by digestion of the cell wall with Zymolyase during 90 minutes. The increase of resistance after treatment of yeast with CFW in the wild type strain was taken as 100% and the resistance of the mutant strains was compared to this value.

As shown in Table 7, an Rlm1p-binding site was common in the promoter regions of the genes upregulated due to CFW action. In this group of genes, we can find mainly genes coding for proteins with well-established functions in the cell wall assembly. This result supports the main role of Rlm1p in the cell wall remodeling in response to CFW.

Table 7: Genes containing in their promoter sequence binding sites for Rlm1p, Crz1p, Swi4p or Msn2/4p, whose expression increased due to Calcofluor white treatment

Regulated gene	Regulatory protein				Description
	Rlm1	Crz1	Swi4	Msn2/4	
CWP1	*		*	p	Cell wall mannoprotein, involved in cell wall organization
HSP12	*			*	Protein that protects membranes from desiccation
PIR1	*			p	O-glycosylated protein required for cell wall stability
PIR2	*			p	O-mannosylated protein required for cell wall stability
PIR3	*			p	O-glycosylated protein required for cell wall stability
SRL3	*	p		p	Uncharacterized
YBR071W	*	*	*	p	Uncharacterized
YLR194C	*	*	p		Protein localized to the cell wall
YNL05Cc	*				Uncharacterized
YOL159C	*				Uncharacterized
KHA1		*		*	Putative K+/H+ antiporter
PEP4	p	*		*	Vacuolar aspartyl protease (proteinase A)
YHR097C		*	p	p	Uncharacterized
YLR414C	p	*			Uncharacterized
YPR197C		*			Uncharacterized
MSB2			*		Osmosensor acting in parallel to the Sho1p pathway
YKR011C			*	p	Uncharacterized
DDR2				*	Multistress response protein
GLK1				*	Glucokinase
YKR005C				*	Uncharacterized
YMR315W	p			*	Uncharacterized
YNL144C				*	Uncharacterized

* -documented binding site; p -potential binding site

2.1.2.3 Discussion

In this part of the work, we characterized the genome-wide transcriptomic response to Congo red and Calcofluor white at the early stages of the applied stress. Despite the apparently different cellular targets (glucan and chitin, respectively), both drugs elicited very similar responses. We showed that the yeast cells needed a relatively long time to elicit changes in the gene expression, since first changes were detected only after 90 min of growth in the presence of either drug. This is most likely due to the fact that either of the tested drugs has been shown to enter the yeast cells where it could cause immediate changes in gene transcription. Another but not conflicting explanation is that the weakening of the cell wall was taking place progressively, reaching in the end the threshold when changes in transcription are induced. This delayed response was confirmed by the biochemical data that showed the occurrence of cell wall remodeling at the same time as induction of the cell wall genes was observed.

Irrespectively of their distinct cellular targets, CR and CFW induced almost identical sets of cell wall related genes (Table A 1, pg 155). Thus, it was not surprising that eventually they caused similar changes in the cell wall composition, i.e. an increase in mannans and chitin levels. This result points to these processes as the general rescue response to this kind of 'mechanical' impairment of the cell wall. Moreover, although we did not detect changes in the expression of genes coding for components of the CWI pathway, we showed that induction of the cell wall genes was accompanied by an increased level of phosphorylated Mpk1 kinase. This observation implied that the observed induction of gene expression was dependent on the CWI pathway. Indeed, the cell wall remodeling induced by both drugs was strongly dependent on the Rlm1p transcription factor that is activated by phosphorylated Mpk1 kinase.

Intriguingly, although the 'non-inhibitory' concentration of the tested drugs induced expression of the cell wall related genes after 90 min of incubation, we did not detect cell wall reinforcement at this time-point. In addition, activation of Mpk1 kinase at these conditions was quite modest. On the other hand, while the 'inhibitory' concentration elicited an almost identical induction of the cell wall genes, it caused intensive remodeling of the cell wall and it was accompanied by a clearly increased amount of phosphorylated Mpk1p. Since the majority of the induced cell wall genes were potentially regulated by the Rlm1 transcription factor, we can assume that active Mpk1p caused this induction. This result implies that even a moderate

increase of the level of phosphorylated Mpk1p is sufficient for transcriptional induction of the cell wall genes. A corollary of this result is the fact that yeast should be equipped with some kind of posttranscriptional regulation to prevent cell wall biosynthesis, even when the transcription of the cell wall genes is elevated. Furthermore, it is noteworthy that we observed remodeling of the cell wall when the growth of the cells was inhibited. Thus, the putative posttranscriptional control of the cell wall-related genes could be linked to the growth inhibition. Recently, the 'cell wall integrity checkpoint' was discovered, which arrests the cell cycle in response to the damage of the cell wall (Suzuki *et al*., 2004b). It is possible that a so far unidentified 'sensor' of the cell wall damage which signals to this checkpoint has a threshold of sensitivity and is not activated by the low concentrations of CR or CFW. Thus, progression of the cell cycle would be stopped only when the cell encounters a severe aggression while ignoring minor ones.

Despite the similarities in the effects of CR and CFW, we observed also some differences. First, while the cell wall remodeling in response to CFW was almost completely dependent on the Rlm1p transcription factor, the response to CR likely involved other transcription factors, like Crz1p. Secondly, CFW affected expression of roughly twice as many genes and only 5% of them were previously identified to be involved in the cell wall assembly. Unlike in the case of CR, the most abundant category of genes influenced by CFW were those of RNA metabolism. However, this result cannot be explained without further investigation, it suggests that CFW may have some additional targets in the cell.

2.1.3 Response of yeast cells to caffeine

2.1.3.1 Caffeine induces remodeling of the cell wall

2.1.3.1.1 Treatment of yeast with caffeine induces changes in the cell wall composition

Caffeine is commonly believed to affect cell wall biosynthesis, since mutants impaired in the CWI pathway are hypersensitive to this compound (Costigan *et al*., 1992; Jacoby *et al*., 1998; Martin *et al*., 2000; Martinez-Pastor *et al*., 1996; Watanabe *et al*., 1997). Consistently, caffeine has been shown to be an inducer of the CWI pathway as demonstrated by the increased amount of phosphorylated Mpk1 kinase in caffeine-treated cells (de Nobel *et al*., 2000; Martin *et al*., 2000; Rajavel *et al*., 1999). Nevertheless, the actual effects of caffeine on the cell wall composition have not been investigated before. In order to see whether caffeine causes changes in the cell wall structure, we tested Zymolyase sensitivity of cells treated with the 'inhibitory' and 'non-inhibitory' concentrations of this drug (Figure 18A, below). These concentrations were previously determined as 2 mM and 20 mM, respectively, similarly as it was done for Congo red and Calcofluor white (Kuranda *et al*., 2006). Yeast cultures were challenged for 90 minutes with caffeine and then cells were resuspended in a solution containing Zymolyase. Cell lysis was followed as a decrease of OD_{600} (method in chapter 3.1.5.1, pg 123). The 'inhibitory' concentration of caffeine elicited Zymolyase resistance, which indicated occurrence of changes in the cell wall structure. The 'non-inhibitory' concentration did not cause changes in the sensitivity to Zymolyase. Although this result was similar to the effects of the typical cell wall damaging agents Congo red and Calcofluor white, the increase of resistance elicited by the 'inhibitory' concentration of caffeine was much more pronounced. After prolonged (5 h) incubation with the 'non-inhibitory' concentration of caffeine, we observed only a moderate increase of cell resistance to Zymolyase (result not shown).

To investigate the exact character of the caffeine-induced changes in the cell wall composition we measured the content of glucan, mannans and chitin in cells growing for 20 h in the presence of caffeine or without it (Figure 18B,C below). The 'non-inhibitory' concentration of caffeine did not cause significant changes in any of the measured compounds while the 'inhibitory' concentration caused a prominent alteration of the cell wall

composition. We confirmed that, as could be expected, the higher resistance of caffeine-treated cells to Zymolyase reflected an increase in the content of glucan. Additionally, chitin level was increased about two-fold when compared to the control conditions. The lack of significant changes in the cell wall composition of the cells treated for 20 h with the 'non-inhibitory' concentration of caffeine was consistent with our assumption that caffeine at this concentration is somehow neutralized by the cell.

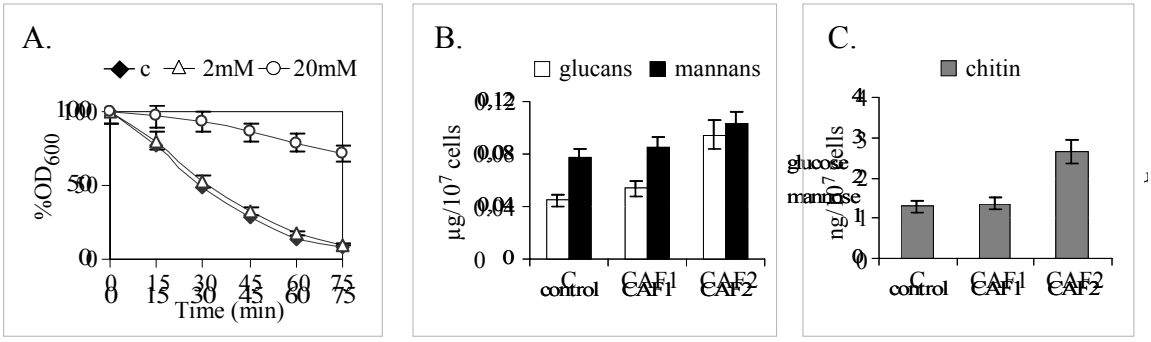


Figure 18: Caffeine-induced cell wall remodeling.

A. Yeast cells (BY4742) were challenged for 90 min with caffeine prior to Zymolyase assay. The cell lysis is expressed as a decrease of OD at 600 nm (percentage of the initial value). **B,C**. Caffeine induces changes of the cell wall composition. Wild-type cells (BY4742) were cultivated for 20 h in the absence (C) or presence of caffeine (CAF1 2 mM; CAF2 20 mM), the cell wall was purified and glucose, mannose and glucosamine were determined by HPAIE. Results represent an average of two independent experiments.

2.1.3.1.2 Induction of Mpk1p phosphorylation in response to caffeine

Several reports have shown that treatment of yeast cells with caffeine leads to the activation of the Pkc1p-dependent MAP kinase signaling cascade, as measured by dual phosphorylation of Mpk1 kinase (de Nobel *et al*., 2000; Martin *et al*., 2000; Rajavel *et al*., 1999). We investigated this event further by evaluating the rate and the potency of this agent to elicit phosphorylation of Mpk1p, and attempted to correlate the phosphorylation level of this MAP kinase with changes in the cell wall structure. To this end, we tested the state of Mpk1p after 5, 15, 90 min, 20 h and before addition of caffeine to the yeast culture (method in chapter 3.1.8.1, pg 128). It turned out that phosphorylation of Mpk1p upon exposure to the 'non-inhibitory' concentration was detectable only after 90 min of incubation (Figure 19, only 1.5 and 20 h time-points are shown), similarly to the case of low concentrations of CR and CFW. In contrast, the 'inhibitory' concentration elicited an increase of phosphorylated Mpk1p already after 5 min of incubation (1.5-fold increase, result not shown). While phosphorylated Mpk1p was no longer detectable after 20 h of incubation with CR or CFW (compare Figure

11, pg 40; and Figure 15, pg 46), caffeine was still able to elicit a strong response (Figure 19). Moreover, after 1.5 and 20 h of incubation with 20 mM caffeine, two additional phosphorylated bands were detected. These bands most likely corresponded to two related MAP kinases, Fus3p and Kss1p, since these proteins, when phosphorylated, are recognized by the same anti-phospho-p44/42 MAPK antibody used to visualize phospho-Mpk1p (Bardwell *et al*., 1998; Sabbagh, Jr. *et al*., 2001). Altogether, treatment of yeast cells with caffeine led to a faster and more potent activation of the Pkc1p-Mpk1p cascade than treatment with CR or CFW. Thus, this higher and sustained activation of the Mpk1p-dependent signaling pathway was at a first glance consistent with the higher resistance of caffeine-treated cells to Zymolyase compared to those treated with the other drugs.

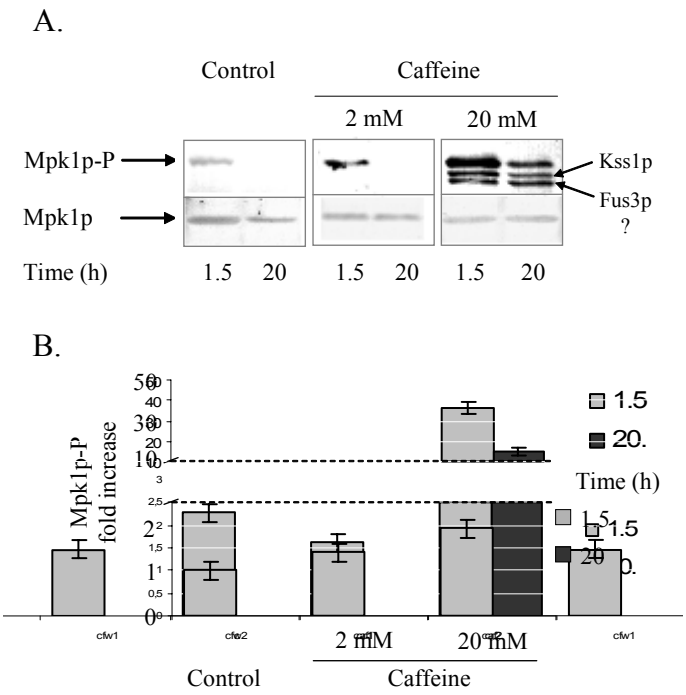


Figure 19: Induction of Mpk1p phosphorylation in response to CAF.

A. Early exponential cultures of wild-type cells (BY4742) were exposed to the 'non-inhibitory' and the 'inhibitory' concentrations of CAF. Samples were taken, at the indicated times, and were assayed for the phosphorylation level of Mpk1p. Anti-phospho-Mpk1p antibodies and the anti-Mpk1p antibodies were used to quantify the phosphorylated Mpk1p (Mpk1p-P) and the total amount of Mpk1p (Mpk1p). Data shown represent one typical experiment that was performed at least twice with consistent results.

B. The ratio between phosphorylated Mpk1p and total Mpk1p was calculated for all Western blots, and this ratio was normalized to 1 at time 0 (not shown) for each of the tested conditions.

2.1.3.1.3 Transcription factors involved in the caffeine-induced cell wall remodeling

In order to verify which transcription factor was responsible for the caffeine-induced cell wall remodeling, we measured the resistance to Zymolyase in mutants impaired in the function of several transcription factors that were previously implicated in the induction of cell wall genes (Figure 20) (Jung and Levin, 1999; Lagorce *et al*., 2003; Zhao *et al*., 1998). We observed a significant involvement of the Crz1p transcription factor since the *crz1*Δ

mutant exhibited only 38% of the resistance of wild-type cells. The *swi4*Δ mutant exhibited 46% of the wild type resistance indicating importance of the Swi4p transcription factor in the remodeling process as well. Interestingly, loss of Msn2/4p function also caused a significant decrease of the resistance. Although it is difficult to explain how the genes regulated by Msn2/4p could contribute to the cell wall reinforcement, as most of them do not encode cell-wall related proteins, they have been associated with the transcriptional response to the cell wall damage (Lagorce *et al*., 2003). Importantly, a typical transcription factor associated with induction of the cell wall genes, Rlm1p, was not significantly involved in the strengthening of the cell wall in response to caffeine. Altogether, these results indicated that caffeine-induced cell wall modifications are largely independent of the main target of Mpk1 kinase, i.e. Rlm1p, but this process is partially mediated by at least three other transcription factors, like Crz1p, Swi4p and Msn2/4p.

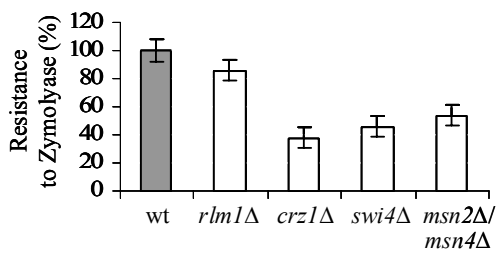


Figure 20: Involvement of chosen transcription factors in the CAF-induced cell wall remodeling.

Cells were treated for 90min with 20 mM CAF, washed and treated with Zymolyase. Resistance was measured as the OD_{600} of cells remaining after lysis provoked by digestion of the cell wall with Zymolyase during 90 minutes. The increase of resistance after treatment of yeast with CAF in the wild type strain was taken as 100% and the resistance of the mutant strains was compared to this value.

2.1.3.2 Response to caffeine as deduced from transcriptome profiling

To study the response of yeast cells to caffeine we analyzed genome-wide transcription levels after 5, 15 and 90 min of growth in the presence of the 'inhibitory' and 'non-inhibitory' concentrations of the drug. According to this analysis, caffeine caused rapid changes in gene transcription which could be observed already after 5 minutes of treatment (Figure 21A). The number of genes responding to caffeine was strongly dependent on the concentration of the drug used. After 5 min, the 'non-inhibitory' concentration caused changes in the expression of approximately 50 genes, and this number decreased upon prolonged incubation, which most likely illustrated neutralization or expulsion of caffeine from cells. In contrast, the number of genes responding to the 'inhibitory' concentration

increased with the duration of treatment and reached 260 differentially expressed genes after 90 minutes.

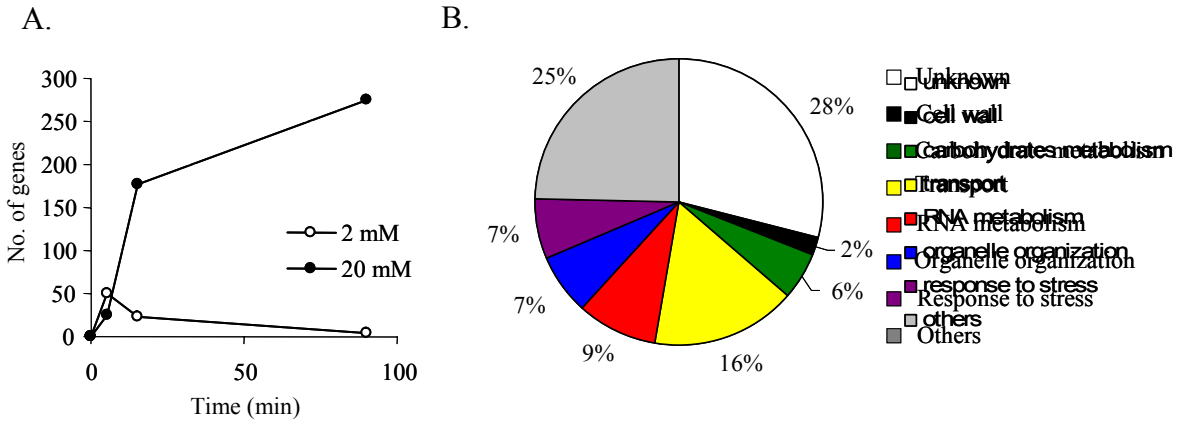


Figure 21: Transcriptomic response of yeast cells to CAF.

A. The number of genes whose expression changed due to CAF treatment. Wild-type cells were treated with indicated concentrations of CAF, and the number of differentially expressed genes was scored after 5, 15 and 90 minutes of growth. **B**. The main functional categories of genes that were affected by CAF treatment. Distribution of genes according to Slim Mapper, SGD.

Genes whose expression changed significantly were distributed among functional categories. As shown in Figure 21B (above), the genes involved in transport were the most affected and represented 16% of all the genes responding to the caffeine treatment. Figure 22 represents particular genes belonging to this category and it can be seen that caffeine probably altered transport of metabolites and ions (sugars, amino acids, protons, Cu^{2+}, Fe^{2+}), as well as the translocation of proteins between cellular compartments. The most affected subgroup of this category was the vesicle-mediated transport. This result was consistent with earlier large scale phenotypic data (~5000 viable deletion mutants tested) indicating that deletions of genes implicated in transport and especially in the vesicle-mediated transport render cells hypersensitive to caffeine (Parsons *et al*., 2004). Interestingly, caffeine activated expression of *SNQ2*, *QDR3* and *MDL2*, genes encoding putative drug transporters. It is possible that these transporters participate in the expulsion of caffeine from the cell, in particular *SNQ2* that has been reported as a multicopy suppressor of caffeine sensitivity of the *mpk1*Δ mutant (Martin *et al*., 2000).

In addition, RNA metabolism, organelle organization and response to stress were the other functional categories affected by caffeine. The function of 28% of affected genes remains unknown.

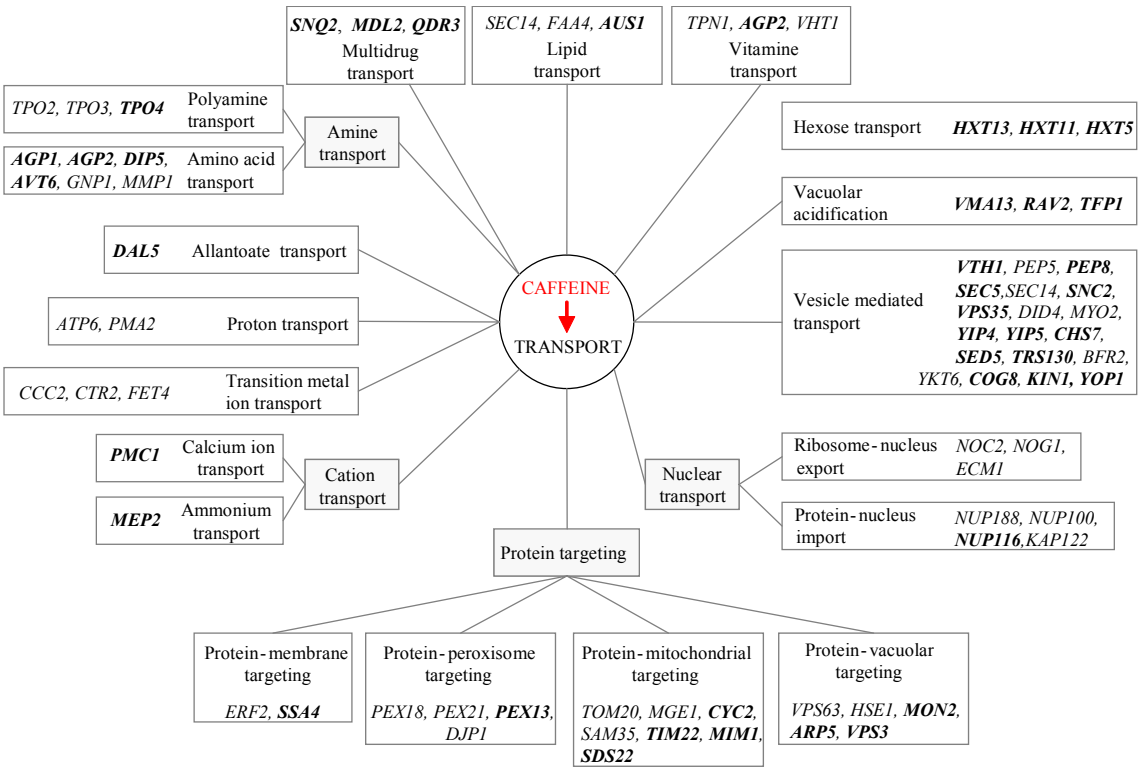


Figure 22: Genes involved in cellular transport whose expression changed due to caffeine treatment. Genes represented in bold were overexpressed while the other genes were underexpressed.

Although caffeine strongly remodeled the composition of the cell wall, genes potentially involved in this process amounted only to 2% of all responding genes. Moreover, the expression of these genes was affected exclusively by the 'inhibitory' concentration of caffeine (Table A 1, pg 155).

Table 8 presents all the genes that were overexpressed due to caffeine treatment whose promoter region contained a binding site for the transcription factors involved in the cell wall remodeling. The majority of those genes (15) could be regulated by Msn2/4p. Fourteen genes were regulated by Crz1p supporting a strong involvement of this transcription factor in the cell wall remodeling. Nearly the same number of genes could be regulated by both Swi4p and Rlm1p (9 and 8). However, the majority of genes potentially regulated by Rlm1p contained also a Crz1p- or Msn2/4p-binding consensus in their promoter region. Thus, we assume that these genes could be activated by these latter transcription factors and not by Rlm1p.

Table 8: Genes containing in their promoter sequence binding sites for Rlmp1, Crz1p, Swi4p or Msn2/4p, whose expression increased due to caffeine treatment

Regulated gene	Regulatory protein				Description
	Rlm1	Crz1	Swi4	Msn2/4	
AFR1	*			p	Alpha-factor pheromone receptor regulator
HSP12	*			*	Protein that protects membranes from desiccation
MCH5	*			p	Protein with similarity to monocarboxylate permeases
PST1	*				Cell wall protein that contains a putative GPI-attachment site
RCK1	*				Protein kinase involved in the response to oxidative stress
YAL053W	*	*		p	Uncharacterized
YGP1	*	*	p	*	Cell wall-related secretory glycoprotein
YSR3	*	*			Dihydrosphingosine 1-phosphate phosphatase
CPS1		*			Vacuolar carboxypeptidase yscS
FUS3		*		p	Protein kinase involved in mating pheromone response
GSC2		*	p		Catalytic subunit of β-1,3-glucan synthase
KRE6	p	*	*	p	Protein required for β-1,6-glucan biosynthesis
PEP4	p	*		*	Vacuolar aspartyl protease (proteinase A)
PMC1		*	*	*	Vacuolar Ca^{2+} ATPase involved in activation of calcineurin
SPI1		*		*	GPI-anchored, serine/threonine rich cell wall protein
YDR370C		*		p	Uncharacterized
YOR220W		*		p	Uncharacterized
YPR170C		*			Uncharacterized
ZSP1		*			Mutation results in a zinc sensitive phenotype
AGP1			*	*	Low-affinity/broad substrate range amino acid permease
APT2			*		Apparent pseudogene
HAP1			*	p	Transcription factor responding to heme and oxygen
HXT11			*	p	Putative hexose transporter that is nearly identical to Hxt9p
MON2			*	p	Protein with a role in endocytosis and vacuole integrity
SLM4			*		Protein involved in the regulation of microautophagy
YLR256W-A	p		*		TyA Gag protein
ACH1				*	Acetyl-coA hydrolase, primarily localized to mitochondria
ALD3				*	Aldehyde dehydrogenase, involved in beta-alanine synthesis
CPR6				*	Peptidyl-prolyl cis-trans isomerase (cyclophilin)
CYB2			p	*	Cytochrome b2 (L-lactate cytochrome-c oxidoreductase)
DNL4		p	p	*	DNA ligase involved in meiosis
ESBP6				*	Protein with similarity to monocarboxylate permeases
GAC1	p			*	Regulatory subunit for Glc7p type-1 protein phosphatase
GPH1				*	Non-essential glycogen phosphorylase
GPT2				*	Glycerol-3-phosphate acyltransferase (lipid biosynthesis)
GRE2				*	Stress induced NADPH-dependent methylglyoxal reductase
GRE3	p			*	Stress induced aldose reductase
GSY2				*	Glycogen synthase
GUT2			p	*	Mitochondrial glycerol-3-phosphate dehydrogenase
HSP31	p			*	Possible chaperone and cysteine protease
HXK1				*	Hexokinase isoenzyme 1
HXT13	p			*	Protein with similarity to hexose transporter
PRX1				*	Mitochondrial peroxiredoxin (1-Cys Prx)
PST2				*	Uncharacterized
SSA4			p	*	Heat shock protein that is highly induced upon stress
TSA2		p		*	Stress inducible cytoplasmic thioredoxin peroxidase
YBR139W		p		*	Putative serine type carboxypeptidase
YER067W	p			*	Uncharacterized
YLR177W			p	*	Uncharacterized
YLR345W				*	Uncharacterized
YNL146W				*	Uncharacterized

* -documented binding site; p -potential binding site

In order to better characterize the differences in the transcriptional responses to caffeine and the two cell wall damaging agents, we compared the transcription profiles elicited by the 'inhibitory' concentrations after 90 minutes. To this end, we analyzed these expression data sets using the software developed by Fellenberg *et al*. that uses correspondence analysis (Fellenberg *et al*., 2001). This method is an explorative statistical tool devoted to investigation of associations between variables (for instance, expression profiles obtained in different conditions) (description of method in chapter 3.1.4.3.1, pg 121). To illustrate the major trends in the data sets, while ignoring minor fluctuations, the data were expressed as a planar projection along axes, whose number corresponded to the number of conditions. Similarities between the conditions were shown as the distance between the axes. It can be seen in Figure 23 that the axes of Congo red and Calcofluor white were closer to each other than to the axis of caffeine. This means that the transcription profiles of CR and CFW were to some extent similar, while they were distinct from the profile elicited by caffeine. Furthermore, the axis of Congo red was the closest one to that of the control conditions, which is consistent with CR inducing the smallest number of differentially expressed genes. This analysis also highlighted association of genes involved in the cell wall assembly mainly to the Congo red and Calcofluor white axes. For instance, *CWP1*, *SRL3, PIR1, PIR2* and *PIR3,* which were induced by both drugs, were located approximately halfway between the axes of these two conditions. On the other hand, the farther away the differentially expressed genes were placed from the centroid, the closer they were associated with the given conditions. For example, a cell wall gene, *HSP12*, associates with the axis of caffeine because of its strong induction by this drug (11-fold) although it was also induced by CR and CFW (1.7- and 4-fold, respectively).

Interestingly, a detailed inspection of genes responding exclusively to caffeine revealed two clusters of genes comprised of 35 upregulated genes (involved mainly in nitrogen discrimination pathway and carbohydrate metabolism) and 21 downregulated genes (involved in ribosome biogenesis). Genes in both clusters are known to be regulated by the TOR pathway.

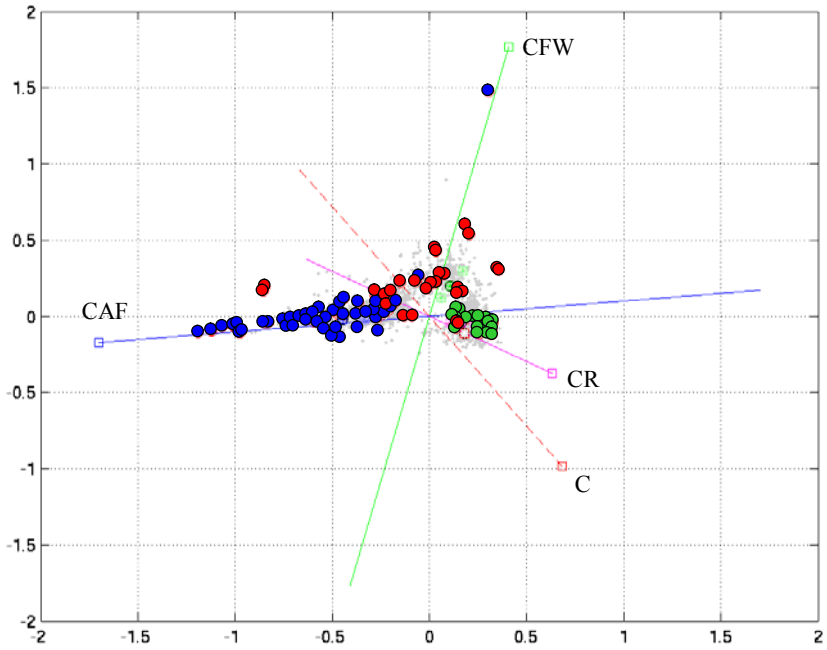


Figure 23: Comparison of transcriptional responses to CAF, CR and CFW.

The planar embedding produced by the Correspondence Analysis of genes (grey dots) whose expression changed after 90 minutes of treatment with 100 µg/ml of Congo red (CR), 100 µg/ml of Calcofluor white (CFW) and 20 mM caffeine (CAF) in comparison to the control conditions (C). Axes represent the major trend according to the condition's medians. Blue and green dots represent genes known to be regulated by TOR that were upregulated anddownregulated, respectively, in response to caffeine. Names of these genes are listed in Figure 24, below. Red dots represent cell wall genes (listed in Table A 1, pg 155) whose expression changed in response to at least one of the three drugs. The centroid is empty because normally expressed genes were filtered out from the analysis.

2.1.3.2.1 Caffeine-affected expression of genes involved in the TOR pathway

The transcriptional changes caused by caffeine suggested that this drug affected the TOR pathway (Figure 23, above). We observed overexpression of some transcription factors which are inhibited by TOR kinases in good growth conditions. Consequently, the expression of genes normally activated by these transcription factors was induced (Figure 24, below). This effect concerned mainly four branches of the TOR pathway controlled by Gln3p, Gat1p, and Rtg1/3p and Msn2/4p transcription factors. Gln3p and Gat1p are transcriptional regulators of the nitrogen catabolite repression (NCR) genes in yeast cells (Shamji *et al.*, 2000). In the presence of a 'good' nitrogen source (e.g. glutamine, glutamate, aspartate), TOR kinases repress nitrogen-regulated genes like *MEP2* (ammonium permease), *DAL5* (allantoate permease), and *GDH2* (glutamate dehydrogenase) by inhibiting the Gln3p and Gat1p transcription factors. All mentioned genes were strongly up-regulated due to caffeine treatment, although yeast were grown in a reach medium (YPD). In addition, treatment of the cells with caffeine resulted in overexpression of the TCA cycle genes: *CIT1* (mitochondrial citrate synthase), *CIT2* (non-mitochondrial citrate synthase) and *PYC1* (pyruvate carboxylase), and retrograde pathway genes, regulated by Rtg1/3p (Crespo *et al.*, 2002).

Furthermore, we observed induction of *MSN4* as well as of genes whose expression depends on this transcription factor. The Msn2 and Msn4 proteins are known to initiate gene expression in case of a general stress response (Martinez-Pastor *et al*., 1996). Interestingly, we detected overexpression of *BMH2* coding for protein that is regulated by the TOR pathway and in turn itself regulates Msn2/4p cellular localization. Moreover, we found an overexpression of the *YBR077C* gene, coding for a protein of unknown function that has been recently identified as a new TOR pathway genetic modifier (Huang *et al*., 2004). Finally, we observed the 5-fold induction of *MAF1*, a gene coding for a inhibitor of polymerase (Pol) III, and underexpression of 17 genes (*YML6*, *RRB1*, *RSA3*, *UTP9*, *MAK5*, *MTR4*, *NOC2*, *NAN1*, *NOG1*, *BMS1*, *NOG2*, *MTG1*, *RRP46*, *UTP21*, *DIM1*, *ECM1*, *RNT1*, *MRT4*, *UTP14*, *NOC3*, *NIP7*, *RPF2*, *TSR1*, *ARX1*) involved in the ribosome biogenesis.

Altogether, these changes in gene transcription clearly indicated inhibition of the TOR pathway by caffeine.

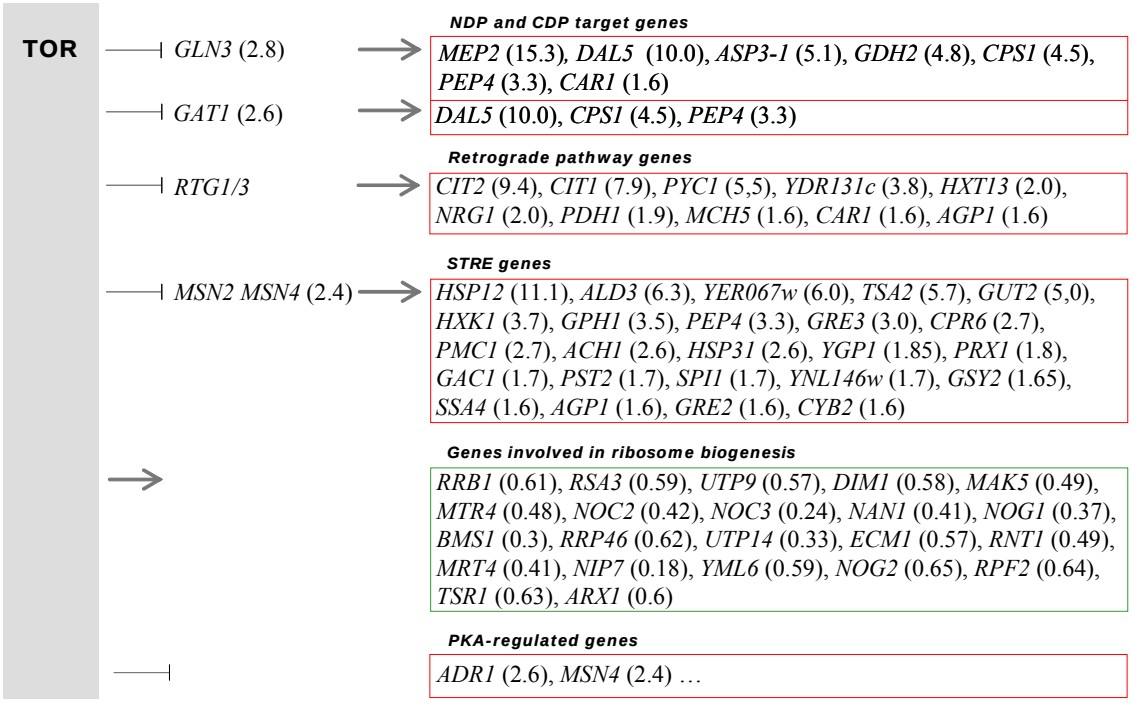


Figure 24: Genes controlled by TOR pathway whose expression changed in response to caffeine.

Change of gene expression after caffeine treatment is given in parentheses. Red - upregulated genes, green - downregulated genes. The PKA-regulated genes are listed in Figure 26, pg 63.

To further provide evidence that caffeine interferes with cellular functions regulated by the TOR pathway, we compared the transcriptomic response to this drug to that to rapamycin, an inhibitor of the Tor1/2 kinases. Using a Venn diagram, we showed that caffeine and rapamycin caused downregulation of the same genes that belonged to the functional

categories of transcription, ribosome assembly and protein synthesis, while they enhanced expression of genes involved in the TCA cycle, retrograde pathway controlled by Rtg1/3p, and Gln3/Gat1p-mediated 'nitrogen catabolite repression' pathway (Figure 25). An additional comparison of the action of rapamycin and caffeine based on the regulatory motifs shared by the induced genes is presented in Table A 2 (pg156). In spite of several similarities, important differences between the responses to caffeine and rapamycin need to be pointed out, which indicated that these two agents do not have an identical spectrum of cellular effects. Firstly, the overlap of the expression data between caffeine and rapamycin amounted only to 25%, although care must be taken with this estimation since the experiments were not carried out under identical conditions (Hardwick *et al*., 1999). Secondly, caffeine caused expression changes of several genes that were regulated by the level of cAMP (Figure 25), while these genes were not found in the transcriptomic response to rapamycin.

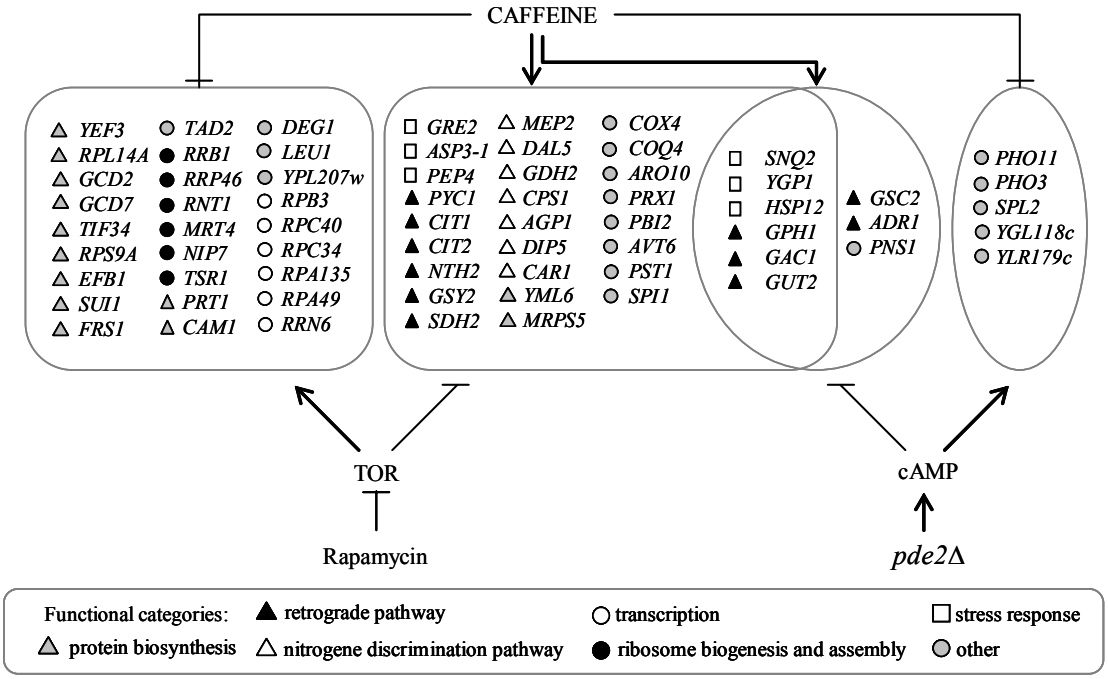


Figure 25: Comparison of expression data for cells treated with caffeine, rapamycin and for the *pde2*Δ mutant.

Venn diagram from the BioPlot software was used to find similarly expressed genes in the data sets of rapamycin (Hardwick *et al*., 1999), caffeine {this work} and the *pde2*Δ mutant (Jones *et al*., 2003). The most significant genes (P value<0.05) that were similarly regulated (upregulation – arrowheads, downregulation - slash heads) in at least two conditions are given in this figure with their functional category.

2.1.3.2.2 Caffeine-affected expression of genes involved in the cAMP signaling cascade

Caffeine has been reported to affect basal cAMP levels in eukaryotic cells. In agreement with these observations, our analysis identified a set of genes known to be regulated by the level of cAMP, whose expression was affected by caffeine (Figure 25, above). More importantly, caffeine affected expression of genes directly involved in the Ras/cAMP signaling cascade (Figure 26, below). Firstly, we detected overexpression of the *CYR1* gene, encoding adenylate cyclase. We observed changes in expression of genes coding for proteins acting upstream of Cyr1p and downstream of PKA. The *ERF2* gene was downregulated. The protein encoded by this gene is required for Ras2p palmitoylation and translocation to the plasma membrane (Dong *et al*., 2003). The *RGS2* gene coding for Gpa2 GAP, thus a negative regulator of adenylate cyclase activity, was downregulated. In contrast, *PLC1*, coding for a protein that enables a physical interaction between the Gpa2 and Gpr1 proteins, was upregulated. Another regulatory gene found to be overexpressed due to caffeine treatment was *RPI1*. It has been reported that overexpression of this gene not only suppressed the effects associated with the mutational hyperactivation of the Ras/cAMP pathway but additionally induced *GSC2*/*FKS2* (1,3-β-glucan synthase) transcription (Sobering *et al*., 2002). Moreover, our analysis indicated that caffeine influenced expression of genes coding for cAMP pathway effectors, involved in storage carbohydrate metabolism (*GSY2*, *GAC1*, *SDS22*) or genes sharing a STRE regulatory motif controlled by the Msn2/4p transcription factors (listed in Figure 24, pg 60). Genes like *GAC1* (regulatory subunit for Glc7p) or *GUT2* (glycerol-3-phosphate dehydrogenase), whose expression was induced by caffeine, are known to be repressed by cAMP but induced by nutrient limitation (Francois *et al*., 1992; Tadi *et al*., 1999). We also found an upregulation of the *ADR1* gene, encoding transcription factor, and some of its targets: *CIT1* (citrate synthase), *GUT2*, *RPM2* (mitochondrial RNase P subunit) and *SNQ2* (plasma membrane ABC transporter). The *ADR1*-encoded protein is phosphorylated by PKA, which inhibits its ability to activate transcription of other genes (Cherry *et al*., 1989). It is worth noting that *SNQ2* has been described as a suppressor of the caffeine sensitive phenotype of the *mpk1*Δ mutant (Martin *et al*., 2000). Altogether, the upregulation of genes that are normally repressed by PKA suggested that caffeine could cause inhibition of this cAMP-dependent protein kinase.

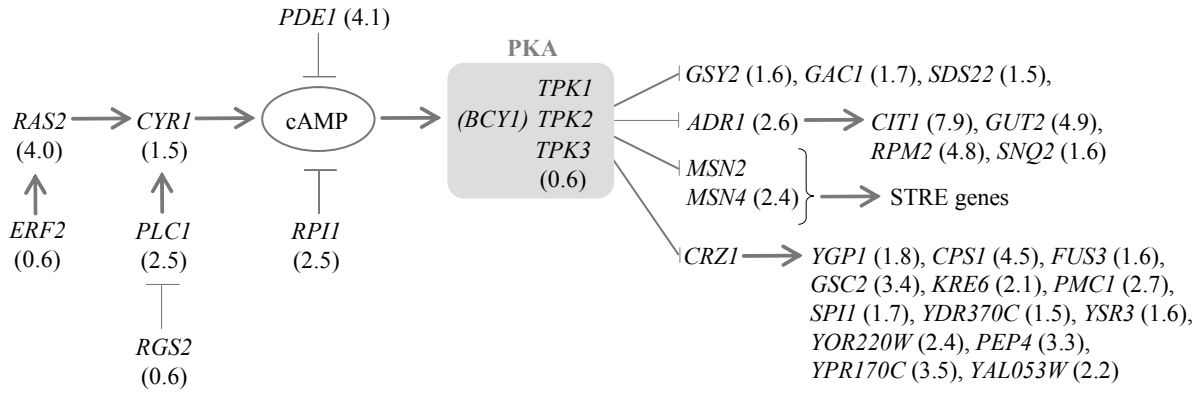


Figure 26: Genes involved in the cAMP signaling cascade whose expression was changed in response to caffeine.

The above representation of cAMP-dependent protein kinase A (PKA) does not reflect the actual structure of the holoenzyme but only shows genes coding for the regulatory (*BCY1*) and the redundant catalytic subunits (*TPK1-3*). Consequently, transcription of genes whose transcription is dependent on PKA was induced. Since Msn2/4p can be also regulated by the TOR pathway, STRE genes are listed in Figure 24, pg 60. Ratios of gene expression after caffeine treatment to that in control conditions are given in parentheses.

2.1.3.2.3 Caffeine-affected expression of genes involved in the Ehrlich pathway

Independently of the concentration used, we observed interesting effects of caffeine treatment on genes encoding proteins involved in amino acid metabolism. Caffeine caused hyperactivation of the Aro80p-regulated pathway implicated in the degradation of phenylalanine into phenylethanol (Figure 27 below). This activation was indicated by a rapid and strong upregulation of *ARO9*, *ARO10* and *ADH5*, which encode the three successive enzymes of the pathway, as well as *ATF1* which encodes the alcohol acetyltransferase that catalyzes the formation of phenylacetate (rose taste) from phenylethanol and acetyl-CoA (Fujii *et al*., 1994; Vuralhan *et al*., 2003). Aro10p, phenylpyruvate decarboxylase responsible for the catalysis of the second step in the Ehrlich pathway has no activity in ammonia-grown cultures and activation of its transcription depends on the presence of aromatic amino acids in the medium and the transcription factor encoded by the *ARO80* gene (Iraqui *et al*., 1999). How caffeine causes this potent activation is not sure. However, a possibility exists that beside the negative regulation of this pathway by Tor1p, caffeine could mimic the effect of the aromatic amino acid tryptophan as a gratuitous inducer of genes of this metabolic pathway.

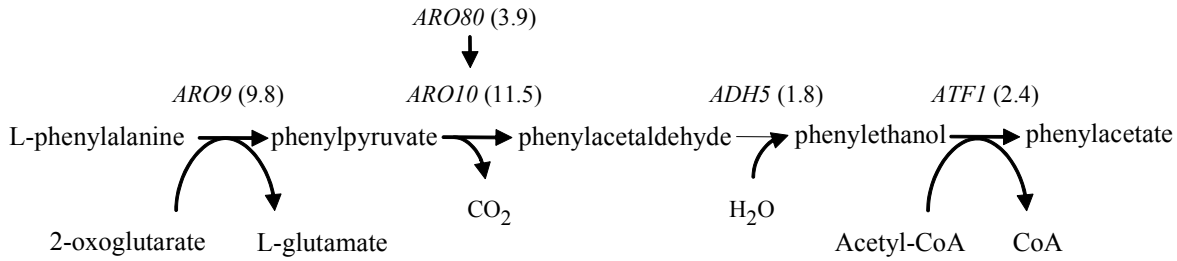


Figure 27: Caffeine-induced transcription of genes in Ehrlich pathway.
Changes in expression levels of the genes involved in degradation of phenylalanine are given in parentheses.

2.1.3.3 Tor1p is the cellular target of caffeine

2.1.3.3.1 Caffeine affects growth of mutants impaired in the TOR pathway

The transcriptional response of yeast to caffeine resembled the situation of depletion of TOR kinases. For this reason, we checked the effect of caffeine on the growth of mutants impaired in the TOR pathway. As the deletion of *TOR2* is lethal, we tested a *tor1*Δ mutant and strains with mutated alleles of TOR kinases *tor1-1* and *tor2-1 tor1*Δ. Two mutations, *tor1-1* (Ser1972Arg) and *tor2-1* (Ser1975Ile), have been shown to render cells rapamycin-insensitive (Heitman *et al*., 1991). This test revealed that the *tor1*Δ mutant was hypersensitive to caffeine even at 2 mM (result not shown), while the rapamycin-insensitive mutant *tor1-1* was less sensitive to caffeine, but more sensitive than the wild type (Figure 28, below). Interestingly, although Tor2p could not rescue the sensitivity of the *tor1*Δ mutant, the Tor2-1p version of this protein from the rapamycin-insensitive *tor2-1 tor1*Δ mutant was able to perform this function. Additionally, we found that *NIR1*, proposed to be an important component of the TOR pathway (Huang *et al*., 2004), was required for growth on caffeine-containing media.

To some extent, the effects elicited by caffeine were similar to those caused by rapamycin. For instance, incubation of yeast cells with 20 mM caffeine promoted nuclear localization of an Rtg1-GFP protein fusion (data not shown), albeit in a less pronounced manner than that caused by rapamycin (Komeili *et al*., 2000). In addition, we found that the deletions of *URE2* or *RTG1* conferred increased sensitivity to caffeine, whereas the *gln3*Δ mutant showed slight resistance to this drug (Figure 28). Respectively, the same phenotypes,

hypersensitivity or resistance, have been reported when rapamycin was used (Cardenas *et al*., 1999; Xie *et al*., 2005).

Interestingly, a mutant deleted for *CRF1*, a gene involved in the inhibition of transcription of ribosomal genes upon TOR inactivation (Martin *et al*., 2004), turned out to be hypersensitive to caffeine. Active TOR negatively regulates the Yak1 kinase that does not allow Crf1p to enter into the nucleus to exert its inhibitory effect on transcription. However, yeast cells deleted for *YAK1* were sensitive at the wild-type level. Sensitivity test also showed caffeine hypersensitivity of the *msn2*Δ*msn4*Δ double mutant. This result was in agreement with the caffeine-induced activation of genes controlled by Msn2/4p. It has been shown that the Msn2 and Msn4 proteins, stimulated by TOR, can bind to the cytoplasmic protein Bmh2p and this interaction prevents their entrance into the nucleus (Beck and Hall, 1999). *BMH2* was also in the group of genes overexpressed upon caffeine treatment.

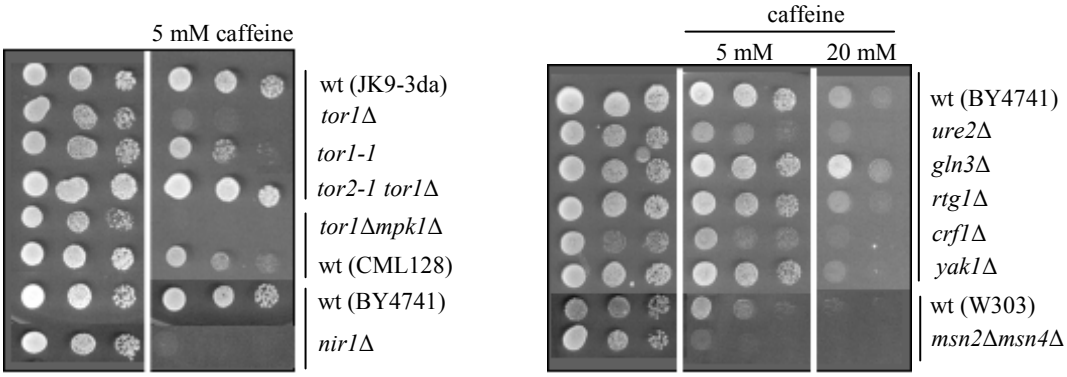


Figure 28: Caffeine sensitivity of mutants in the *TOR* pathway.

Serial dilutions of exponentially growing culture of each mutant were spotted onto YPD agar plates containing various concentrations of caffeine as indicated in the figure. Growth was scored after 2 days of incubation at 28°C.

2.1.3.3.2 Presence of Tor1p is crucial for induction of Mpk1 kinase phosphorylation in response to caffeine treatment

As our results suggested that caffeine could inhibit the TOR pathway, we sought to verify whether the observed caffeine-induced Mpk1p phosphorylation was dependent on the TOR kinases. It can be seen in Figure 29 that the caffeine-induced phosphorylation of Mpk1p was lost in the *tor1*Δ mutant (method in chapter 3.1.8.1, pg 128). Interestingly, the mutated Tor1-1p and Tor2-1p were able to relay the caffeine stress to the Mpk1 kinase. These biochemical data were at a first glance consistent with the genetic data showing that the *tor1*Δ

mutant was hypersensitive to caffeine, whereas introduction of the mutated genes *tor1-1* or *tor2-1* alleviated this phenotype (Figure 28), suggesting a possible correlation between the levels of Mpk1p phosphorylation and caffeine sensitivity. However, this suggestion turned out to be wrong since we found that phosphorylation of Mpk1p was induced in a mutant defective in *NIR1*, a gene encoding a protein proposed to be one of the components of the TOR pathway (Huang *et al*., 2004), while this mutant was hypersensitive to caffeine.

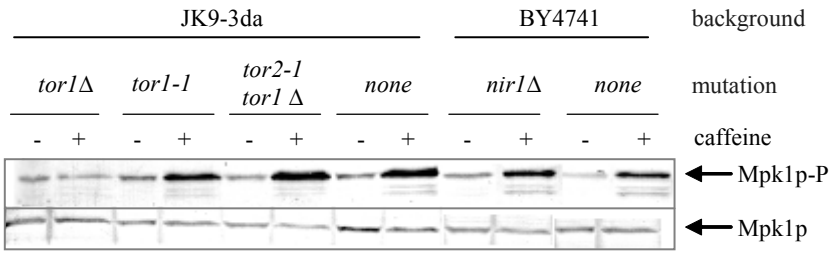


Figure 29: Tor1p is necessary for Mpk1p phosphorylation in response to CAF.

Exponential cultures of the *tor1*Δ, *tor1-1*, *tor2-1 tor1*Δ mutants and their isogenic wild type JK9-3a, and the *nir1*Δ mutant and its wild type BY4741 were treated with 20 mM caffeine for 90 min. Level of Mpk1p phosphorylation was compared to the analogous samples without caffeine added. Phosphorylated Mpk1p and total Mpk1p levels were determined by Western blotting as in Figure 19.

2.1.3.3.3 Caffeine-derived signaling to Mpk1p is mediated through Rom2p

Next step of our investigation was to elucidate at which level the cross talk between the TOR and CWI pathways occurrs in caffeine signaling. To achieve this goal, we decided to identify the component of the CWI pathway that is required for the signaling to Mpk1 kinase. Since we knew that sometimes phenotypes caused by deletion of a given gene might differ depending on the genetic background of the yeast strain, first we confirmed that the CWI mutants tested were caffeine sensitive (Figure 30A below). Importantly, like other groups (e.g. (Parsons *et al*., 2004)), we showed that the *rlm1*Δ mutant was not hypersensitive to caffeine, which supports our previous result of its redundancy in the caffeine-induced cell wall remodeling. Thanks to the work of Martin and colleagues (Martin *et al*., 2000) we knew that caffeine-induced phosphorylation of Mpk1p was dependent on Rho1p and the Mkk1 kinase that directly activates Mpk1p. In addition, we found that the caffeine-induced Mpk1p phosphorylation was abolished upon the deletion of *ROM2* but it was not altered by deletion of the two major cell surface sensors encoded by *MID2* and *WSC1* (Ketela *et al*., 1999; Lodder *et al*., 1999) (Figure 30B). This result contrasted with the fact that mutants defective in either one or both sensors were hypersensitive to caffeine (Figure 30A). We then elaborated

upon this study by investigating a mutant downstream of Pkc1p. As indicated in Figure 30B, the *bck1*Δ mutant did not show a detectable level of phosphorylated Mpk1p either before or after treatment with caffeine. As a conclusion, while these experiments allowed the identification of the components in the CWI pathway needed for Mpk1p phosphorylation in response to caffeine, they also provided a clear indication that the activation of this pathway does not protect cells from the detrimental effects of caffeine.

The finding that the major cell wall sensors Mid2p and Wsc1p were not involved in the caffeine-mediated activation of the Pkc1p-Mpk1p pathway favored the idea that caffeine needs to be internalized to exert its effects.

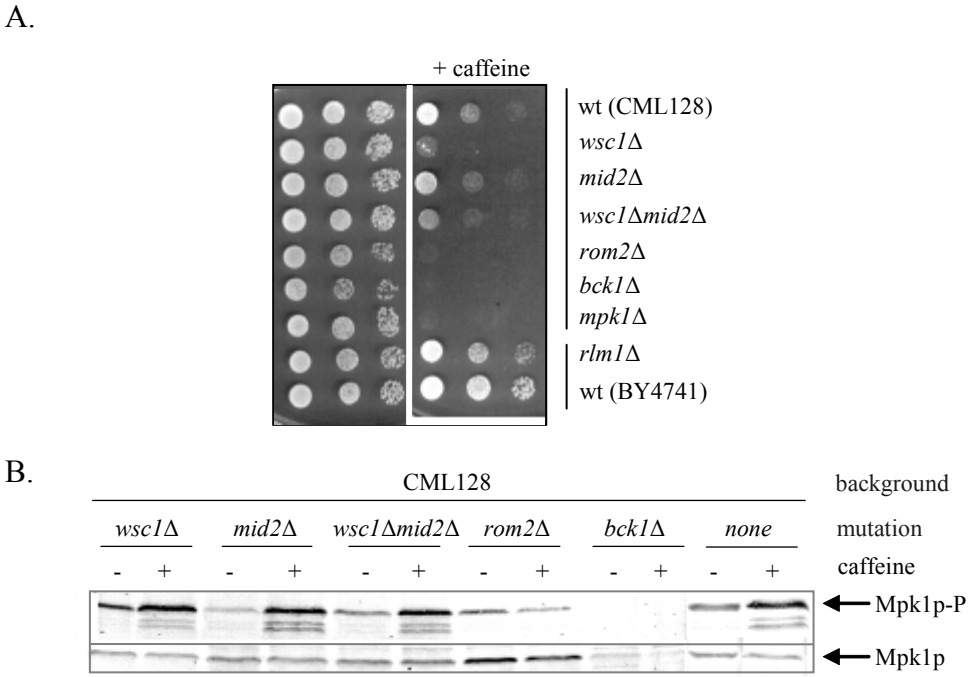


Figure 30: Rom2p is required for caffeine-induced Mpk1p phosphorylation.

A. Serial dilutions of exponentially growing culture of each mutant on YPD were spotted onto YPD agar plates containing 0 or 5 mM caffeine. **B**. Mutants of the CWI pathway (*wsc1*Δ, *mid2*Δ, *wsc1*Δ*mid2*Δ, *rom2*Δ, *bck1*Δ) and their isogenic wild type CML128 were treated with 20 mM caffeine for 90 min. The level of Mpk1p phosphorylation was compared to analogous samples without caffeine added. Phosphorylated Mpk1p and total Mpk1p levels were determined by Western blotting as in Figure 19

2.1.3.3.4 Inhibition of growth caused by caffeine can be alleviated by addition of adenine or sorbitol to culture medium, except for the tor1Δ *mutant.*

Previously, it was proposed that caffeine was actively imported into yeast cells by an unknown adenine permease (Bard *et al*., 1980). To verify this proposal, we supplemented the

medium with an excess of adenine, expecting that if adenine and caffeine were transported via the same permease we would be able to decrease the uptake of caffeine and consequently to decrease its growth-inhibiting effect. Therefore, we tested the effect of adenine on the caffeine sensitivity of mutant strains impaired in the TOR as well as the CWI pathway (Figure 31A below). Given that the cell lysis caused by caffeine is an osmoremedial effect, we used sorbitol-supplemented medium as a control in this experiment. It turned out that addition of adenine as well as addition of sorbitol rescued the caffeine-sensitive phenotype of all the strains tested with the exception of the *tor1*Δ mutant. This result could reflect a competition between caffeine and adenine while they were transported through the same plasma-membrane permease. In yeast, the only one known adenine permease is encoded by the *FCY2* gene (Weber *et al*., 1990). We tested the sensitivity to caffeine and the level of caffeine-induced Mpk1p phosphorylation in the *fcy2*Δ mutant (results not shown). However, the mutant strain reacted like the wild type, which suggests that the *FCY2*-encoded adenine permease was not involved in the uptake of caffeine and other transporters must have performed this function.

In order to investigate further the effect of adenine on the caffeine sensitivity we sought to examine if adenine would decrease the level of caffeine-induced phosphorylation of Mpk1 kinase. Contrary to our expectation, this was not the case, instead, adenine similarly to caffeine induced phosphorylation of Mpk1p (Figure 31B). This effect could be explained by adenine, an analogue of caffeine, mimicking its presence in the cell. Thus, adenine can induce Mpk1p phosphorylation but is not toxic to the cells. Apparently, this effect merits further investigation, although it showed that the mode of adenine and sorbitol action to release the growth inhibition of mutants by caffeine were evidently different.

As a corollary of these data, we showed that the hypersensitivity to caffeine was not correlated with the ability of the mutant strains to induce Mpk1p phosphorylation. In fact, our experiments allowed us to distinguish two groups of the mutants tested in this study. The first group comprised mutants that were caffeine sensitive and unable to phosphorylate Mpk1p in response to this drug (*rom2*Δ, *bck1*Δ, *mpk1*Δ, *tor1*Δ). To the second group we qualified those caffeine-sensitive mutants that were able to activate Mpk1p as well as the wild type (*wsc1*Δ, *mid2*Δ, *wsc1*Δ*mid2*Δ, *TOR1-1*, *nir1*Δ). This idea was further supported by the fact that adenine rescued cells growing on caffeine-containing medium, while adenine itself induced Mpk1p phosphorylation rather than repressing it, as it was in the case of sorbitol. Moreover, sorbitol rescued also the growth of the *nir1*Δ mutant that was not impaired in the caffeine-

induced phosphorylation of Mpk1p. Consequently, the hypersensitivity to caffeine could be ascribed to the loss of Tor1p function, independently of the Pkc1p-Mpk1p pathway. Thus, it remains to be identified which Tor1p-regulated cellular functions are responsible for the sensitivity to caffeine.

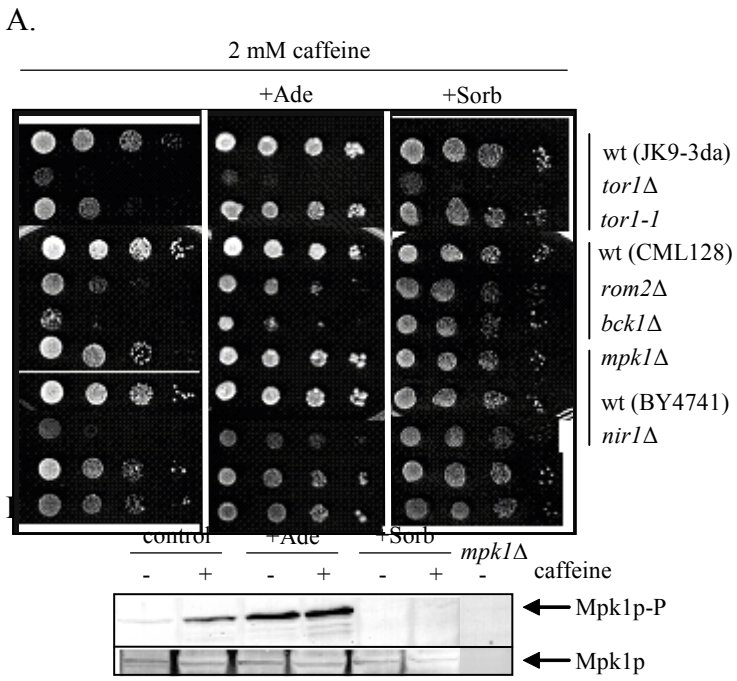


Figure 31: Effect of adenine and sorbitol on caffeine sensitivity of mutants in the CWI and TOR pathways.

A. The effects of adenine and sorbitol on the sensitivity to caffeine. Serial dilutions of yeast cultures were spotted onto YPD agar plates without additions or containing 2 mM caffeine and supplemented with either 20 mM adenine (Ade) or 1 M sorbitol (Sorb). Growth was scored after 2 days at 28°C. **B**. Effects of adenine and sorbitol on caffeine-induced phosphorylation of Mpk1p. Early exponential cultures of BY4742 cultivated in the absence (control), or in the presence of either 20 mM adenine (Ade) or 1 M sorbitol (Sorb), were treated for 90 min with 20 mM caffeine. Level of Mpk1p phosphorylation was compared to analogous samples without caffeine added. Phosphorylated Mpk1p and total Mpk1p levels were determined by Western blotting as in Figure 19, pg 53.

2.1.3.3.5 Loss of Tor1p function results in strengthening of the cell wall

To study further the cell wall remodeling in response to caffeine we asked whether the changes in the cell wall composition caused by caffeine are mediated through its effect on the TOR pathway. To this end, we measured the sensitivity of the *tor1*Δ mutant to Zymolyase (Figure 32). As we showed before, the *tor1*Δ mutant was unable to activate the MAP kinase cascade measured as Mpk1p phosphorylation (Figure 29, pg 66), thus we expected that it would be also unable to elicit caffeine-induced cell wall remodeling. Contrary to expectation, it turned out that the mutant strain was less sensitive to cell wall digestion than wild-type cells. This result showed that deletion of *TOR1*, like the inhibition of its function caused by caffeine, stimulated strengthening of the cell wall. Moreover, it showed that the caffeine-

derived signal for the cell wall remodeling was not mediated through Tor1p, since the cell wall strengthening was still possible in the *tor1*Δ mutant. Therefore, in the *tor1*Δ mutant the MAP kinase cascade was not responsive to caffeine and the alteration of the cell wall composition should depend on other transducers.

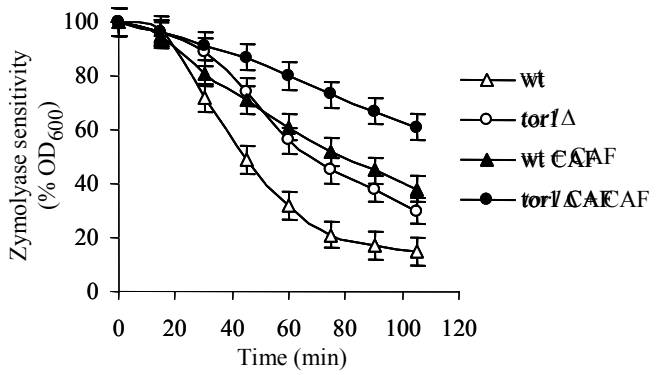


Figure 32: Loss of Tor1p function results in a decrease of Zymolyase sensitivity.

Wild-type yeast cells and the *tor1*Δ mutant (JK9-3da background) were challenged for 90 minutes with 20 mM caffeine prior to Zymolyase assay. Every 15 minutes cells lysis was measured as a decrease of OD at 600 nm (percentage of the initial value). Presented results are an average of three independent experiments ± SD.

2.1.3.4 Caffeine causes a transient Rom2p-dependent decrease in cAMP level

As indicated by the Venn diagram in Figure 25 (pg 61), we identified a set of 14 genes whose expression was affected both upon deletion of *PDE2*, encoding cAMP phosphodiesterase (Jones *et al*., 2003), and upon exposure to caffeine, but the genes that were induced by the increased cAMP level in the *pde2*Δ mutant were downregulated in the presence of caffeine. Moreover, we observed changes in the expression of genes directly involved in the biosynthesis of this signaling molecule. These results suggested that caffeine might cause an inhibition of the Ras/cAMP cascade. In accordance with this idea, we showed that addition of caffeine to early exponentially growing cells triggered a rapid but transient decrease in the intracellular level of cAMP, with an effect that was really significant at 20 mM caffeine (Figure 33A, method in chapter 3.1.8.2, pg 129). Our result was thus at variance to the effects of this drug reported for mammalian cells (Belibi *et al*., 2002; Jafari and Rabbani, 2000; Naderali and Poyser, 1997). Remarkably, we found that this caffeine-induced decrease of cAMP was defective in a mutant deleted for *ROM2*, encoding a GDP/GTP exchange factor whose main target is Rho1p that activates the Pkc1p-Mpk1p cascade (Figure 33B, below). This latter result was in agreement with the recent proposition of Park and co-workers that Rom2p downregulates PKA activity by decreasing the levels of cAMP (Park *et al*., 2005).

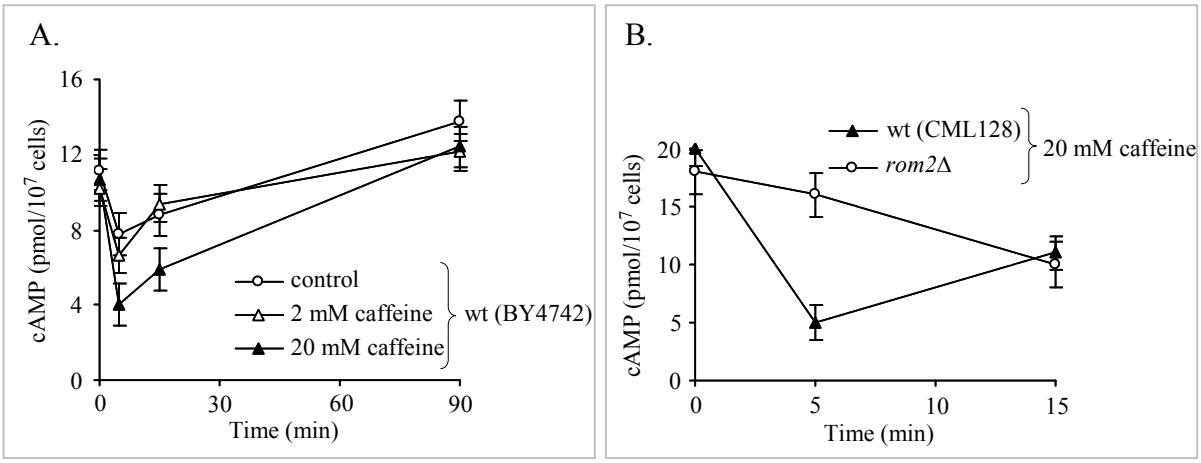


Figure 33: Caffeine induced a transient decrease in intracellular level of cAMP that is abolished in the *rom2*Δ mutant.

A. Early exponential cultures (OD600 ≈ 0.2-0.4) of the wild type strain (BY4742) in YPD medium were treated with caffeine at a final concentration of 0, 2, or 20 mM. Samples were taken at indicated times to measure cAMP. Bars indicate standard deviation derived from duplicates of three independent experiments. **B**. Similar as in panel A, except that cAMP was measured in the wild type (CML128) and in the *rom2*Δ mutant after addition of 20 mM caffeine.

2.1.3.5 Caffeine and growth regulation

It has been shown in various organisms that the TOR pathway is implicated in the temporal and spatial control of growth (for a review see (Jacinto and Hall, 2003; Martin and Hall, 2005)). While Tor1p and Tor2p share the function of the 'temporal' control, only Tor2p takes part in the 'spatial' control of growth via regulating the cell cycle-dependent polarization of the actin cytoskeleton. The inhibition of both Tor1p and Tor2p by rapamycin or the deletion of respective genes causes growth arrest in the early G1 phase. The deletion of *TOR2* causes growth arrest randomly at any phase of cell cycle, while the deletion of *TOR1* causes only mild growth defects (Barbet *et al*., 1996; Heitman *et al*., 1991; Kunz *et al*., 1993). Taking into account these data, we decided to verify whether caffeine could also influence the yeast cell cycle (phases of the yeast cell cycle can be seen in Figure 1, pg 4). To this end, to early-exponential yeast cultures various concentrations of caffeine were added and after 24 h of growth budding and non-budding cells (G1 cells) were counted. As shown in Table 9, addition of caffeine did not cause an arrest in the G1 phase of the cell cycle, since we observed roughly the same percentage of budded and unbudded cells. This result suggested that caffeine did not inhibit both Tor proteins, which is necessary for the G1 arrest of cell

cycle. The fact that only Tor2p has been shown to be required for G1 progression (Kunz *et al*., 1993) further supports our biochemical and genetic data indicating that caffeine affects Tor1p only.

Table 9: Caffeine does not arrest the cell cycle

Caffeine concentration	*G1 cells* (%)	*Budding cells* (%)
-	59	41
2 mM	62	38
20 mM	63	37

Additionally, we used a Nikon Eclipse E800 microscope with the Lucia G/F Screen Measurement System (Nikon) to measure the size of cells treated with caffeine. It turned out that caffeine caused an increase in the cell size (Figure 34, method in chapter 3.1.8.4, pg 130) and cells seemed to be more round than oval. An average of the cell radius from two independent experiments (in each ~200 cells were counted) was 2.96 µm (±0.41) for the control cells and 3.59 µm (±0.35) for caffeine-treated cells. An enlargement of yeast cells could be also seen in the case of rapamycin treatment, although this fact was not carefully studied (Cardenas and Heitman, 1995; Schmelzle *et al*., 2004). Eventually, it is not sure whether this effect of caffeine is mediated through the TOR function or not.

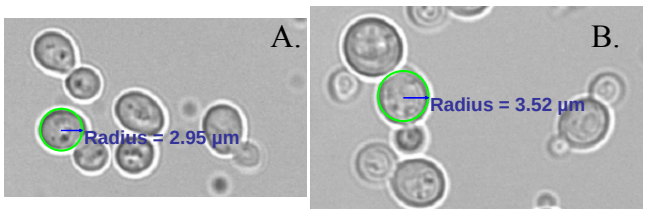


Figure 34: Caffeine affected the size of yeast cells.

Light microscopy of yeast cells (Nikon Eclipse E800 microscope, objective magnification × 100, oil immersion). Radius of cells that gives approximation of the cell size was scored after 24 h of growth without (**A**) or with 20 mM caffeine added (**B**). At least 200 cells were measured from two independent experiments. The radius of cells growing in the presence of caffeine was by 14% higher then the radius of untreated cells.

2.1.3.6 Discussion

In this part of the work, we present a number of genetic and biochemical results characterizing the action of caffeine in a lower eukaryotic cell. A model based on these experiments is shown in Figure 35. Overall, we can propose that caffeine may affect at least three pathways TOR, Ras/cAMP and CWI, and it also causes pronounced reinforcement of the cell wall. Generally, there is increasing evidence that these pathways as well as the cell

wall remodeling are functionally linked to each other and our data brings a new insight into the interdependencies among them.

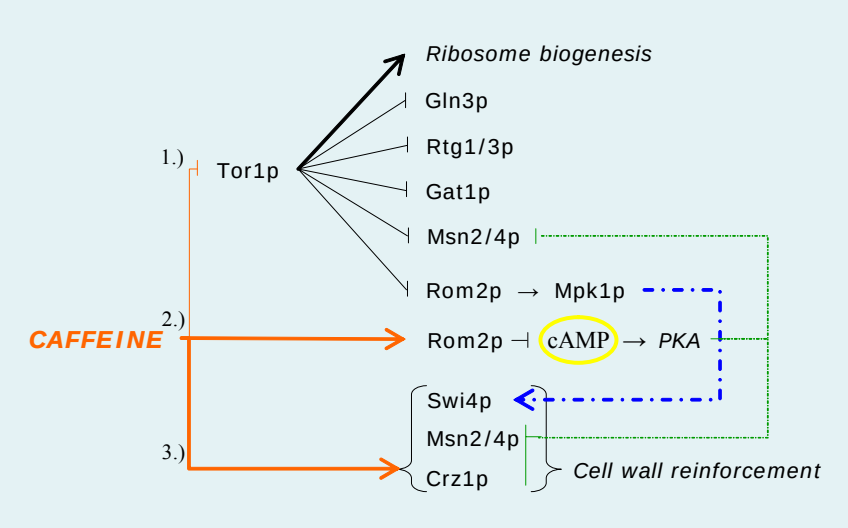


Figure 35: Model of caffeine action in the yeast cell.

1.) Caffeine inhibits the function of Tor1p, which causes downregulation of ribosome biogenesis and activation of gene expression dependent on Gln3p, Rtg1/3p, Gat1p and Msn2/4p. In addition, the inhibition of Tor1p activates Rom2p GDP/GTP exchange factor that activates the Pkc1p-Mpk1p signaling cascade. **2.)** Caffeine also elicited inhibition of the Ras/cAMP signaling cascade however, it is not sure whether Tor1p mediated this effect. **3.)** Independently of Tor1p, caffeine causes reinforcement of the cell wall that is mediated by at least three transcription factors, i.e. Swi4p, Crz1p and Msn2/4p. Additional regulations are indicated with the dashed lines although it is not verified if they were functional in the presence of caffeine. First, showing that Msn2/4p can be regulated by PKA as well as by the TOR pathway (Beck and Hall, 1999; Boy-Marcotte *et al*., 1998). Second, showing that Crz1p is negatively regulated by PKA (Kafadar and Cyert, 2004). Third, showing that Swi4p can be activated by the Mpk1 kinase (Baetz *et al*., 2001).

Caffeine and the TOR pathway. Our results allow proposing the Tor1 kinase as the main target of caffeine in yeast, whose inhibition leads to the activation of the Pkc1p-Mpk1p signaling cascade, and indicate that this activation is not important for the cell survival in the presence of caffeine. These conclusions are based on the genome-wide expression analysis, complemented by genetic and biochemical data, showing that the response of yeast cells to caffeine resembled the response to rapamycin, an inhibitor of Tor1/2 kinases. With respect to the response of cells to caffeine, *TOR1* could be apparently replaced by the *tor2-1* dominant allele, as it was able partially to overcome the caffeine sensitivity of the *tor1*Δ mutant and restore the caffeine-induced Mpk1p phosphorylation. This result can be explained if we assume that the Tor2-1p variant could exhibit kinetic properties similar to those of Tor1p. Whatever the exact mechanism, the relative specificity of caffeine action against Tor1p is likely the reason for the narrower spectrum of the transcriptional response induced by this drug when compared to rapamycin, which inhibits both kinases. On one hand, both caffeine and rapamycin induced the Gln3p-dependent nitrogen catabolite-repressed genes, activated

Rtg1/3p-regulated genes involved in specific TCA and anaplerotic reactions, and downregulated genes involved in ribosome biogenesis (Cardenas *et al*., 1999; Hardwick *et al*., 1999; Shamji *et al*., 2000). This result suggests the predominant role of Tor1p over Tor2p in controling these functions, a conclusion that could not be reached using rapamycin since this drug inhibits both Tor proteins (Barbet *et al*., 1996). Moreover, this result supports the work of Reinke and colleagues (Reinke *et al*., 2004) who proposed that Tor2p becomes a component of the TORC1 complex only upon Tor1p depletion. On the other hand, unlike rapamycin, caffeine did not affect processes that are supposed to be mainly dependent on the Tor2p function (Helliwell *et al*., 1998a; Kunz *et al*., 1993), like growth arrest at the G1 phase of the cell cycle (Table 9, pg 72).

While we observed effects consistent with Tor1p inhibition, the exact mechanism by which caffeine could exert this effect remains unknown. Since cells bearing the rapamycin-insensitive Tor1-1p were still more sensitive to caffeine than wild-type cells, this mechanism should be distinct from the action of rapamycin, i.e. independent of the binding between Tor1p and the yeast FKBP12 protein. Additionally, one cannot exclude that the caffeine effect on Tor1p requires the presence of one of its specific protein partners. Since we assume that Tor2p is not implicated in the caffeine effect, protein members of the TORC2 complex can be excluded. Likewise Lst8p, which is present in both TORC1 and TORC2 complexes, probably is not involved either (Loewith *et al*., 2002). An involvement of Tco89p, which interacts specifically with Tor1p, is very unlikely since the loss of its function did not induce the transcriptional readouts associated with the inhibition of the TOR function (Reinke *et al*., 2004), leaving Kog1p as a potential candidate. Finally, the most probable scenario is that caffeine in yeast, similarly to mammalian cells, directly inhibits the Tor1 kinase (McMahon *et al*., 2005). This action of caffeine could be likely extended to other PI3K-related kinases, like Tel1p or Mec1p proposed previously to be involved in yeast response to caffeine (Moser *et al*., 2000; Saiardi *et al*., 2005), since also the mammalian counterparts of these kinases, ATM and ATR, were shown to be inhibited by caffeine *in vitro* (Blasina *et al*., 1999; Hall-Jackson *et al*., 1999). Thus, it will be interesting to examine further the sensitivity of these three PI3K-related kinases to the action of caffeine in order to evaluate which of them is the most sensitive target of this drug in yeast and in mammalian cells.

Lack of correlation between caffeine sensitivity and the activation of Mpk1 kinase. One of the important findings of this study was to show that the hypersensitivity to caffeine was not correlated with the phosphorylation/activation of Mpk1p. Actually, our experiments allowed distinguishing two groups of caffeine-dependent phenotypes. The first

group comprised mutants that were caffeine hypersensitive and unable to phosphorylate Mpk1p in response to this drug (*rom2*Δ, *bck1*Δ, *mpk1*Δ, *tor1*Δ), while the second group contained caffeine-hypersensitive mutants that were still able to activate Mpk1p like wild-type cells (*wsc1*Δ, *mid2*Δ, *wsc1*Δ*mid2*Δ, *tor1-1*, *nir1*Δ). Moreover, addition of adenine to the growth medium could overcome the caffeine hypersensitivity of mutants impaired in the CWI pathway, despite the fact that it acted oppositely to sorbitol on the level of phosphorylated Mpk1p, i.e. adenine induced phosphorylation of the kinase, while sorbitol diminished this effect. In addition to these data, we show that caffeine-induced Mpk1p phosphorylation was fruitless in terms of Rlm1p activation, which is the main transcription factor inducing the cell wall genes in case of the cell wall damage. Therefore, we propose that increased phosphorylation of Mpk1p was just a 'side effect' of Tor1p inhibition by caffeine, and it is not necessary for the cell's survival in the presence of this drug. In favour of this idea, it was shown that expression of a constitutively activated allele of *RHO1* could not overcome caffeine hypersensitivity of the *rom2*Δ mutant (Park *et al*., 2005).

Caffeine and the cell wall remodeling. Since caffeine was used in yeast to identify mutants impaired in cell wall biosynthesis we verified its action concerning this cellular structure by comparing its effects with those of two cell wall damaging agents, Congo red and Calcofluor white (Figure 23, pg 59; Table A 1, pg 155). These agents are known to disturb the cell wall directly by interacting with it, and the defense of the cells includes activation of the CWI pathway (de Nobel *et al*., 2000; Kopecka and Gabriel, 1992; Roncero and Duran, 1985). Thus, these two compounds shared with caffeine one common feature, i.e. the induction of Mpk1p phosphorylation. When we proved the occurrence of caffeine-induced changes in the cell wall we addressed the question whether the cell wall remodeling induced by caffeine was a direct consequence of the Mpk1p activation. Our results clearly demonstrated that this was not the case. On the contrary, the caffeine-induced phosphorylation of Mpk1p was not accompanied by induction of the Rlm1-dependent genes encoding the cell wall proteins, and remodeling of the cell wall could occur independently of the *RLM1* gene. On the other hand, we identified three mutants *swi4*Δ, *crz1*Δ and *msn2*Δ*msn4*Δ, which were impaired in the caffeine-induced cell wall remodeling, showing that each of them was contributing to this process. An involvement of these transcription factors was consistent with the induction of their targets in the transcription profile of the cells treated with caffeine. In the tested conditions, Swi4p could be activated by phosphorylated Mpk1p (Baetz *et al*., 2001), although some other way of activation cannot be excluded. The Crz1p and Msn2/4p transcription

factors were both shown to be negatively regulated by PKA (Beck and Hall, 1999; Boy-Marcotte *et al*., 1998). Hence, it would be interesting to verify whether Crz1p and Msn2/4p were activated because of the decreased cAMP level or independently of this effect.

The cell wall gene most affected by caffeine treatment was *HSP12* (11-fold induction). This gene was shown to be required for biofilm formation (Zara *et al*., 2002), cell wall flexibility as well as growth on caffeine-containing media (Motshwene *et al*., 2004). It was also shown to be negatively regulated by PKA (Siderius *et al*., 1997), which is consistent with our observation of inhibited Ras/cAMP pathway.

In addition, typical sensors of the cell wall damage like Wsc1p and Mid2p were not involved in transmitting the caffeine-derived signal to Mpk1p. The transcriptional response of yeast cells, when compared to the response to cell wall damaging agents, was rapid and transient at a low concentration of caffeine. Altogether, these results support the idea that caffeine does not act directly at the cell surface to trigger its effects but it must enter the cell first.

Caffeine inhibits the Ras/cAMP pathway. In this work, we report genetic and biochemical results showing that unlike in mammalian cells, in yeast caffeine causes a rapid and transient decrease in the intracellular level of cAMP. Then a new question emerged, whether this effect was a consequence of Tor1p inhibition by caffeine. In a previous work from M. Hall's group, it was reported that the rapamycin-induced inhibition of TOR resulted in the translocation of one of the PKA catalytic subunits to the nucleus (Schmelzle *et al*., 2004). This translocation reflects the inactivation of PKA that is usually caused by a decrease of cAMP. However, the question whether TOR could control PKA directly via a protein-protein interaction or by decreasing cAMP level was not addressed in that study.

Furthermore, we identified Rom2p as required for the observed drop of cAMP. This result was consistent with another report in which Rom2p was proposed to regulate negatively PKA by decreasing cAMP level (Park *et al*., 2005). Importantly, that report suggested that Rom2p acted on cAMP independently of downstream components of the Pkc1p-Mpk1p cascade, as a constitutive allele of *RHO1* did not rescue the caffeine-sensitivity of the *rom2*Δ mutant, additionally supporting the redundancy of this pathway in resistance to caffeine. Moreover, we found that while Wsc1p, a protein that directly interacts with Rom2p to activate it, was not necessary for mediating the caffeine-derived signal to Mpk1 kinase, a mutant lacking *WSC1* was still hypersensitive to caffeine. This result could be explained by the putative role of the *WSC1-3* genes in the Ras/cAMP signaling that was shown in the work of Verna and colleagues (Verna *et al*., 1997). In that report, it was shown that the heat shock

sensitivity of cells carrying a hyperactive allele of *RAS2* was rescued by overexpression of the *WSC* genes. Moreover, the heat shock sensitivity of the *wsc*Δ mutants was rescued by deletion of *RAS2*. Hence, now it would be interesting to verify if the caffeine-induced decrease in cAMP level was also dependent on the *WSC* genes.

In addition, we showed that the 'inhibitory' concentration of caffeine induced phosphorylation of Mpk1 kinase but also of two other MAP kinases, Kss1p and Fus3p. These two proteins have also been reported to regulate Ras/cAMP signaling. Fus3p represses cAMP accumulation regardless of its phosphorylation status, while Kss1p represses accumulation of cAMP only when it is not phosphorylated, otherwise it is a positive regulator of cAMP (Cherkasova and Hinnebusch, 2003). Interestingly, in cells treated with caffeine we detected overexpression of *FUS3* but not of *KSS1*. It is possible that the caffeine-induced phosphorylation of Fus3p and Kss1p could be linked to the observed decrease of the cAMP level. In future studies it will be important to verify whether Fus3p could act in concert with Rom2p to repress cAMP accumulation. However, our data imply rather that Fus3p should be under control of Rom2p, since deletion of *ROM2* was sufficient to abolish the caffeine-induced drop of cAMP level. How Rom2p affects cAMP is still unknown, although it was reported that its action was independent of Ira2p, a GAP for Ras2p (Park *et al*., 2005). On the other hand, Fus3p and Kss1p were shown to affect cAMP through interaction with Cdc25p, a GEF for Ras2p (Cherkasova and Hinnebusch, 2003).

In conclusion, we present here a comprehensive study of caffeine action in yeast at the cellular level and we propose that Rom2p, a component of the CWI pathway, is a convergence point for conveying the caffeine-induced signal to the Ras/cAMP pathway and the Pkc1p-Mpk1p signaling cascade.

2.2 REGULATION OF THE ISOPRENOID PATHWAY

2.2.1 Transcriptional response of yeast to inhibitors of isoprenoid pathway

In order to investigate regulation of the IP pathway, we chose to manipulate isoprenoid metabolism using two inhibitors, lovastatin and zaragozic acid, that have the same effect on the cellular ergosterol level but act differently on the availability of FPP (see Figure 6, pg 21). While lovastatin would decrease the level of FPP, zaragozic acid, by inhibition of SQS, the main FPP-consuming enzyme, increases its availability for other branching pathways. We have chosen to use 25 μg/ml lovastatin and 10 μg/ml zaragozic acid, as these concentrations had been reported, respectively, to inhibit and induce Hmg2p degradation, which is dependent on FPP availability in the yeast cell. Moreover, addition of 25 μg/ml lovastatin to the medium was sufficient to abolish the enhancing effect of 10 μg/ml zaragozic acid on Hmg2p degradation (Gardner *et al*., 2001). According to our measurements both drugs at the chosen concentrations inhibited the growth rate by only ~5% {unpublished data}, therefore we had a good chance of observing primary effects of these drugs and not secondary processes typically associated with the growth inhibition and death of the cell. As the effects of these inhibitors were earlier studied only after long incubation times, to complement those studies, we have decided to focus on the short-term response to these agents. To this end, we analyzed the genome-wide transcriptional response of yeast cells after 10, 30 and 60 minutes of treatment with lovastatin and zaragozic acid.

2.2.1.1 Short-term response of yeast cells to lovastatin

In order to examine yeast response to lovastatin, a HMG-CoA reductase (HMGR) inhibitor, we performed genome-wide expression analysis in cells treated with lovastatin (25 μg/ml) and compared it with expression pattern of the cells grown without any additions (see details in chapter 3.1.4, pg 117). Samples were taken after 10, 30 and 60 minutes.

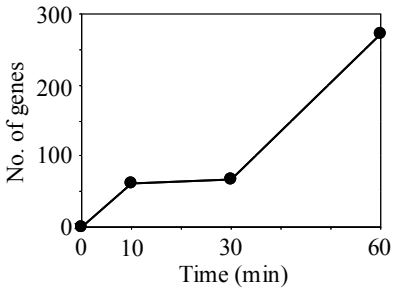


Figure 36: Number of genes whose expression was changed in response to lovastatin.

Genes whose ratio of expression has changed, at indicated time-points, by at least 1.5-fold (P value 0.05) were treated as significantly changed.

Then, all differentially expressed genes were ascribed to the SGD functional categories. The numbers of upregulated (red histograms) or downregulated (green histograms) genes in each category are shown in Figure 37, reflecting a relative activation or repression of a given category. The majority of genes fell into the categories of transport and organelle organization. Overal effect of lovastatin stress was to induce gene expression. This concerned especially genes involved in meiosis and cell cycle as well as in protein catabolism, protein biosynthesis and ribosome biosynthesis.

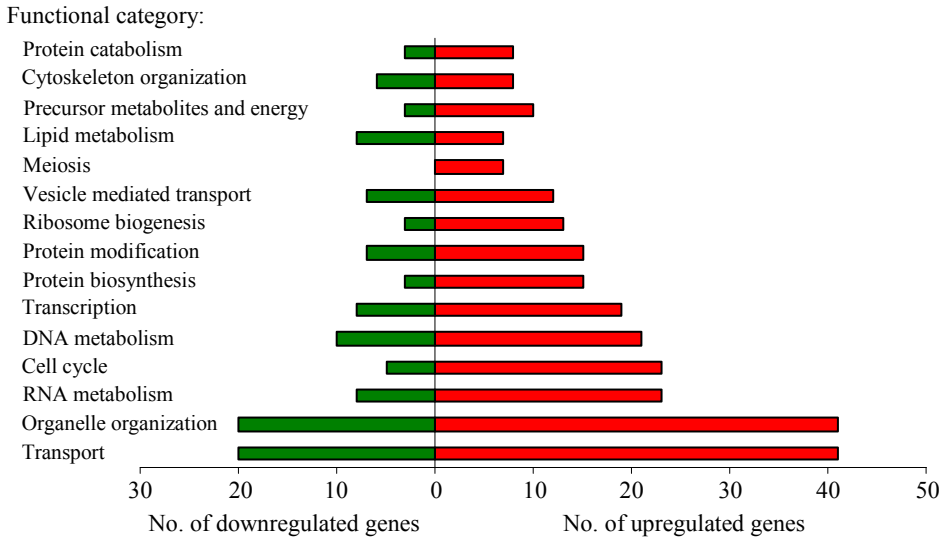


Figure 37: Distribution of lovastatin-responsive genes among functional categories according to Slim Mapper.

In the tested conditions, lovastatin did not cause expression changes of genes that were directly involved in the IP pathway. We did not observe significant changes in the expression of genes involved in dolichylphosphate-dependent protein glycosylation, and expression of only two genes coding for prenylated proteins was increased, i.e. *RCY1* involved in the recycling of plasma membrane proteins and *YNL234W* coding for a protein of unknown function and possessing a heme-binding domain. In agreement with the previous work of

Dimster-Denk and colleagues who did not observe any changes in the *HMG1* transcript in the mevalonate-starved or lovastatin-treated cells (Dimster-Denk *et al*., 1994), there was no change in the expression of this gene upon lovastatin treatment.

Nevertheless, we detected overexpression of *DAN1* that is responsible for uptake of sterols in aerobic conditions, which suggested an increased demand of the cell for these compounds (Alimardani *et al*., 2004). In agreement with the fact that the level of sterols affects the plasma membrane we observed overexpression of 13 genes coding for plasma membrane proteins (*SSY5, SRO7, HIP1, DUR3, ASG7, MEP1, OPT2, TNA1, LSB6, PUT4, AZR1, HXT14, MMP1*) and underexpression of six genes involved in membrane lipid metabolism (*PLB1, TSC3, OPI3, YDC1, SCS3, CDS1*).

Lovastatin affected expression of some genes encoding proteins of which activity could be dependent on FPP level. For instance, *COQ8*, upregulated in response to lovastatin, was initially isolated as a multicopy suppressor of a defect in cytochrome b mRNA translation (Bousquet *et al*., 1991). The protein encoded by this gene as well as FPP are required for the biosynthesis of ubiquinone Q. Upon depletion of *COQ8*, cells accumulate a prenylated intermediate (prenyl-4-hydroxybenzoic acid) of ubiquinone Q biosynthesis and have a decreased activity of cytochrome c oxidase (Do *et al*., 2001). Two other genes involved in cytochrome C oxidase activity, *OXA1* and *COX20*, were downregulated due to lovastatin treatment. Changed expression of these genes can reflect a problem with FPP availability since cytochrome c oxidase uses heme A as a cofactor, which is synthesized from FPP (Keyhani and Keyhani, 1978). In fact, earlier data from our laboratory suggest that Coq8p could interact with Erg20p (FPP synthase), as evidenced by a yeast two-hybrid screen {K. Grabinska, PhD thesis}. In the same screen, we identified Hir3p and Yta7p. Remarkably, we found genes coding for these proteins overexpressed in response to lovastatin. Interestingly, we detected an overexpression of *YAL011W*. This gene was identified in a screen for Hmg1p-sensitive mutants (Wright *et al*., 2003), as cells lacking *YAL011W* and overexpressing *HMG1* are characterized by abnormal karmellae assembly.

In the tested conditions, we did not observe changes of expression of genes belonging to the sterol branch of the IP pathway, which obviously does not exclude response at the protein level. Altogether, these data imply that the first reaction of the cell to lovastatin is to increase the uptake of sterols and activation of genes involved in sterol biosynthesis could be the next step of this response. However, this assumption is based only on the transcript data and should be further investigated.

2.2.1.2 Short-term response of yeast cells to zaragozic acid

To investigate the transcriptional response to zaragozic acid, a squalene synthase (SQS) inhibitor, we treated yeast cells with this drug (10 μg/ml final concentration) for 10, 30 and 60 min. According to this analysis, zaragozic acid rapidly changed the level of transcription of a large number of genes; already after 10 min there were 229 such genes, and their number remained approximately unchanged after 30 and 60 minutes (Figure 38).

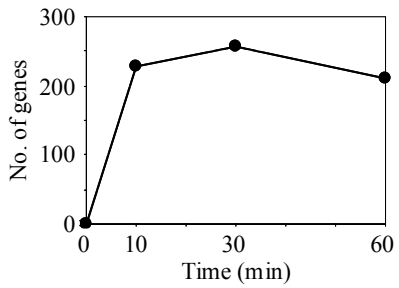


Figure 38: Number of genes whose expression was changed in response to zaragozic acid.

Genes whose ratio of expression has changed by at least 1.5-fold (P value 0.05) were treated as significantly changed.

To get a systematic view of this transcriptomic response, all the differentially expressed genes were assigned to the SGD functional categories. The majority of differentially expressed genes fell into the categories of transport and organelle organization. In the categories like meiosis, protein catabolism, ribosome biogenesis and protein biosynthesis repression of expression prevailed.

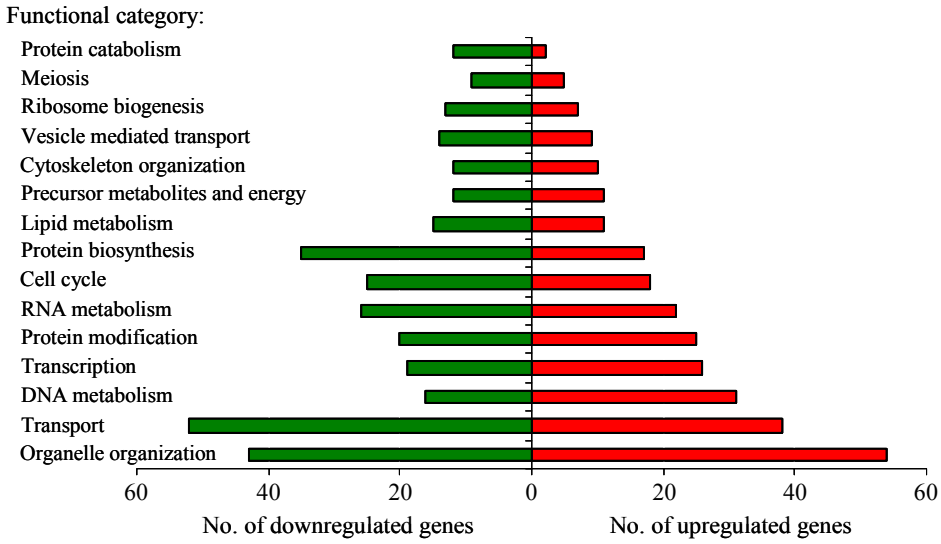


Figure 39: Distribution of zaragozic acid-responsive genes among functional categories according to Slim Mapper.

Zaragozic acid induced expression of two genes involved directly in ergosterol biosynthesis, i.e. *ERG6* and *ERG26*. *ERG26* encodes C-3 sterol dehydrogenase, an enzyme that catalyzes the second of three steps required to remove two C-4 methyl groups from an intermediate in ergosterol biosynthesis (Gachotte *et al*., 1998). The enzyme removes 3-alpha-hydrogen, resulting in the decarboxylation of the 3-ketocarboxylic acid sterol intermediate. *ERG6* encodes delta (24)-sterol C-methyltransferase which converts zymosterol to fecosterol. This result suggests that expression of these genes was the most sensitive to the decreased efficiency of squalene synthase. We also observed changes in the expression of other genes involved in sterol metabolism, like *ARE2*, *OSH1*, *DAN3*, *UGT51*, *YLR101C* and *ECM22*. The product of *ARE2*, which was downregulated, is an enzyme that contributes the major sterol esterification activity in the presence of oxygen and this esterified form of sterol is considered to serve as a storage of sterols in the cell (Zweytick *et al*., 2000). Since the action of this enzyme reduces the amount of free sterol that could be integrated in the plasma membrane, repression of *ARE2* upon depletion of the cellular sterol pool seems reasonable. *OSH1* encodes one of the seven oxysterol binding proteins identified in yeast. It has been shown that the level of oxysterol in cells decreases after treatment with zaragozic acid (Gardner *et al*., 2001). Thus, repression of *OSH1* could be a response to the presumable decrease in oxysterol content. *ECM22*, which was downregulated in response to zaragozic acid, encodes a protein belonging to the fungus-specific Zn(2)-Cys(6) binuclear cluster family of transcription

factors. These proteins bind sterol regulatory element and regulate transcription of the sterol biosynthetic genes, like *ERG2* or *ERG3*. Recently it has been shown that the amount of Ecm22p decreases upon sterol depletion, consistently with our observations (Davies *et al*., 2005). Additionally, Ecm22p was shown to regulate expression of the *DAN3* and *DAN2* genes but not of other *DAN*/*TIR* genes (Abramova *et al*., 2001). Accordingly, we observed overexpression of *DAN3* in the tested conditions. We also found the *UGT51* gene to be overexpressed. *UGT51* encodes sterol glucosyltransferase and although we know that sterol glycosides can be integrated in the yeast membranes their detection is difficult and the function remains unknown (Warnecke *et al*., 1999). Another gene which was overexpressed was *YLR101C*. The function of this gene is unknown but it could potentially affect the sterol pathway as its open reading frame overlaps the 3' end of the essential *ERG27* gene encoding 3-keto sterol reductase.

Zaragozic acid also affected expression of genes involved in protein prenylation including *BET4* (2.63-fold increase) coding for one of the geranylgeranyltransferases in yeast. Additionally, we observed overexpression of genes coding for two prenylated proteins, Ypr092wp of unknown function and Ras2p, a small GTP-binding protein. Consistently with *RAS2* overexpression, we observed changes in expression of other genes involved in cAMP/Ras signaling, i.e. upregulation of *ERF2* and *IRA2*, required for regulation of Ras2p function, upregulation of *SRV2* coding for a regulatory subunit of adenylate cyclase, and downregulation of *BCY1*, a regulatory subunit of cAMP-dependent kinase (PKA).

Moreover, we found repression of some genes involved in heme metabolism. First, *COX10* coding for farnesyltransferase of heme A, which is the prosthetic group required for cytochrome c oxidase activity. *COX18* that is required for the assembly of cytochrome c oxidase and *HMX1* encoding peroxidase involved in the degradation of heme, were downregulated. On the other hand, *PAU2* coding for seripauperin that is normally repressed by heme was overexpressed.

Additionally, the process of glycosylation dependent on dolichyl phosphate, which is synthesized from FPP, seemed to be affected. We detected underexpression of *PMT2* (dolichyl phosphate-D-mannose protein mannosyltransferase), *ALG2* (mannosyltransferase involved in N-linked glycosylation, *alg2*Δ mutants accumulate truncated oligosaccharides linked to dolichyl phosphate), *OST5* (zeta subunit of the protein oligosaccharyltransferase complex) and *SAC1* (depending on the dolichyl phosphate mannose synthase Dpm1p for its attachment to the ER membrane).

Interestingly, we found *GOS1* that was identified in a screen for Hmg1p-sensitive mutants (Wright *et al*., 2003) to be underexpressed due to zaragozic acid treatment. Cells lacking this gene are additionally characterized by abnormal karmellae assembly.

2.2.1.3 Genes regulated both by lovastatin and zaragozic acid

While we were analyzing further transcriptional data, we found a large group of genes (59 genes) whose transcription rate was oppositely regulated upon lovastatin and zaragozic acid treatment, suggesting that there was an inverse correlation between expression levels of these genes and FPP availability. Theoretically, the genes presented in Table 10 and Table 11 could be regulated by the changing level of FPP or its derivatives. Importantly, except for a few genes (overexpressed- *SRD1, NFT1, HXT14, YPR116w*; underexpressed- *VPH1, YNL100w*) we did not find genes that would be regulated in the same way by both inhibitors. Forty-four genes whose expression was activated by lovastatin and repressed by zaragozic acid were mainly involved in RNA metabolism, transcription, protein catabolism and protein biosynthesis (Table 10). These genes seemed to be positively regulated by a decreased availability of FPP or its derivatives and negatively regulated by an increased availability of FPP or its derivatives. Among these genes, we found *YTA7* whose supposed role in the IP pathway will be further examined.

Moreover, we found the *OPT2* gene coding for an oligopeptide transporter to be overexpressed and underexpressed upon treatment with lovastatin and zaragozic acid, respectively. An involvement of Opt2p in the IP pathway is still uncertain but it is supported by another work where this gene was found to be downregulated in a strain with reduced susceptibility to azoles (Barker *et al*., 2003). Another gene in this category was *AZR1*, encoding a plasma membrane transporter that is required for resistance to azoles (Tenreiro *et al*., 2000). A direct involvement of other identified genes in the IP pathway remains unknown and requires further investigation.

Table 10: Genes whose expression was activated by lovastatin and repressed by zaragozic acid

Functional category	Gene	Ratio of expression		Description
		Lo	ZA	
RNA metabolism	*YGR251W*	2.31	0.43	Putative protein of unknown function; deletion mutant has defects in pre-rRNA processing
	YGR272C	2.25	0.58	Putative protein of unknown function; deletion mutant has

				defects in pre-rRNA processing
	JSN1	2.32	0.53	Member of the Puf family of RNA-binding proteins, interacts with mRNAs encoding membrane-associated proteins
	LSM7	1.92	0.22	Lsm (Like Sm) protein; part of heteroheptameric complexes involved in mRNA decay and in processing tRNA, snoRNA, and rRNA
	UTP8	2.16	0.28	Nucleolar protein required for export of tRNAs from the nucleus and in processing of pre-18S rRNA
Protein catabolism	*NAS2*	1.62	0.41	Protein with similarity to the p27 subunit of mammalian proteasome modulator; not essential; interacts with Rpn4p
	CIC1	2.0	0.21	Essential protein that interacts with proteasome components and has a potential role in proteasome substrate specificity; also copurifies with 66S pre-ribosomal particles
	YTA7	2.0	0.61	Protein of unknown function, member of CDC48/PAS1/SEC18 family of ATP-ases, potentially phosphorylated by Cdc28p
Transcription	*POL2*	2.34	0.59	Catalytic subunit of DNA polymerase epsilon, one of the major chromosomal DNA replication polymerases
	YAP6	1.54	0.29	Putative basic leucine zipper (bZIP) transcription factor; overexpression increases sodium and lithium tolerance
	RPB3	1.66	0.59	RNA polymerase II third largest subunit B44, part of central core; similar to prokaryotic alpha subunit
	RPC25	2.0	0.57	RNA polymerase III subunit C25
Protein biosynthesis	*RPL39*	1.61	0.22	Protein component of large (60S) ribosomal subunit, required for ribosome biogenesis; exhibits genetic interactions with SIS1 and PAB1
	RPL14B	2.8	0.37	Protein component of large (60S) ribosomal subunit
	YHR181W	1.53	0.63	(*SVP26*); Integral membrane protein of the early Golgi apparatus, may function to promote retention of proteins in early Golgi compartment; mutation affects protein N-glycosylation and cell wall integrity
Cell cycle	*SWE1*	2.27	0.36	Protein kinase that regulates G2/M transition by inhibition of Cdc28p kinase activity; potential Cdc28p substrate
	SPC72	1.57	0.48	Component of cytoplasmic Tub4p (gamma-tubulin) complex, binds spindle pole bodies and links them to microtubules
	PMS1	1.57	0.4	ATP-binding protein required for mismatch repair in mitosis and meiosis
Other	*MST28*	1.63	0.45	Putative integral membrane protein, involved in vesicle formation; forms complex with Mst27p; binds COPI and COPII vesicles
	AZR1	1.82	0.37	Plasma membrane transporter of the major facilitator superfamily, involved in resistance to azole drugs such as ketoconazole and fluconazole
	COB	1.95	0.2	Cytochrome b, mitochondrially encoded subunit of ubiquinol-cytochrome c reductase complex
	VMA21	2.33	0.6	Integral ER membrane protein that is required for vacuolar H+-ATPase (V-ATPase) function, functions in the assembly of V-ATPase
	CIN2	1.92	0.24	Tubulin folding factor C (putative) involved in beta-tubulin (Tub2p) folding
	DAL7	2.2	0.46	Malate synthase, role in allantoin degradation unknown
	RHR2	1.51	0.56	Constitutively expressed isoform of DL-glycerol-3-phosphatase; involved in glycerol biosynthesis, induced in response to both anaerobic and osmotic stress
	PUT4	1.95	0.55	Proline permease, required for high-affinity transport of proline
	OPT2	2.3	0.36	Oligopeptide transporter; member of OPT family, with potential orthologs in *S. pombe* and *C. albicans*
	MTM1	3.04	0.52	Mitochondrial protein of the mitochondrial carrier family, involved in activating mitochondrial Sod2p
	ATX1	1.87	0.44	Cytosolic copper metallochaperone that transports copper to secretory vesicle copper transporter Ccc2p for eventual insertion

				into Fet3p
	BCY1	1.54	0.43	Regulatory subunit of cyclic AMP-dependent protein kinase (PKA)
	YJL171C	2.17	0.3	GPI-anchored cell wall protein of unknown function; induced in response to cell wall damaging agents and by mutations in cell wall genes
	YJR096W	1.77	0.51	Putative xylose and arabinose reductase
	PGA1	1.66	0.53	Essential protein required for maturation of Gas1p and Pho8p; has synthetic genetic interations with secretory pathway genes
	YOR129C	2.41	0.4	Putative component of the outer plaque of spindle pole body; may be involved in cation homeostasis or multidrug resistance
Unknown	*YBR191WA*	1.61	0.25	Dubious open reading frame unlikely to encode a protein
	YGR126W	2.45	0.54	Protein of unknown function; localized to the cytoplasm and nucleus
	YOL035C	1.85	0.65	Hypothetical protein
	YIL015CA	2.53	0.63	Hypothetical protein
	YOR268C	1.54	0.6	Hypothetical protein
	YJL182C	2.51	0.39	Hypothetical protein
	YPL109C	1.51	0.55	Hypothetical protein
	YPL073C	1.62	0.49	Hypothetical protein
	YML101CA	2.35	0.42	Hypothetical protein
	YCR049C	2.0	0.42	Hypothetical protein

Fifteen genes whose expression was activated in response to zaragozic acid and repressed in response to lovastatin were involved mainly in RNA metabolism, transcription and cytokinesis. With the exception of *MAF1*, these genes had not been functionally linked to the IP pathway by other reports. The role of *MAF1* will be discussed in the next chapter (2.2.1.3.2).

Table 11: Genes whose expression was activated by zaragozic acid and repressed by lovastatin

Functional category	Gene	Ratio of expression		Description
		Lo	ZA	
RNA metabolism	*AI2*	0.58	6.0	Reverse transcriptase required for splicing of *COX1* pre-mRNA, encoded by a mobile group II intron within mitochondrial *COX1* gene
	NOP14	0.57	2.75	Nucleolar protein involved in maturation and nuclear export of 40S ribosomal subunits; and also in processing of pre-18S rRNA
	DBP3	0.56	2.43	Putative ATP-dependent RNA helicase of DEAD-box family involved in ribosomal biogenesis
Cytokinesis	*SAC6*	0.63	2.53	Fimbrin, actin-bundling protein
	BUD6	0.62	2.39	Actin- and formin-interacting protein, involved in actin cable nucleation and polarized cell growth; potential Cdc28p substrate
Transcription	*MAF1*	0.22	2.16	Negative regulator of RNA polymerase III; targets initiation factor TFIIIB
	DNA43	0.63	2.1	(MCM10); Essential, chromatin-associated protein involved in initiation of DNA replication
Other	*FDH1*	0.64	3.78	NAD(+)-dependent formate dehydrogenase, may protect cells from exogenous formate; involved in purine fermentation
	RPN6	0.59	2.72	Essential, non-ATPase regulatory subunit of 26S proteasome lid required for the assembly and activity of 26S proteasome
	JEN1	0.43	2.0	Lactate transporter, required for uptake of lactate and pyruvate; expression is repressed in the presence of glucose, fructose, and mannose

	MDH1	0.66	2.4	Mitochondrial malate dehydrogenase, catalyzes interconversion of malate and oxaloacetate; involved in tricarboxylic acid (TCA) cycle
Unknown	*YCL020W*	0.59	2.23	TyA Gag protein; main structural constituent of virus-like particles (VLPs)
	YLL055W	0.46	2.0	Putative ion transporter activity localized to ER
	YDL068W	0.58	1.69	Hypothetical protein
	YLR124W	0.59	2.41	Hypothetical protein

2.2.1.3.1 Rpn4p transcription factor and proteasome-regulated protein degradation

To identify potential transcription factors involved in the early response to inhibitors of the IP pathway we looked for regulatory motifes in the promoters of the responsive genes. We have restricted our search only to documented interactions between a gene and a given transcription factor. The most striking and common feature of the genes responsive to lovastatin or zaragozic acid was the fact that many of them contained in their promoter region a sequence binding the Rpn4p transcription factor (Figure 40). Potentially, Rpn4p could regulate approximately 15% of the genes overexpressed due to lovastatin and 15% of those overexpressed due to zaragozic acid. Interestingly there was no overlap between those two groups of genes, suggesting that Rpn4p could act as a condition-altered transcription factor, i.e. targeting different genes depending on the stimuli. Although Rpn4p is known to acts as an activator of transcription, it is important to mention that ~12% of the genes underexpressed in the presence of zaragozic acid and ~4% repressed by lovastatin also contained an Rpn4-binding sequence in their promoters. This suggests an additional regulation of these genes in the tested conditions, likely by a distinct transcription factor.

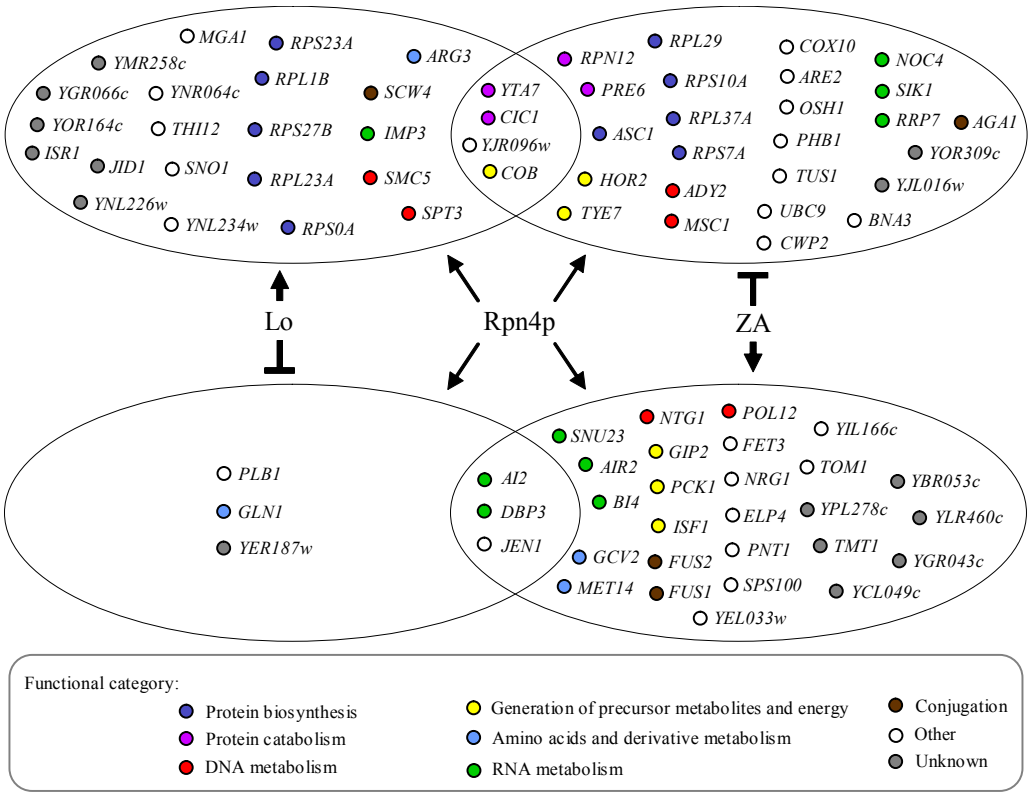


Figure 40: Genes responsive to lovastatin or zaragozic acid potentially regulated by Rpn4 transcription factor.

The Rpn4 protein is known to be required for normal expression of 26S proteasome subunits, however, a global analysis of expression in the *rpn4*Δ mutant has shown much wider effects of its depletion (Fleming *et al*., 2002; Xie and Varshavsky, 2001). The 26S proteasome is a giant protein complex (comprising more than 30 proteins) devoted to ATP-dependent degradation of proteins, which were earlier ubiquitinated. When we compared the distribution of the genes responsive to lovastatin and zaragozic acid we could notice that while the response to lovastatin was characterized by activation of protein catabolism (upregulated genes- *YTA7, AMA1/SPO70, CIC1, GRR1, CNE1, NAS2, PNG1, DOC1*; downregulated genes- *YDR306C, ATE1, RPN6*), zaragozic acid caused rather a suppression of genes representing this category (upregulated genes- *RPN6, JEM1*; downregulated genes- *NAS2, SCJ1, RPN12, CIC1, PEP4, YTA7, GGA2, APC2, BAR1, MNS1, PUP3, PRE6*) (see Figure 37, pg.79 and Figure 39, pg 82). Some of these genes, which are involved in proteasome-dependent protein degradation, are described in Table 12. It can be seen that zaragozic acid caused repression of five proteasome subunits while lovastatin induced expression of two.

These transcriptional effects are important since we know that 26S proteasome-dependent degradation of proteins is one of the ways to regulate the performance of the IP

pathway. Remarkably, expression of an essential gene, *RPN6*, coding for a proteasome subunit was activated by zaragozic acid and repressed by lovastatin. This subunit is responsible for recognition of a subset of substrates of the proteasome.

An independent study has shown that expression of *RPN4* and consequently 26S proteasome-dependent proteolysis are regulated by Pdr1p, a transcription factor that mediates multiple drug response (Owsianik *et al*., 2002). We detected overexpression of *PDR1* in cells treated with zaragozic acid. Additionally, we observed changes in the expression of genes involved in proteasome function but apparently lacking a binding site for Rpn4p, e.g. *RPN6* and *NAS1*. These genes and others that are involved in ubiquitination that precedes protein degradation are presented in Table 12.

Altogether, these results suggest that the activity and substrate specificity of 26S proteasome could be inversely affected by the presence of lovastatin and zaragozic acid.

Table 12: Genes involved in protein ubiquitination and degradation in 26S proteasome, whose expression changed in response to lovastatin (Lo) or zaragozic acid (ZA)

Gene	Ratio of expression*		Description
	Lo	ZA	
RPN6	0.59	2.72	Essential, non-ATPase regulatory subunit of 26S proteasome lid required for assembly and activity of 26S proteasome
CIC1	2.02	0.21	Essential protein that interacts with proteasome components and has potential role in proteasome substrate specificity
NAS2	1.62	0.49	Protein with similarity to p27 subunit of mammalian proteasome modulator; not essential; interacts with Rpn4p
PRE6	-	0.33	20S proteasome subunit
RPN12	-	0.42	Subunit of 19S regulatory particle of 26S proteasome lid; synthetically lethal with *RPT1*, which is an ATPase component of 19S regulatory particle; physically interacts with Nob1p and Rpn3p
PUP3	-	0.45	Beta subunit of 20S proteasome involved in ubiquitin-dependent protein catabolism
PNG1	1.53	-	Conserved peptide N-glycanase required for deglycosylation of misfolded glycoproteins during proteasome-dependent degradation
GID7	-	2.08	Protein of unknown function, involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase
APC2	-	0.58	Subunit of Anaphase-Promoting Complex/Cyclosome (APC/C), which is a ubiquitin-protein ligase required for degradation of anaphase inhibitors
DOC1	1.52	-	Processivity factor required for the ubiquitination activity of APC, mediates activity of APC by contributing to substrate recognition; involved in cyclin proteolysis
GRR1	1.94	-	F-box protein component of SCF ubiquitin-ligase complex, required for Cln1p and Cln2p degradation
HSE1	1.75	-	Subunit of endosomal Vps27p-Hse1p complex required for sorting of ubiquitinated membrane proteins into intralumenal vesicles prior to vacuolar degradation, as well as for recycling of Golgi proteins and formation of lumenal membranes
UBC11	1.59	-	Ubiquitin-conjugating enzyme not required for degradation of mitotic cyclin Clb2
UBP8	0.63	-	Ubiquitin-specific protease that is a component of SAGA (Spt-Ada-Gcn5-Acetyltransferase) acetylation complex; required for SAGA-mediated deubiquitination of histone H2B

BUL1	-	1.61	Ubiquitin-binding component of Rsp5p E3-ubiquitin ligase complex, functional homologue of Bul2p
PIB1	-	1.52	RING-type ubiquitin ligase of endosomal and vacuolar membranes, binds phosphatidylinositol(3)-phosphate
TOM1	-	1.74	E3 ubiquitin ligase of hect-domain class; has a role in mRNA export from nucleus and may regulate transcriptional coactivators
RPL40A	-	0.45	Fusion protein, identical to Rpl40Bp, that is cleaved to yield ubiquitin and a ribosomal protein of large (60S) ribosomal subunit with similarity to rat L40; ubiquitin may facilitate assembly of the ribosomal protein into ribosomes
RPL40B	-	0.47	Fusion protein, identical to Rpl40Ap, that is cleaved to yield ubiquitin and a ribosomal protein of large (60S) ribosomal subunit with similarity to rat L40; ubiquitin may facilitate assembly of the ribosomal protein into ribosomes
CSN9	-	1.76	Subunit of Cop9 signalosome, which is required for removal of ubiquitin-like protein Rub1p from Cdc53p (cullin); involved in adaptation to pheromone signaling

* '-' indicates no significant changes of expression level

2.2.1.3.2 RNA polymerase III transcription

Several genes putatively regulated by FPP availability play a role in transcription (compare Table 10 & Table 11). A detailed list of genes involved in various aspects of tRNA metabolism responsive to lovastatin or zaragozic acid is presented in Table 13. Interestingly, only zaragozic acid affected tRNA methylation (P value 0.0095) which is the most prevalent modification of yeast tRNA. However, the function of this modification remains unclear. On the other hand, lovastatin caused downregulation of *THG1* gene whose depletion results in accumulation of uncharged $tRNA^{His}$ in the nucleus and activation of the GCN4 pathway (Gu *et al*., 2005). This activation is to some extent dependent on *GCN2* whose expression was induced by lovastatin treatment. In these circumstances, it is possible that the GCN4 pathway was activated due to lovastatin action especially that we observed an overexpression of 14 genes (*ACO2, ADE8, ARG3, BAT1, FOL2, GIP1, MUP3, PCL5, PEX21, SNO1, TDH3, THR1, TPK1, YHR020W*) that are known targets of this pathway.

Table 13: Genes involved in tRNA metabolism that were responsive to lovastatin (Lo) or zaragozic acid (ZA)

Gene	Ratio of expression		Description
	Lo	ZA	
			Repressor of Pol III
MAF1	0.22	2.16	Negative regulator of RNA polymerase III; targets initiation factor TFIIIB
			TF III
TFC3	-	2.0	Largest of six subunits of RNA polymerase III transcription initiation factor complex (TFIIIC)
BRF1	-	0.49	TFIIIB subunit, 70 kDa, interacts with Maf1p

		Pol III	
RPC25	1.99	0.57	RNA polymerase III, 25 KD subunit
RPO26	-	1.66	RNA polymerase I, II, III 18 KD subunit
RPC11	-	1.53	RNA polymerase III subunit C11, required for RNA cleavage activity and transcription termination
		tRNA metabolism	
UTP8	2.16	0.28	Nucleolar protein required for export of tRNAs from nucleus
GRS1	-	0.61	Cytoplasmic and mitochondrial glycyl-tRNA synthase
YMR087w	-	0.60	Putative ADP-ribose-1"-monophosphatase that converts ADP-ribose-1"-monophosphate to ADP-ribose; may have a role in tRNA splicing
SEN15	0.66	-	Subunit of tRNA splicing endonuclease, which is composed of Sen2p, Sen15p, Sen34p, and Sen54p
DED81	-	0.42	Cytosolic asparaginyl-tRNA synthetase, required for protein synthesis
RNH70	-	0.5	3' exoribonuclease, required for 5S and tRNA-Arg3 maturation
PUS6	-	0.36	Pseudouridine synthase responsible for modification of cytoplasmic and mitochondrial tRNAs at position 31
ATE1	0.64	-	arginyl-tRNA-protein transferase
THG1	0.53	-	tRNAHis guanylyltransferase, adds guanosine residue to 5' end of tRNAHis after transcription and RNase P cleavage
GCN2	1.74	-	Protein kinase, phosphorylates eIF2 (Sui2p) in response to starvation; activated by uncharged tRNAs and Gcn1p-Gcn20p complex
		tRNA methylation	
TRM12	-	1.91	S-adenosylmethionine-dependent methyltransferase of seven beta-strand family; required for wybutosine formation in phenylalanine-accepting tRNA
TRM7	-	1.62	2'-O-ribose methyltransferase, methylates nucleotides at positions 32 and 34 of tRNA anticodon loop
TRM8	-	1.51	Subunit of tRNA methyltransferase complex composed of Trm8p and Trm82p that catalyzes 7-methylguanosine modification of tRNA
TRM82	-	0.32	Subunit of tRNA methyltransferase complex composed of Trm8p and Trm82p that catalyzes 7-methylguanosine modification of tRNA
GCD10	-	1.64	Subunit of tRNA (1-methyladenosine) methyltransferase, required with Gcd14p for modification of adenine at position 58 in tRNAs

Among genes inversely regulated by IP pathway inhibitors and involved in tRNA metabolism we can find *MAF1*, a gene encoding repressor of polymerase (pol) III and *RPC25*, encoding a subunit of pol III that is required for initiation of transcription (Desai *et al*., 2005; Pluta *et al*., 2001; Zaros and Thuriaux, 2005) as well as *UTP8* required for export of tRNAs from the nucleus. Pol III is responsible for the transcription of tRNAs as well as many other essential small untranslated RNAs.

So far, there is only one established connection between tRNA biosynthesis and the IP pathway since both pathways use DMAPP as a substrate. DMAPP, apart from being required for FPP synthesis, is also used by Mod5p to create in tRNA molecules N^6-(Δ^2-isopentenyl) adenosine (i^6A tRNA) that alters tRNA-mediated nonsense suppression (Benko *et al*., 2000). The amount of Mod5p is co-regulated with the amount of tRNA available for modification and consequently with the general rate of tRNA synthesis (Boguta *et al*., 1997). Importantly, when Maf1p is depleted, the levels of tRNA and Mod5p increase while the level of ergosterol goes down (Kaminska *et al*., 2002). This is consistent with an earlier hypothesis regarding

competition between the Mod5 and Erg20 proteins for the limited pool of DMAPP (Benko *et al.*, 2000).

Figure 41 gives a general comparison between the effects of lovastatin and zaragozic acid on the genes involved in tRNA synthesis. It can be seen that while lovastatin could positively regulate the transcription and nuclear export of mature tRNAs, zaragozic acid had an opposite effect.

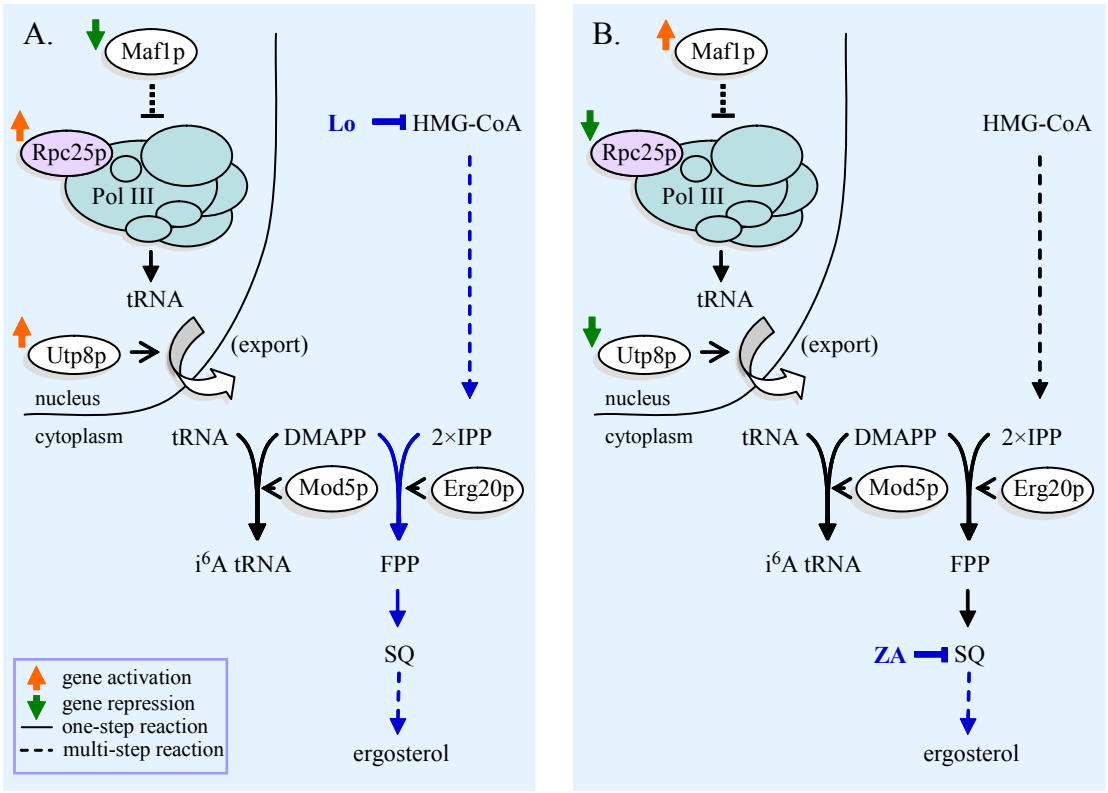


Figure 41: Effects of lovastatin and zaragozic acid on tRNA biosynthesis.

A. Model of lovastatin (Lo) action. **B**. Model of zaragozic acid (ZA) action. Known affected pathways are marked in blue. Dashed lines indicate pathways of which the components are not shown. Other genes involved in tRNA metabolism are listed in Table 13, pg 90.

Generally, the main difference between the two compared conditions, i.e. lovastatin or zaragozic acid stress (Figure 41), would be the availability of FPP that is expected to decrease in the presence of lovastatin and to increase in the presence of zaragozic acid (details in chapter 1.2.2, pg 25). Our transcriptomic data implies a positive feedback regulation of the *MAF1* gene expression by increased availability of FPP or DMAPP. The observed upregulation of *MAF1* might result in the inhibition of tRNA biosynthesis. It is known that tRNA synthesis, as an energy-costly event, is coupled to the cell growth. When FPP accumulates (as in the case of SQS inhibition) toxic farnesol is produced which results in growth inhibition (Machida *et al.*, 1999). Thus, it is reasonable that cells treated with

zaragozic acid would decrease tRNA biosynthesis because of toxic farnesol. Accordingly, tRNA synthesis has been shown to be repressed in the various conditions that are not favoring the growth and this repression is dependent on Maf1p (Upadhya *et al*., 2002).

On the other hand, a decrease of FPP availability in the cell, by the repression of *MAF1* expression, could increase the rate of tRNA synthesis and consequently lead to an increase of protein synthesis, including the proteins of the IP pathway. By this means, the cell would be able to biosynthesize more FPP. Nevertheless, the level of tRNA after treatment of yeast with IP pathway inhibitors remains to be verified.

It has been suggested before that Maf1p could be involved in the control of the IP pathway (Kaminska *et al*., 2002). Our data imply that, reciprocally, compounds of this pathway might regulate *MAF1* expression and consequently tune tRNA synthesis with the cell demand. Potential candidates for playing this role would be signals derived from DMAPP, a non-sterol FPP offshoot or FPP itself. It is important to note that ergosterol is unlikely to have a regulatory function on *MAF1* expression as the action of both tested inhibitors should result in a decreased metabolite flux of the ergosterol branch of the IP pathway.

2.2.1.4 Discussion

To enlarge upon earlier studies of the IP pathway we treated yeast cells with two agents, lovastatin, an inhibitor of HMG-CoA reductase, and zaragozic acid, an inhibitor of squalene synthase. Both these inhibitors are known to have the same reducing effect on the amount of cellular sterols while having an opposite effect on the FPP availability. Analysis of the response to lovastatin showed that the first reaction of the cells at the transcriptional level was to increase the expression of genes engaged in the uptake of sterols from the medium, while transcription of other genes directly involved in the IP pathway was not changed. The first response to zaragozic acid was upregulation of two genes coding for components of the sterol branch of the IP pathway. Also other genes, known to regulate sterol metabolism were affected. Remarkably, we noticed that approximately 15% of the genes whose expression changed due to lovastatin or zaragozic acid treatment contained in their promoter a region binding site for the Rpn4p transcription factor that is involved in expression of the 26S proteasome subunits. Moreover, both inhibitors caused changes in the expression of genes involved in ubiquitination and proteasomal degradation of proteins. Importantly, while lovastatin preferably caused activation of genes involved in protein catabolism, this functional

category was suppressed by zaragozic acid. These data together with the fact that Hmg2p is regulated by proteasomal degradation support the notion that this kind of cellular control, in response to changing availability of FPP, could affect more proteins belonging to the IP pathway.

Additionally, the transcription profiles of the response to lovastatin and to zaragozic acid indicated that availability of FPP could affect tRNA biosynthesis. Probably because of this regulation, lovastatin caused activation of genes involved in ribosome biogenesis and protein biosynthesis while zaragozic acid tended to suppress both groups of genes. Both these transcriptional responses, concerning the 26S proteasome and tRNA biosynthesis, should be investigated further.

Overall, we have identified 59 genes whose expression was presumably regulated by the changing availability of FPP. Among these genes, there was a nonessential gene coding for Yta7p of unknown function. Since it had previously been suggested that this protein could be involved in the IP pathway {K. Grabinska, PhD thesis} we decided to focus our further investigation on the *YTA7* gene.

2.2.2 Involvement of Yta7p in the isoprenoid pathway

YTA7, a gene coding for a protein of unknown function, was previously found to interact with *ERG20*, coding for one of the IP pathway's enzymes, FPP synthase. Cells lacking *YTA7* had an increased level of squalene. This increase could be explained in two ways: by increased squalene synthase activity or a decreased turnover of squalene. The latter explanation is less probable since the levels of other sterol products were not changed in the *yta7*Δ mutant {Grabińska, unpublished}. In this study, we discovered that expression of *YTA7* was regulated in opposite ways by the two inhibitors of the mevalonate pathway investigated in this work, acting oppositely on the intracellular availability of FPP. Altogether, these results suggested an involvement of Yta7p in the IP pathway. To establish the function of Yta7p, in the next step we measured the enzymatic activities of proteins belonging to the IP pathway upon depletion of *YTA7*.

2.2.2.1 Enzymatic activities of FFP synthase, **cis***-prenyltransferase and dolichol level in the* **yta7***Δ mutant*

Since earlier data showed a 2-fold increase in the level of squalene in the *yta7*Δ mutant {K. Grabinska, PhD thesis} we decided to investigate the effect of *YTA7* depletion on other enzymes of the IP pathway beside squalene synthase. *Cis*-prenyltransferase, as the first enzyme committed to dolichol biosynthesis and one of the enzymes consuming FPP in the cell, was chosen for further investigation. It turned out that Yta7p apparently had an inhibitory effect on FPP as we observed a moderate increase of this enzyme's activity in the *yta7*Δ mutant (Figure 42B; see the method in chapter 3.1.7.1, pg 127). Although this increase was not statistically significant, in cells treated with lovastatin the increase of the activity reached 31% of the wild type level and was significant. This result suggests that the inhibitory effect of Yta7p on *cis*-prenyltransferase activity should be more pronounced upon lovastatin treatment, i.e. when the level of FPP is decreased. This view would be in agreement with the higher demand of cells for Yta7p activity when the level of FPP is decreased and could explain *YTA7* overexpression in response to lovastatin. In agreement with the very moderate increase in *cis*-prenyltransferase activity in the *yta7*Δ mutant (by 19% of the wt level),

dolichol level showed a similar increase (by 23% of the wt level) (Figure 42D; see the method in chapter 3.1.7.3, pg 128). Lovastatin decreases the efficacy of the mevalonate pathway and in agreement with this well-established fact we observed a decrease in the total amount of dolichol in cells treated with this inhibitor. A similar decrease was observed in the *yta7*Δ mutant upon lovastatin treatment, suggesting that the process leading to this decrease was not affected by *YTA7* deletion.

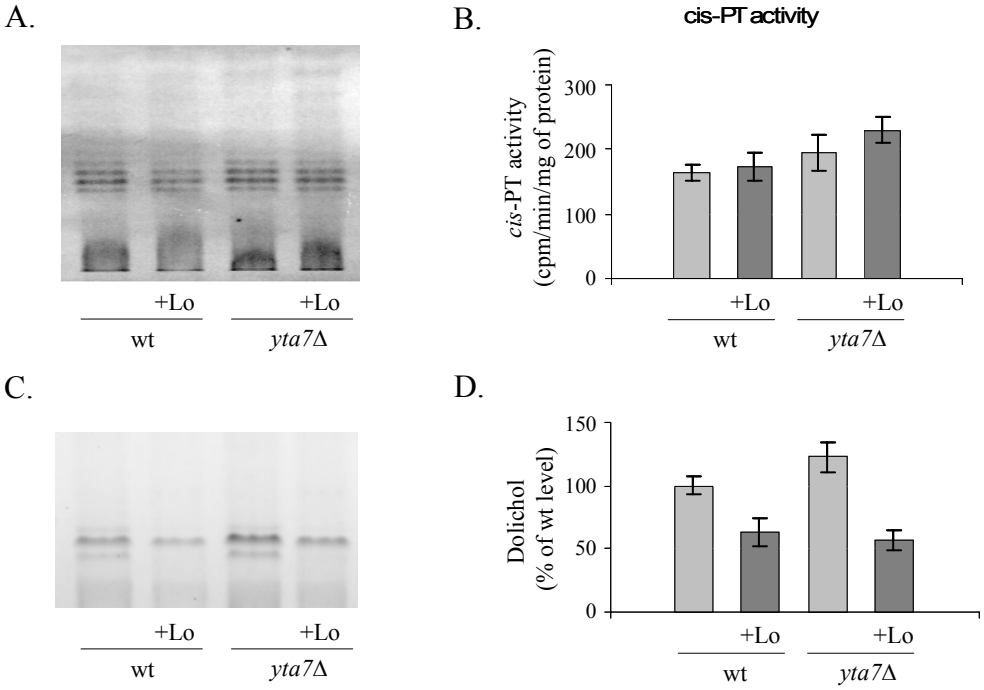


Figure 42: *Cis*-prenyltransferase activity and dolichol level.

A. Autoradiogram of polyprenols synthesized *in vitro*. In order to measure the activity of *cis*-prenyltransferase, {^{14}C} IPP was used as the radiolabeled substrate. The products of the reaction were developed on RP18 HPTLC plates and detected by autoradiography. **B**. Activity of *cis*-prenyltransferase. Regions of RP18 HPTLC plates, from panel A, containing radiolabeled products were scraped and quantified by liquid scintillation counting.
C. Dolichols on RP18 HPTLC plates stained with iodine vapor. To measure dolichol levels, lipid fraction was isolated from cellular membranes, developed on an RP18 HPTLC plate and revealed with vapor of iodine.
D. Intracellular level of dolichol. The intensity of dolichol signal was quantified and compared to that in the wild type strain, taken as 100%. Results shown in panel B and D are averages of three independent experiments ± SD. Where indicated, 25 μg/ml of lovastatin (Lo) was added to the growth medium.

Since we assumed that Yta7p could inhibit the activity of *cis*-prenyltransferase our next move was to check if deletion of *YTA7* could affect the activity of FPP synthase, acting upstream of both *cis*-prenyltransferase and squalene synthase. To verify this, we measured FPP synthase activity in the *yta7*Δ mutant (Figure 43; see the method in chapter 3.1.7.2, pg 127). Our experiment clearly showed a lack of any significant effects of the *YTA7* deletion on the activity of FPP synthase. Moreover, this result showed that the activity of FPP synthase in

wild-type cells was moderately affected by lovastatin treatment while it was not affected in the *yta7*Δ mutant.

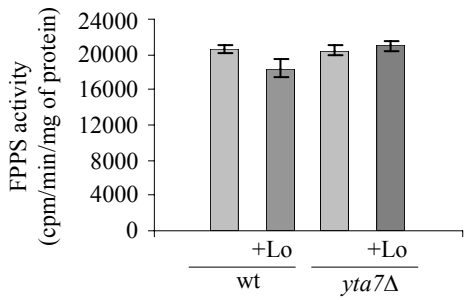


Figure 43: FPPS activity *in vitro*.

The assay was performed with DMAPP as the allylic starter and {^{14}C} IPP as a radiolabeled substrate and yeast cell extract as the source of the enzyme. Where indicated, 25 μg/ml lovastatin (Lo) was added to the growth medium. The results shown are averages of three independent experiments ± SD.

2.2.2.2 HMG2 *but not* HMG1 *is a multicopy supressor of lovastatin sensitivity of the* yta7*Δ mutant*

Since we observed overexpression of the *YTA7* gene in the cells with the lovastatin-impaired IP pathway, we decided to verify the effects of decreased FPP availability in the *yta7*Δ mutant. Thus, in an attempt to decrease the cellular level of FPP we added lovastatin to yeast cultures of the wild type and *yta7*Δ mutant. Additionally, we tested cells overexpressing HMGR encoding genes. It can be seen in Figure 44 that *HMG1* or *HMG2* expressed from a multicopy plasmid alleviated the growth inhibition of the wild type strain caused by lovastatin.

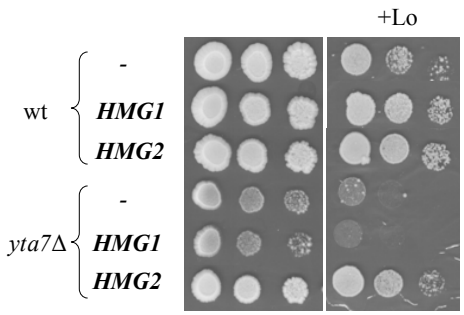


Figure 44 : *HMG2* but not *HMG1* is a multicopy supressor of lovastatin sensitivity of the *yta7*Δ mutant.

Serial dilutions of exponentially growing cultures of each mutant were spotted onto SD agar plates containing 0 or 300 μg/ml lovastatin (Lo). Growth was scored after 5 days of incubation at 28ºC. *HMG1*, strain bearing pNEV*HMG1*; *HMG2*, strain bearing pNEV*HMG2*; - strain bearing empty vector.

However, in the *yta7*Δ mutant only overexpression of *HMG2* was able to exert this effect. This result suggests that Yta7p is either needed for one of the steps of *HMG1* expression or it is required further at the level of FPP-consuming enzymes. The fact that the

YTA7 deletion did not affect FPP synthase activity suggests that Yta7p acts downstream of this enzyme in the IP pathway Since it was proposed that Yta7p was involved in chromatin silencing, we considered the possibility that Yta7p could play a role in *HMG1* silencing. Importantly, this effect could not occur in our experimental design because the multicopy plasmids we used bear only ORF of the gene without the surrounding regions where the Dpb4p complex guarding the boundary of silent chromatin could potentially bind. Nevertheless, we verified whether this regulation might occur in the genome, i.e. we compared coordinates of the *HMG1* and *HMG2* loci to the coordinates of regions binding Dpb4p complex (Tackett *et al*., 2005). Surprisingly, it turned out that *HMG1* could be potentially regulated in this way because it was placed just upstream of the boundary region of heterochromatin (Chromosome XIII, coordinates 118898 bp to 120089 bp) while *HMG2* was not located in the neighborhood of such regions. On the other hand, it cannot be excluded that overexpression of HMG1 was not functional in the *yta7*Δ cells for some other reasons.

The different effects of *HMG1* and *HMG2* overexpression in the *yta7*Δ mutant in terms of lovastatin sensitivity additionally support the notion of distinct regulation of these two genes.

2.2.3 Characterization of molecular properties of Yta7p

2.2.3.1 Yta7 protein localization

As we decided to focus our study on the Yta7 protein function, we first investigated its cellular localization. In parallel, we localized Erg20p, a protein that had earlier been proposed to interact physically with Yta7p {K. Grabinska, PhD thesis}. In order to visualize Yta7p two independent methods were used, subcellular fractionation and immunofluorescence microscopy.

2.2.3.1.1 Subcellular fractionation

Subcellular fractionation is a method based on the separation of intracellular organelles according to their density by centrifugation (for details see chapter 3.1.6.1, pg

125). Obviously, this method allows only an approximation of a protein's localization due to the inevitable minor contaminations between fractions. To trace the Yta7 and Erg20 proteins in individual fractions, they were fused with a ProtA tag. This experiment showed that Yta7p was not freely dispersed in the cytoplasm since it was absent in the 100 000×g supernatant fraction containing soluble cytoplasmic proteins (Figure 45). Yta7p was present in the 500×g, 3000×g and 100 000×g pellet fractions, showing exactly the same pattern of localization as Dpm1p (dolichyl phosphate mannose synthase), which is known to localize to the ER membrane. The 3000×g pellet fraction is supposed to contain nuclei, but as the ER membrane differentiates directly from the nucleus membrane, this fraction is by its nature contaminated with the ER membrane. Erg20p showed an ubiquitous pattern of localization. The protein was most abundant in the 100 000×g pellet containing the ER compartment and 100 000×g supernatant containing the cytosol, while its amount was reduced in the nuclei.

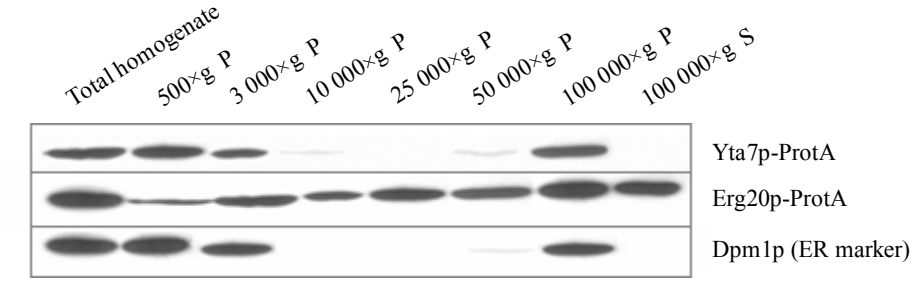


Figure 45: Localization of Yta7 and Erg20 proteins – *subcellular fractionation*.

Yeast spheroplasts from cells expressing ProtA-Erg20p or ProtA-Yta7p from a single-copy vector were homogenized and subjected to a series of centrifugations: 500×g, 3000×g, 10 000×g, 25 000×g, 50 000xg and 100 000×g. Aliquots were taken from pellets (P) and supernatant (S) (100000×g) fractions and analyzed by immunoblotting. Dpm1p (dolichyl phosphate mannose synthase) detected with anti-Dpm1p antibody was used as an ER membrane marker.

2.2.3.1.2 Immunofluorescence microscopy

As previously, to trace the Yta7 and Erg20 proteins they were fused with ProtA tag, which was visualized with an antibody conjugated to Cy3-fluorophore. It can be seen in Figure 46A that Yta7p exhibited high concentration in and around the nucleus and a punctate pattern in the cytoplasm (see the method in chapter 3.1.6.2, pg 126). Erg20p was barely detected in the nucleus but it was present in the cell zone tightly surrounding this structure. Additionally, Erg20p not only showed a punctate pattern but was also dispersed in the cytoplasm (Figure 46A). We also performed control staining of ER membranes in the tested

strain using Dpm1p as an ER-marker protein (Figure 46B). ER was localized to the thin layer surrounding the nucleus.

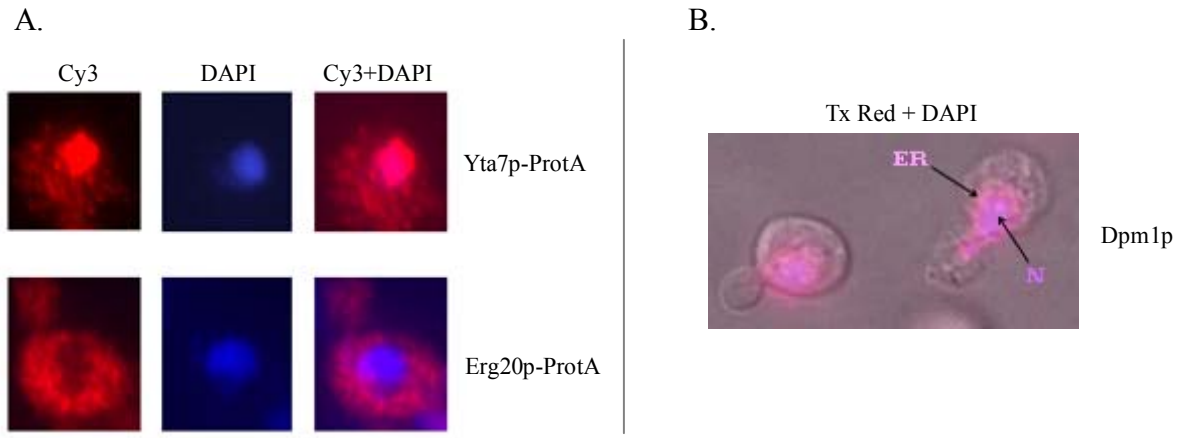


Figure 46: Localization of Yta7 and Erg20 proteins – *immunofluorescence microscopy*.

A. Yeast cells expressing ProtA-Erg20p or ProtA-Yta7p from single-copy vectors were fixed with formaldehyde cross-linking. Fusion proteins were detected with an antibody conjugated with Cy3-fluorophore. After DAPI staining of DNA, cells were subjected to immunofluorescence microscopy. **B**. A control experiment done in order to visualize ER in BY4742 cells. As an ER marker protein, Dpm1p was used. Nikon Eclipse E800 microscope was used for the visualization of yeast (magnification × 100, oil immersion).

2.2.3.1.3 Binding of Yta7p to cellular membranes

After having localized Yta7p to the ER membrane we decided to verify whether Yta7p was an integral or a peripheral protein. According to SGD PepPlot analysis of hydrophobic properties of the protein sequence, two transmembrane regions were predicted (533-550aa, 838-857aa). However, careful analysis showed that these hydrophobic regions lie within predicted ATP-binding domains (Figure 47B) which possess some hydrophobic regions likely buried in the protein's core (Beyer, 1997). To investigate this problem, we isolated membrane fraction from logarithmically growing yeast cells expressing Yta7p fused with a TAP-tag, and treated it with various polar reagents (NaCl, Na_2CO_3, and urea) and a detergent (TritonX-100) (method in chapter 3.1.8.6, pg 131). Then the samples were centrifuged and the efficacy of Yta7p solubilzation was determined by assessing the translocation of the protein from the membrane fraction (pellet) to the aqueous solution (supernatant). As a control, the same experiment was performed tracing Dpm1p, an integral protein of the ER membrane (not shown). As shown in Figure 47A, all the reagents used were able to break the association of

Yta7p with the membrane which indicates that it is a peripheral protein linked to the membrane indirectly, probably by an interaction with another protein.

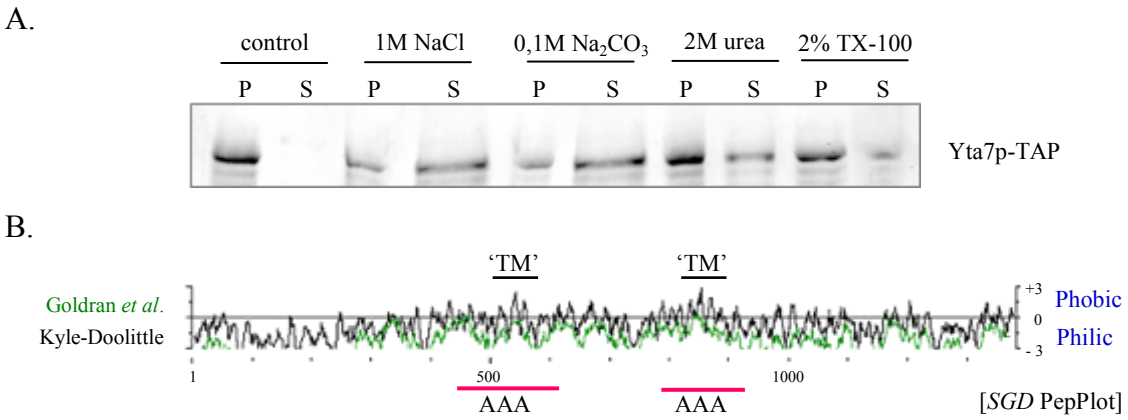


Figure 47: Yta7p is a peripheral membrane protein.

A. Western blot analysis of the membrane fraction isolated from a yeast strain carrying fusion Yta7-TAP protein-encoding gene integrated in the genome. The membrane fraction was treated with the indicated compounds and after centrifugation, the presence of the protein was checked in the pellet (P) and in the supernatant (S) fraction. **B**. Hydropathy plot of Yta7 protein, putative transmembrane (TM) regions and ATP-binding (AA) domains are indicated.

2.2.4 Involvement of Yta7p in ion homeostasis

2.2.4.1 Global gene expression changes in the yta7Δ *mutant*

To gain a broader perspective of the cellular role of Yta7p we analyzed a genome-wide transcription pattern in a mutant strain lacking the *YTA7* gene. Deletion of *YTA7* caused only moderate changes in the gene expression and after statistical analysis (see the method in chapter 3.1.4, pg 117), we accepted 20 transcripts as significantly changed when compared to the levels of expression in the wild type. These genes, their expression ratio and brief descriptions are listed in Table 14. This experiment clearly showed that deletion of *YTA7* did not cause changes in the genes directly involved in the isoprenoid biosynthesis, suggesting that the previously observed effects on the enzymes of the IP pathway do not occur at the transcriptional level. The most induced gene turned out to be *TRK1*, coding for a high affinity potassium pump. Three genes were involved in cell wall biosynthesis and organization, namely *GSC2*, coding for a subunit of glucan synthase, *KNR4*, whose gene product interacts with Mpk1 kinase and *CRZ1*, encoding a transcription factor positively regulating *GSC2*

expression. Among the downregulated genes we observed *SDL1* coding for serine dehydratase, which controls the major route of serine catabolism. Serine, besides its occurrence in proteins, is a component of glycerophospholipids and is used for sphingosine and ceramide biosynthesis. *BIO3*, encoding 7,8-diamino-pelargonic acid (DAPA) aminotransferase, was also downregulated. The product of this gene takes part in biotin synthesis, whose essential function is to transfer carboxyl groups. Biotin serves as a cofactor of enzymes such as acetyl-CoA carboxylase, methylmalonyl-CoA carboxyltransferase and methylmalonyl-CoA decarboxylase. Almost half of the genes whose expression was changed are uncharacterized, which makes interpretation of this data very difficult.

Table 14: Genes whose expression was changed in the *yta7*Δ mutant

Rang	ORF	Gene *	Ratio	Description	P-value
1	YJL129C	*TRK1*	2.43	Potassium transporter I	0.01549
2	YGL093W	*SPC105*	1.75	Protein required for accurate chromosome segregation	0.01350
3	YJL218W	*YJL218W*	1.74	Protein similar to *E.coli* galactoside O-acetyltransferase	0.04978
4	YNL027W	***CRZ1***	1.68	Calcineurin responsive zinc-finger transcription facor	0.03819
5	YNL225C	*CNM67*	1.65	Component of the spindle pole body outer plaque	0.03766
6	YPL207W	*YPL207W*	1.63	Protein of unknown function	0.03944
7	YGR032W	***GSC2***	1.62	1,3-beta-D-glucan synthase subunit	0.01075
8	YGR120C	*COG2*	1.57	Component of conserved oligomeric golgi complex	0.02594
9	YNL266W	*YNL266W*	1.54	Hypothetical protein	0.00618
10	YGR229C	***KNR4***	1.51	Protein involved in cell wall biosynthesis	0.01934
11	YPL125W	*KAP120*	0.67	Karyopherin with a role in export of 60S ribosomal subunits	0.01279
12	YDL094C	*YDL094C*	0.66	Hypothetical protein	0.03290
13	YIL100W	*YIL100W*	0.65	Hypothetical protein	0.02970
14	YHL010C	*YHL010C*	0.65	Homologue of human breast cancer-associated protein BRAP2	0.03875
15	YIL037C	*PRM2*	0.65	Pheromone-regulated protein	0.02940
16	YHR11W	*UBA4*	0.62	Protein that activates Urm1p before its conjugation to proteins	0.01431
17	YJR128W	*YJR128W*	0.62	Hypothetical protein	0.03112
18	YIL168W	*SDL1*	0.6	L-serine dehydratase	0.04716
19	YNR058W	*BIO3*	0.47	DAPA aminotransferase involved in biotin synthesis	0.04827
20	YIL015W	*BAR1*	0.44	Barrierpepsin precursor, cleaves and inactivates alpha factor	0.02993

* The genes involved in the cell wall biosynthesis are shown in bold.

Next, we used the obtained transcriptional data to validate the assumption on the role of Yta7p in chromatin silencing (described earlier in chapter 1.2.3, pg 27). Only two genes *SMI1/KNR4* and *SLD1* out of the 20 whose expression was changed in the *yta7*Δ mutant were located near the boundaries of chromatin regions enriched in the Dpb4p complex and

therefore could potentially be regulated by this complex. The positions of those genes in the chromosome next to the regions of Dpb4p-complex binding are presented in Figure 48. However, no simple conclusion can be drawn from this observation as while the amount of the *KNR4* transcript increased in the *yta7*Δ mutant, the amount of the *SDL1* transcript decreased. Moreover, when one considers that 368 regions enriched in the Dpb4p complex have been identified in the whole yeast genome (Tackett *et al*., 2005), the probability of founding two genes in a group of 20, according to the z-score calculation, does not exeed that of a random event. To sum up, our data are inconclusive as to the involvement of Yta7p in chromatin silencing and in fact suggest no such involvement.

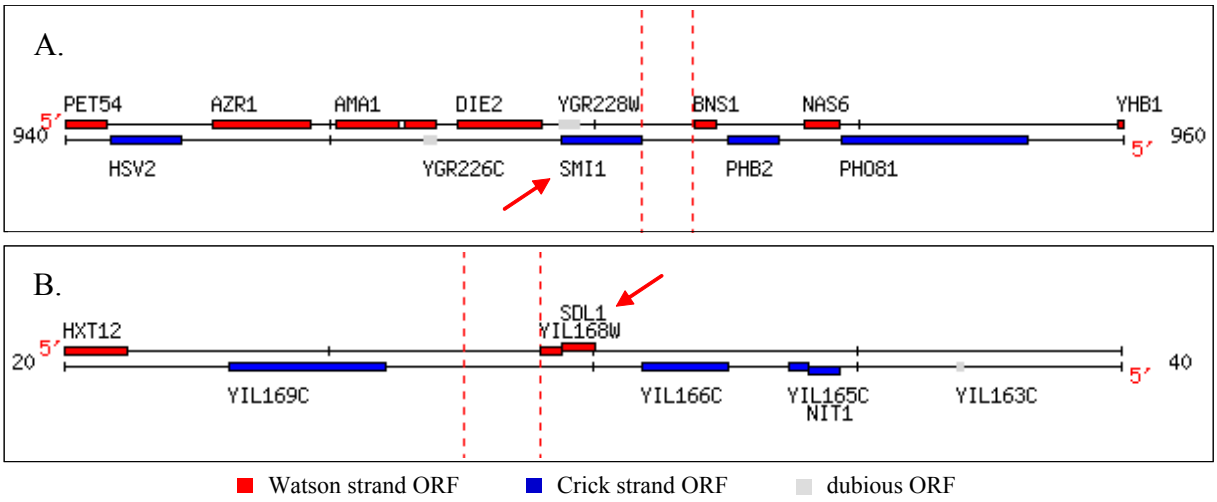


Figure 48: Chromosomal position of *KNR4* and *SDL1* near the boundary regions of heterochromatin.

A. Chromosome VII features spanning coordinates 940000 - 960000 bp. The region enriched in Dbp4 complex is located between two vertical red dashed lines (coordinates 950890 bp to 951885 bp). *KNR4/SMI1* locus is indicated by an arrow. **B**. Chromosome IX features spanning coordinates 21000 - 37000 bp. The region enriched in Dbp4 complex is located between two vertical red dashed lines (coordinates 27569 bp to 29032 bp). The *SDL1* locus is indicated by an arrow. Represented chromosomal maps were obtained at SGD Database (Cherry *et al*., 1997).

2.2.4.2 YTA7-TRK1 *genetic interaction*

2.2.4.2.1 The yta7Δ *mutant has a decreased level of intracellular potassium*

The gene with the most strongly affected expression in the *yta7*Δ mutant was *TRK1* coding for a potassium pump (Gaber *et al*., 1988). In yeast there are two proteins responsible for specific potassium uptake, i.e. Trk1p, a high affinity pump, and Trk2p with a much lower

affinity for potassium. Nevertheless, deletion of both genes is not lethal because yeast have other non-specific systems of cation uptake (Bihler *et al*., 2002; Ko and Gaber, 1991).

To investigate the putative genetic interaction between the *YTA7* and *TRK1* genes we constructed a double mutant *trk1*Δ*yta7*Δ (see the method in chapter 3.1.2, pg. 114) and we measured the content of intracellular K^+. Analysis of the mutant strains showed that *yta7*Δ and *trk1*Δ*yta7*Δ exhibited a decreased potassium level, similar to that of the *trk1*Δ mutant (Figure 49B; see the method in chapter 3.1.8.7, pg 131). According to our measurements, deletion of the *TRK1* gene alone caused a ~10% decrease in the cellular potassium content, in the cells grown on YPD or SC supplemented with potassium, while mutant cells cultivated in SC without additions contained 22% of potassium less than the wild type (only results obtained on SC+0.1 M KCl are shown). Recently, it has been shown that, depending on the strain background, loss of both *TRK1* and *TRK2* genes caused a 10 to 27% decrease in the cellular potassium level (Merchan *et al*., 2004; Ruiz *et al*., 2004). The uptake of potassium in this situation was attributed to unspecific transport systems, which remain to be identified.

When the *yta7*Δ mutant was grown on the synthetic complete (SC) medium its growth was severely impaired comparing to that on YPD (Figure 49A), an effect similar to that observed by others for a *trk1*Δ strain (Bertl *et al*., 2003). It is known that the reduced growth of the *trk1*Δ mutant on the SC medium is caused by potassium deficit and can be rescued by addition of potassium to the medium. Therefore we tested the growth of the mutant strains on the SC medium with or without addition of 0.1 M KCl as well as on the rich YPD medium (Figure 49A). It could be seen that the *trk1*Δ, *yta7*Δ and *trk1*Δ*yta7*Δ mutants exhibited a growth defect on the SC medium which could be overcome by addition of potassium only in *trk1*Δ and *trk1*Δ*yta7*Δ but not in the case of the *yta7*Δ mutant.

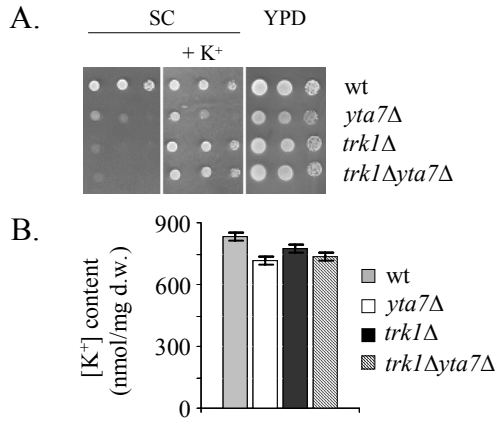


Figure 49: Growth characteristics and potassium content in the *trk1*Δ, *yta7*Δ and *trk1*Δ*yta7*Δ mutants.

A. Serial dilutions of mutants were spotted onto agar plates containing SC, SC supplemented with 0.1 M KCl or YPD. Growth was scored after 2 days at 28°C. **B**. Measurement of cellular potassium level using flame atomic absorbtion spectrometry (FAAS). Cells were grown in SC supplemented with 0.1 M KCl. The results shown are averages of two measurements ± SD.

Interestingly, we observed a negative effect of the KCl supplementation in the SC medium on the *cis*-prenyltransferase activity. Our measurements showed that in wild type cells, during growth on SC medium supplemented with 0.1 M KCl, the activity of *cis*-prenyltransferase decreased to ~60% of that seen in the cells grown on SC without additions (Figure 50; see the method in chapter 3.1.7.1, pg 127). This result, although very difficult to explain without further investigation, reflects the same tendency as was observed in the *yta7*Δ mutant: upon deletion of *YTA7*, i.e. when the level of intracellular K^+ was lower comparing to the level in wild-type cells, the activity of *cis*-prenyltransferase was increased. Addition of potassium to the SC medium did not enhance the growth of the *yta7*Δ mutant. Interestingly, additional deletion of *TRK1* in this strain led to an improvement of the growth in response to KCl supplementation. Moreover, in these conditions, all three mutants contained comparable levels of cellular K^+. These results suggested that the *yta7*Δ mutant growth defect was not linked to the decreased level of potassium.

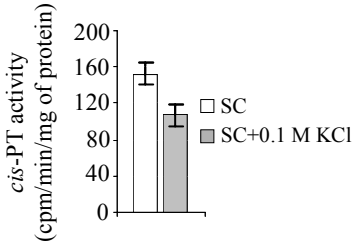


Figure 50: Effect of the KCl supplementation on *cis*-prenyltransferase activity.

Wild-type cells were grown in SC medium supplemented with 0.1 M KCl or without additions. The isolated membrane fraction served as a source of the enzyme. The activity of *cis*-prenyltransferase was measured using {^{14}C} IPP as the radiolabeled substrate. Products of the reaction were developed on RP18 HPTLC plates and quantified by liquid scintillation counting. The result shown is an average of three measurements ± SD.

The non-specific uptake of potassium into cells is impaired in the presence of high extracellular concentrations of competing monovalent cations. Thus, when grown on media containing high concentrations of monovalent cations, yeast cells are expected to exhibit a greater dependence on specific potassium uptake mechanisms. Recently, it has been shown that specific potassium uptake in wild-type cells grown in the presence of 100 mM LiCl depends exclusively on Trk1p activity (Bertl *et al*., 2003). Given that, we studied the sensitivity of mutants to Li^+ (Figure 51) and Na^+ (results not shown). It turned out that *yta7*Δ was more sensitive to Li^+ than the wild type but less sensitive than *trk1*Δ and the *trk1*Δ*yta7*Δ double mutant. Tests with Na^+ did not indicate an increased sensitivity of the *yta7*Δ mutant.

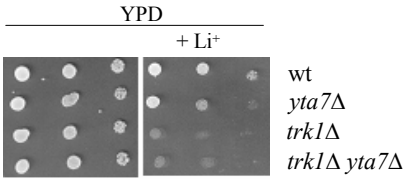


Figure 51: Li^+ sensitivity assay.

Serial dilutions of yeast cultures were spotted onto YPD agar plates without additions or with 0.15 M LiCl added. Growth was scored after 2 days at 28°C.

As a decreased level of K^+ renders cells more sensitive to positively charged antibiotics we tested the sensitivity of our mutants to Hygromycin B (Figure 52A). Consistently with an earlier study (Madrid *et al*., 1998) the *trk1*Δ mutant was hypersensitive to this antibiotic. Also the double mutant *trk1*Δ*yta7*Δ turned out to be hypersensitive to Hygromycin B but *yta7*Δ was as sensitive as the wild type. To be sure that the observed increased sensitivity was not due to the possible increase of cell wall permeability we tested also the sensitivity to other antibiotics like chloramphenicol (Figure 52A) and cycloheximide (result not shown). None of the tested strains was more sensitive to these antibiotics. On the other hand, the hypersensitivity to Hygromycin B can also indicate defects in protein glycosylation (Dean, 1995). For this reason, we tested the efficiency of protein glycosylation in our mutants. To this end, we examined glycosylation of carboxypeptidase Y (CPY), which is a good marker protein of this process (see the method in chapter 3.1.8.8, pg 132). When glycosylation is impaired, several forms of partially glycosylated CPY can be observed in Western analysis due to distinct gel mobilities of proteins carrying different numbers of sugar residues. It can be seen in Figure 52B, that all the mutants tested contained only the mature fully glycosylated form of CPY, indicating that the process of glycosylation was not disturbed. These results suggest that the hypersensitivity to Hygromycin B of the *trk1*Δ and *trk1*Δ*yta7*Δ strains was caused by hyperpolarization of the plasma membrane.

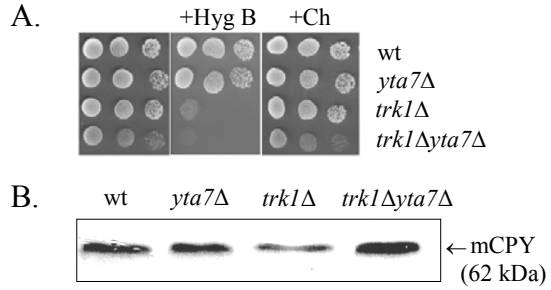


Figure 52: Hygromycin B sensitivity and CPY glycosylation.

A. Serial dilutions of yeast cultures were spotted onto YPD agar plates containing 50 µg/ml of Hygromycin B (Hyg B) or 500 µg/ml of Chloramphenicol (Ch). Growth was scored after 2 days at 28°C. **B**. Western analysis of CPY glycosylation. Only a single band (62 kDa) was detected corresponding to the mature form of CPY (mCPY).

2.2.4.2.2 *Deletion of* YTA7 *alters the cell wall structure and rescues CR- and CFW-sensitive phenotype of* trk1Δ *mutant*

The genome-wide analysis of gene expression levels in the *yta7*Δ mutant indicated that Yta7p could influence the cell wall biosynthesis (Table 14, pg 102). Observing overexpression of the *GSC2* gene, coding for glucan synthase, and its transcriptional activator *CRZ1*, we decided to verify if deletion of *YTA7* could cause changes in the cell wall features. To this end, we performed a test of Zymolyase sensitivity (see method in chapter 3.1.5.1, pg 123). We found out that the *yta7*Δ and *trk1*Δ*yta7*Δ mutants were more resistant to the digestion of the cell wall with Zymolyase than the wild type strain suggesting an increased level of glucan in the cell wall of these mutants (Figure 53A). Then, we tested the sensitivity of the mutant strains to two cell-damaging agents, Congo red and Calcofluor white (Figure 53B). Interestingly, the *trk1*Δ mutant was hypersensitive to both tested drugs and this effect was abolished after deletion of the *YTA7* gene. Altogether, these results implied that a lack of *YTA7* led to changes in the cell wall composition.

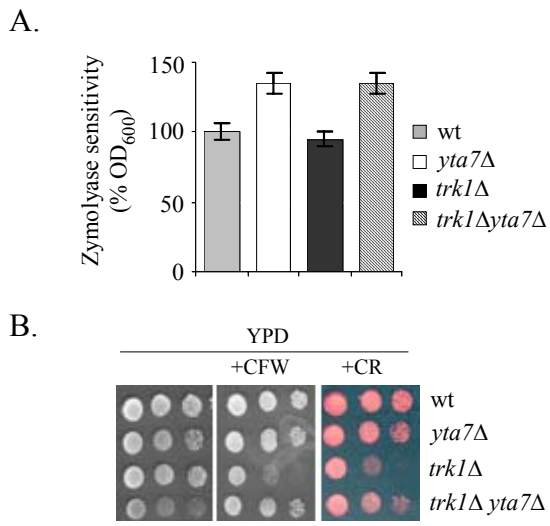


Figure 53: Deletion of *YTA7* changes the properties of the cell wall.

A. Deletion of *YTA7* caused a decrease in the sensitivity to Zymolyase. The fraction of wild-type cells that resisted lysis after 90 min of digestion with Zymolyase 20-T (50 µg/ml) was taken as 100%. Values given are averages ± SD of three independent experiments. **B**. Deletion of *YTA7* rescues the sensitivity of *trk1*Δ mutant to Calcofluor white (CFW) and Congo red (CR). Serial dilutions of yeast cultures were spotted onto YPD agar plates containing 50 µg/ml Calcofluor white or 50 µg/ml Congo red. Growth was scored after 2 days at 28°C.

To further support these results, we isolated the cell wall fractions from the mutant strains and using high-performance anionic exchange chromatography measured the content of the three main carbohydrate compounds (see the method in chapter 3.1.5.2, pg 124). As can be seen in Figure 54, only the levels of glucans were significantly increased in the *yta7*Δ mutant (by ~11%), while in the case of mannans and chitin a similar, but statistically non-

significant tendency was found. Additionally, we observed that deletion of *YTA7* in the *trk1*Δ mutant also caused an elevation of the amount of glucan in the cell wall, which could explain the decreased sensitivity of the double mutant to the cell wall damaging agents compared to the *trk1*Δ strain.

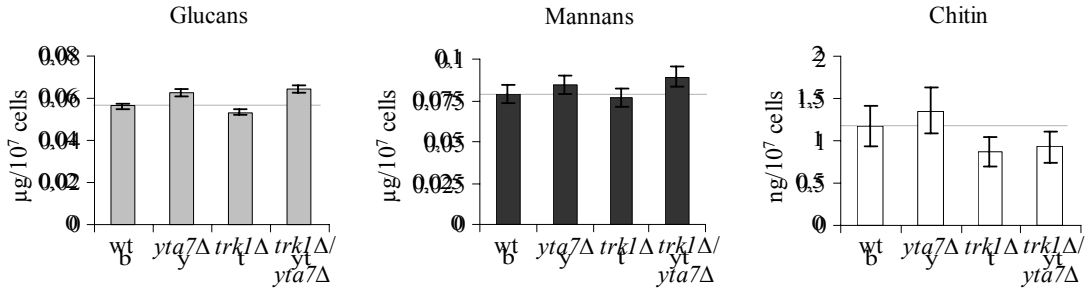


Figure 54: Content of the cell wall components.

Glucose, mannose and glucosamine were released from the cell wall polysaccharides glucans, mannans and chitin, respectively, by sulfuric acid hydrolysis of purified cell wall fraction and quantified by high-performance anionic exchange chromatography (HPAEC). The results are averages of two independent experiments ± SD.

Since induction of *GSC2* expression can be also caused by Rlm1 the transcription factor, which is activated by the CWI pathway (Jung *et al*., 2002; Zhao *et al*., 1998), we investigated if this pathway was activated in the *yta7*Δ mutant (see description of the CWI pathway in chapter 1.1.2.2.1, pg 9). To examine this possibility we analyzed the amount of phosphorylated Mpk1 kinase (Figure 55; method in chapter 3.1.8.1, pg 128). It turned out that *yta7*Δ had a normal level of phosphorylated Mpk1p, thus we could exclude an involvement of the CWI pathway in the observed effects of the *YTA7* deletion on the cell wall.

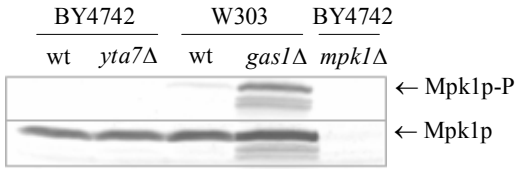


Figure 55: Mpk1p phosphorylation in *yta7*Δ mutant.

Yeast were cultivated in YPD medium to reach OD_{600} 0.5 and were assayed for phosphorylation level of Mpk1p using anti-phospho-p44/42 antibodies (Mpk1-P) and anti-Mpk1p antibodies (Mpk1p). The *gas1*Δ mutant was used as a positive control of Mpk1p phosphorylation and *mpk1*Δ as a negative control. The experiment was performed twice with consistent results.

2.2.4.3 Discussion

To summarize our results up to this point we can say that deletion of *YTA7* affected activity of at least two enzymes of the IP pathway, i.e. *cis*-prenyltransferase and squalene synthase, resulting in an increased level of dolichol and squalene in the *yta7*Δ mutant. Expression of the *YTA7* gene changed upon lovastatin or zaragozic acid treatment suggesting its dependency on FPP level. Yet, we showed that the effects on the IP pathway elicited by *YTA7* do not occur at the transcriptional level. Moreover, we discovered that for some unknown reasons overexpression of the *HMG1* gene was not functional in the cells lacking *YTA7* while overexpression of *HMG2* was. All these data strongly support the importance of Yta7p for the IP pathway. We also showed, with two independent methods, that Yta7p was localized mainly to the nucleus, the endoplasmic reticulum (ER) membranes and it could be seen in the cytosol in a punctate pattern. Localization to the ER compartment was in agreement with the previously discovered ER retention signals in Yta7p sequence {M. Grynberg, unpublished}. These properties of Yta7p strongly indicated a role in the cell additional to the one putatively played in chromatin silencing, which was proposed by Tackett and coworkers (Tackett *et al*., 2005). If Yta7p exerted its function only through binding to chromatin, how could we explain its other cellular localizations? Among the genes whose expression changed upon *YTA7* depletion we found only two located to the immediate proximity of the silent regions and only one of them was downregulated. The fact that we did not detect downregulation of *HMG1* in the *yta7*Δ mutant argues against the idea that deletion of *YTA7* could cause silencing of this gene. Although our data does not support an involvement of Yta7p in the silencing we also cannot exclude it as one of the functions of this protein that could be performed in the nucleus.

It is relevant that Yta7p was localized also to the ER compartment where many reactions of the IP pathway take place. Since Erg20p is also localized to the ER membranes, it is likely that the previously proposed physical interaction between Yta7p and Erg20p, shown by a two-hybrid screen, could occur in this compartment. Additionally, we showed that Yta7p was not a transmembrane protein but it was likely associated with an unknown membrane protein.

A genome-wide transcriptome analysis of the *yta7*Δ strain pointed to the *TRK1* gene, coding for a potassium pump, as the most strongly induced gene. Our further experiments supported this genetic interaction as we showed that the *yta7*Δ mutant had a decreased cellular level of K^+. Additionally, the *yta7*Δ mutant was sensitive to Li^+ while it was not sensitive to

Ca^{2+}. Since in the presence of high concentrations of Li^{+} uptake of K^{+} is dependent exclusively on Trk1p (Bertl *et al*., 2003), these results implied that the Trk1p-dependent specific uptake of potassium could be impaired in the *yta7*Δ mutant. This impairment of potassium uptake could be reflected by observed overexpression of the *TRK1* gene in this mutant. Although cells depleted for *YTA7* contained less K^{+}, apparently they were not hyperpolarized as shown by their normal Hygromycin B sensitivity.

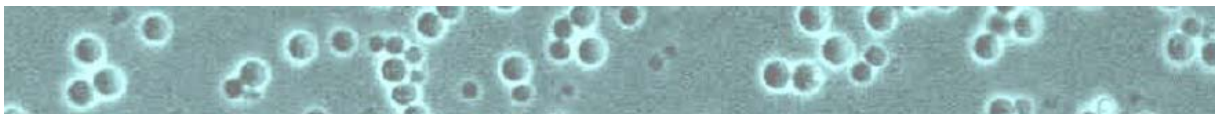
Although it is not known what could be the precise function of *YTA7* in the IP pathway and ion homeostasis, for the first time we have reported here a dependency between the activity of *cis*-prenyltransferase and cellular K^{+} content.

Interestingly, we found out that the deletion of *TRK1* gene rendered cells Congo red and Calcofluor white sensitive. It is not clear why the deletion of *TRK1* could cause the sensitivity to the cell wall damaging agents. Nevertheless, since K^{+} is the cation responsible for the turgor of the cell (Serrano, 1996), it is likely that changes in the tension of the plasma membrane can influence the structure of the cell wall.

Deletion of *YTA7* also influenced expression of a few genes involved in the cell wall remodeling (*CRZ1*, *GSC2*, *KNR4*) and consistently with this induction we have detected changes in the cell wall structure in the *yta7*Δ cells. Deletion of *YTA7* in the *trk1*Δ strain caused the same effects on the cell wall structure, rescuing the Congo red and Calcofluor white sensitivity of this strain. We verified that the observed cell wall remodeling in the *yta7*Δ cells was independent of the typical CWI pathway. Nevertheless, it is worth noting that a physical interaction of Mpk1p with the Knr4 protein is necessary for correct signaling to the Rlm1p transcription factor (Martin-Yken *et al*., 2003) and *KNR4* was upregulated in cells lacking *YTA7*.

Altogether, these data show that Yta7p is involved in both the IP pathway and potassium homeostasis. So far, it is not known if this due to two separate activities played by this protein or to co-regulation of both pathways in the cell. However, our preliminary data suggest that the cellular potassium level can influence the activity of enzymes of the IP pathway. It needs further investigation whether the observed changes in the cell wall structure were the effects of an impairment of the IP pathway and potassium homeostasis or were caused by a direct effect of Yta7p depletion.

EXPERIMENTAL PROCEDURES



3 EXPERIMENTAL PROCEDURES

Abreviations that are commonly used in this chapter:

BSA - Bovine serum albumin
EDTA – ethylenediaminetetraacetic acid
HEPES – N-2-hydroxyethylpiperazine-N'-ethanosulfonic acid
PMSF - phenylmethylsulfonyl fluoride
RT – room temperature (~20°C)
SDS – sodium dodecyl sulfate
SSC – solution containing 0.15 M NaCl and 15 mM sodium citrate, pH 7.0.
TBS – Tris buffered saline (50 mM Tris and 0.9% NaCl, pH 8.0)
Tris - Tris(Hydroxymethyl)aminomethane

3.1.1 Bacterial and yeast strains, plasmids and media used in this study

Bacterial strain

E.coli strain DH5αF' was used for cloning and plasmid propagation.
DH5αF' genotype; *F' supE44 ΔlacU169 {ϕ80 lacZΔM15} hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (Hanahan, 1983)

Yeast strains

For the majority of the experiments in this study, we have chosen a haploid, BY4742, which is widely used laboratory strain. This strain originated from S288C strain that was sequenced, which facilitates genetic manipulations. Exceptionally, in some experiments other yeast backgrounds were used. All strains used in this study are listed in Table 15. Since some studies reported that in the individual cases using different yeast strains might lead to discrepancies in the results, the special care was taken while interpretating the obtained data.

Table 15: *S. cerevisiae* strains used in this study

Strain name/ Euroscarf acc. no.	Relevant genotype	Reference or source
BY4741/Y00000	*MATa* [a]	(Brachmann *et al*., 1998)
BY4742/Y10000	*MATα* [b]	(Brachmann *et al*., 1998)
JK9-3da	*MATa* [c]	(Helliwell *et al*., 1998a)
MML378	*MATa tor1::kanMX4* [c]	(Torres *et al*., 2002)
MML380	*MATa tor2-1 tor1::kanMX4* [c]	(Torres *et al*., 2002)
JH11-1c	*MATa tor 1-1* [c]	(Helliwell *et al*., 1998a)
CML128	*MATa* [d]	(Torres *et al*., 2002)
MML447	*MATa tor1::kanMX4 mpk1::URA* [d]	(Torres *et al*., 2002)
MML382	*MATα wsc1::CaURA3* [d]	(Torres *et al*., 2002)
MML387	*MATa mid2::kanMX4* [d]	(Torres *et al*., 2002)
MML393	*MATa wsc1::CaURA3 mid2::natMX4* [d]	(Torres *et al*., 2002)
MML391	*MATα rom2::kanMX4* [d]	(Torres *et al*., 2002)
MML200	*MATα bck1::kanMX4* [d]	(Torres *et al*., 2002)
Y00993	*MATa mpk1::kanMX4* [a]	(Brachmann *et al*., 1998)
Y02739	*MATa rlm1::kanMX4* [a]	(Brachmann *et al*., 1998)
Y03214	*MATa ybr077c::kanMX4* [a]	(Brachmann *et al*., 1998)
Y01983	*MATa ure2::kanMX4* [a]	(Brachmann *et al*., 1998)
Y00173	*MATa gln3::kanMX4* [a]	(Brachmann *et al*., 1998)
Y01759	*MATa rtg1::kanMX4* [a]	(Brachmann *et al*., 1998)
Y03921	*MATa crf1::kanMX4* [a]	(Brachmann *et al*., 1998)
Y07006	*MATa yak1::kanMX4* [a]	(Brachmann *et al*., 1998)
Y00191	*MATa fcy2::kanMX* [a]	(Brachmann *et al*., 1998)
Y06109	*MATa swi4::kanMX4*[a]	(Brachmann *et al*., 1998)
W303	*MATa* [e]	(Martinez-Pastor *et al*., 1996)
W303msn	*MATa msn2-3::HIS3 msn4-1::TRP1* [e]	(Martinez-Pastor *et al*., 1996)
W303gas1	*MATa gas1::LEU2* [e]	(Popolo *et al*., 1997)
Y21258	*Mat a/α erg20::kanMX4/ERG20* [f]	(Brachmann *et al*., 1998)
Y11296	*MATα trk1::kanMX4* [b]	(Brachmann *et al*., 1998)
Y15922	*MATα yta7::kanMX4* [b]	(Brachmann *et al*., 1998)
KY1	*MATα yta7::HIS3* [b]	This study
KTY1	*MATα trk1::kanMX4 yta7::HIS3* [b]	This study
K314	*MAT a TAPtag-N-YTA7* [g]	This study
YDL401	*MAT a* [g]	(Lafontaine and Tollervey, 1996)

a) background: *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*

b) background: *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*

c) background: *leu2-3 112 ura3-52 trp1 his4 GAL+ HMLa*

d) background: *leu2-3 112 ura3-52 trp1 his4 can1r*

e) background: *ade2-1 ura3-1 leu2,3-112 trp1-1 his3-11 15 can1-100*

f) background: *his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; met15Δ0/MET15; LYS2/lys2Δ0; ura3Δ0/ura3Δ0*

g) background: *ura3-52 trp leu2Δ0 his3-Δ200 gal2 galΔ108*

Yeast and bacterial media

- Yeast strains were grown in solid or liquid media at 28ºC.

1) YPG - rich medium:

1% yeast extract (Difco), 1% bacto peptone (Difco), 2% glucose as a carbon source

2) SD - synthetic minimal medium:

0.7 g/l yeast nitrogen base, 2% glucose, supplemented with amino acids as required.

Growth supplements- amino acids and nucleotides:

adenine (2 mg/ml), uracil (2 mg/ml), histidine (2 mg/ml), leucine (3 mg/ml), lysine (2 mg/ml), methionine (2 mg/ml), tryptophan (2 mg/ml), tyrosine (1 mg/ml).

3) SC - synthetic complete medium:

0.7 g/l yeast nitrogen base, 2% glucose, supplemented with all required amino acids.

- *E. coli* was grown in solid or liquid LB medium containing 2% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, the pH was adjusted to 7.5. When necessary LB was supplemented with ampicilin (100 μg/ml).

- Solid media were prepared with 20 g/l of bacto-agar (Difco). For preparation of selective medium purified bacto-agar was used (Sigma)

3.1.2 Yeast genetics procedures

Construction of the *trk1*Δ*yta7*Δ mutant (KTY1). In order to obtain double *trk1*Δ*yta7*Δ mutant we deleted *YTA7* in the *trk1*Δ strain (*trk1*::*MX4*- Y11296). To delete *YTA7*, the *HIS3* gene was amplified from pF6aHIS3 (Longtine *et al*., 1998) by PCR using primers:

YTAfHIS:

5’GGTCTATTTCTCTCTTCTCGCTATTTATTCACAATTGGTGCGGATCCCCGGGTTAATTAA3’

YTArHIS:

5’GTAAGAGCCAGTAATGGGCTTTGCATACGGTCTTGTTCACTGAATTCGAGCTCGTTTAAAC3’

The linear product was transformed into yeast. Transformants were screened on the selective medium and genomic DNA was isolated using Wizard Genomic Purification kit (Promega) according to manufacturer instructions. The presence of both the *YTA7* and *TRK1* deletions was confirmed by diagnostic PCR using primers:

diagTRK1f- 5’ TATTGCTACGGCCATCGACT 3’

diagTRK1r- 5’ ATGGCGTTGACGATGACGAA 3’

diagYTA7f- 5’ CTGAAGGATCTTGCGTACTC 3’

diagYTA7r- 5’ ACGTATGCCAGAACAGTGAC 3’

Construction of the strains used for the Yta7 and Erg20 proteins localization. The *yta7*Δ mutant (Y15922) was transformed with a centromeric plasmid expressing Yta7p-ProtA protein fusion (pRS316 YTA7-ProtA). As the *ERG20* deletion is lethal, to obtain yeast cells expressing Erg20p-ProtA the heterozygous *erg20*Δ mutant (Y21258) was transformed with a centromeric plasmid pRS316 ERG20-ProtA. Resulting transformants were sporulated in medium containing 1% potassium acetate and 2% bacto-agar. Then yeast spores were treated for 15 min with 0.22 mg/ml cytohelicase and for 20 min with ethyl ether. Obtained haploid cells were selected based on the *ERG20* deletion marker (Kan^R) and pRS316 ERG20-ProtA maker (*URA3*). Additionaly, to ensure that Erg20p-ProtA was indispensable for the cells survival we performed the test with FOA (5-fluoroorotic acid).

Construction of the YTA7-TAP mutant (K314). In order to introduce N-terminal TAP (Tandem Affinity Purification) tag into the *YTA7* gene the procedure described by Puig *et al.* (Puig *et al*., 2001) was thoroughly followed. Briefly, the pBS1761 plasmid was used as a template to amplify PCR product for yeast transformation. PCR primers contained regions of homology to the *YTA7* gene:

YTA7NA -

5’TCTCTTCTCGCTATTTATTCACAATTGGTGCAGAAAGAACGAACAAAAGCTGGAGCTCAT3’

YTA7NB -

5’CAACGTCGCTACCGCGTCTATTCCTTAAATTTCGTGCCATCTTATCGTCATCATCAAGTG3’

YDL401 strain that does not contain *GAL1* promoter was a recipient for transformation with the tagging cassette that comprised also the *GAL1* promoter and *TRP1* marker nedded for the selection of transformants. Finaly, the *GAL1* promoter and the *TRP1* marker were removed from the genome by Cre recombinase leaving tagged *YTA7* under the control of its natural promoter. As the deletion of *YTA7* was reported to cause sensitivity to 6% ethanol (Agostoni Carbone *et al*., 1998), we used this phenotype to verify functionality of Yta7-TAP protein.

Transformation

Transformation of the yeast strains was performed according to the lithium acetate (LiAc)/single-stranded DNA (SS-DNA)/polyethylene glycol (PEG) procedure (Gietz *et al*., 1995). Transformation of *E. coli* was performed with the use of Gene Pulser (BioRad) according to the supplied instruction.

3.1.3 General molecular biology procedures

3.1.3.1 Yeast extract preparation

In order to prepare yeast extract, suspension of cells in appropriate buffer was lysed by vigorous shaking with glass beads (Ø 45mm) using a vortex (8 × 30 sec with 30 sec-interval on ice). Cell extracts were separated from glass beads and cell debris, collected in a new Eppendorf tube by centrifugation, and clarified by a 13 000 × *g* spin for 15 min at 4°C. The obtained supernatant served as the source of proteins.

Further, in particular methods the compositions of the lysis buffers are indicated.

3.1.3.2 Western blot analysis

To the samples a sample buffer was added (60 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 0.025% Bromophenol Blue) and they were denaturated for 5 min at 100°C. Aliquots

corresponding to the 60 µg of proteins were loaded into the wells of the 10% SDS-polyacrylamid gel (SDS-PAGE). Electrophoresis was performed for 4 h at 80V then proteins were transferred by a semi-dry system (1h, 1.2 mA/cm^2 of the gel) to the Immobilon-P (Milipore) membrane. After protein transfer, membrane was shortly immersed in the methanol and dried. For the incubation times and dilutions of antibodies instructions of the manufacturers were followed. Usually, in order to visualize a given protein the secondary antibodies conjugated with alkaline phosphatase were used (DakoCytomation, 1:2000, 2h, RT) and the activity of this protein was detected with BCIP/NBT solution (Sigma).

3.1.3.3 Estimation of protein concentration

Protein concentration was determined according to Lowry *et al*. (LOWRY *et al*., 1951) or with Bradford reagent (Sigma). Bovine serum albumin (Promega) was used as a standard of the protein quantification.

3.1.4 DNA microarrays

The microarray datasets of Congo red, Calcofluor white and caffeine are available in a query-format at http://biopuce.insa-toulouse.fr/jmf/cellwallgenomics. This work is fully MIAME-compliant and has been deposited at GEO website with accession number GSE4049. All statistically treated datasets described in this work are presented in the separately printed SUPPLEMENT.

Figure 56 represents a general overview of the method that we used to perform analysis of the genome-wide transcription pattern elicited by the tested drugs. Particular steps of this method are described in the following chapters.

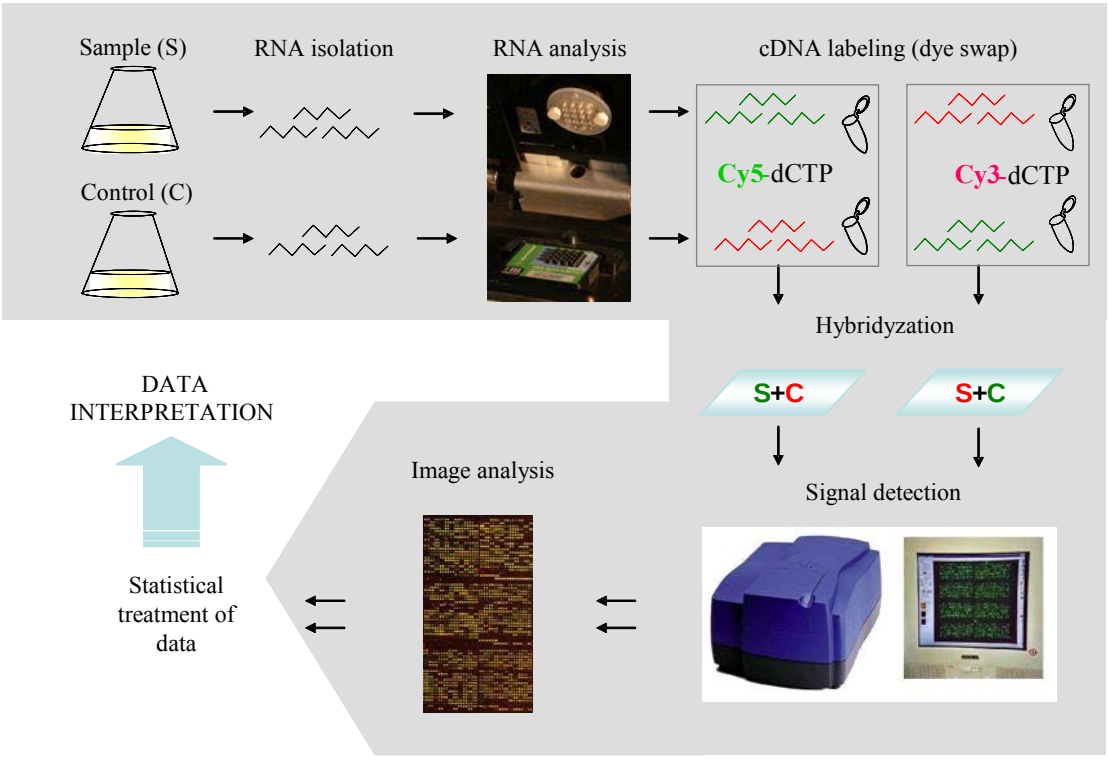


Figure 56: DNA microarray – a scheme of the method.

Yeast cells were grown without (C) or with a given drug added (S), and the respective RNAs were isolated. The obtained RNA was assayed for its quality and quantity (Bioanalyzer 2100) and transcribed into the labeled DNA using Cy5- and Cy3-dCTP. In this procedure dye swap was applied. After hybridization of the *control* DNA and *sample* DNA to the DNA chip, slides were scanned (GenePix 4000B) and the image analysis was performed. The obtained signal intensities were treated statistically prior to the interpretation.

3.1.4.1 Experimental design and drug treatment of yeast cultures

The used strains, drugs concentrations and other details of the experimental design are presented in Table 16. Papulacandin B was a generous gift from A. Duran (Ciba-Geigy, Basel, Switzerland). A stock solution was prepared at concentration 20 mg/ml Papulacandin B in methanol and stored at -20°C. Caffeine and Congo red were purchased from Sigma, and Calcofluor white was from ICN Biomedicals. Lovastatin was purchased from A.G. Scientific and zaragozic acid from Merck. Lovastatin, prior to use was changed into its active form by alkaline lysis. For 25 mg of the powdered lovastatin 0.5 ml of 95% EtOH was added and it was placed at 55ºC for 5 min. Than 62.6 µl of 1 M NaOH and 0.5 ml of H_2O was added and it was incubated for 10 min at 55ºC. Again 62.6 µl of 1M NaOH was added and mixture was further incubated with periodic swirling for 30 min at 55ºC. Finaly, mixture was brought to the room temperature, 40 µl of 1M Tris/HCl pH 8.0 and 20 µl of 1 M HCl were added.

Solution was brought to the final volume of 2 ml. Prepared stock was stored at -20 ºC. At the same time a vehicle solution that lack lovastatin was prepared and it was later on added to the media of the control cultures. Stock solutions of other drugs were prepared with water.

Drug treatment of the yeast cultures was carried out as follows. Yeast cells were cultivated in YPD medium at 28°C to the logarithmic phase of growth ($0D_{600}$ 1.0). Then they were diluted to an initial OD_{600} 0.05 and grown until OD_{600} 0.2. The culture was then quickly divided in several parts. One part was allowed to grow under the same conditions (control), while others were treated with the various concentrations of the appropriate drugs. After indicated times (Table 16) yeast cells were collected by centrifugation, rapidly frozen in a liquid nitrogen and kept at -80°C until use.

Table 16: DNA microarrays – Sumary of the experimental design

Rank	Strain	Additions		Time of incubation (min)	Number of Experiments (cultures)	Number of slides per experiment
		Control	Sample			
1	BY4742	-	2 mM CAF	5, 15, 90	2	1
2	-//-	-	20 mM CAF	5, 15, 90	2	1
3	-//-	-	20 µg/ml CR	5, 15, 90	2	1
4	-//-	-	100 µg/ml CR	5, 15, 90	2	1
5	-//-	-	20 µg/ml CFW	5, 15, 90	2	1
6	-//-	-	100 µg/ml CFW	5, 15, 90	2	1
7	W303	vehicle	0.25 µg/ml PAP	60	2	1
8	-//-	vehicle	2.5 µg/ml PAP	60	2	1
9	-//-	vehicle	25 µg/ml PAP	60	2	1
10	BY4742	vehicle	25 µg/ml Lo	10, 30, 60	2	2
11	-//-	-	10 µg/ml ZA	10, 30, 60	2	2
12	BY4742 (wt and *yta7*Δ)	-	-	5h	2	2

3.1.4.2 RNA isolation, cDNA synthesis and hybridization to microarrays

Frozen cells (10 units of OD_{600}) were mechanically disrupted (MicroDismembrator Braun, Melsungen) and total RNA was isolated using RNeasy Mini kit (Qiagen) following the protocol of the manufacturer. The quantity and the quality of the extracted RNA were determined by microcapillary electrophoresis using Bioanalyzer 2100 and the RNA6000 Nano LabChip Kit (Agilent Technologies, Wilmington, DE, USA). Incorporation of Cyanine

3- or Cyanine 5-dCTP (Amersham Bioscience) was performed during reverse transcription of 20 µg of denaturated RNA using Label Star Reverse Transcriptase (Qiagen). Labeled cDNA was purified with MinElute spin columns (Qiagen) and was hybridized on the dendrimer-activated glass slides which represented whole yeast genome by covalently attached DNA probes (70-mer oligonucleotides) (Le, V *et al*., 2003). In the each slide a single gene was represented four times. Prehybridisation was performed with solution of 1% BSA, 2 ×SSC, 0.2% (v/v) SDS over 1hour at 42°C. After automatic washing according to manufacturer's instructions, the slides were hybridised for 10 h in 200 µl of ChipHybe™ buffer (Ventana Medical System, Inc). After hybridisation, the slides were washed for 2 min in 2 ×SSC/0.2% (v/v) SDS, for 2 min in 0.2 ×SSC/0.2% (v/v) SDS and for 2 min in 0.2 ×SSC, immersed briefly in isopropanol and then dried under a stream of air.

To reduce biological and systematic variability, total RNA from two independent cultures were extracted, labeled with dCTP-Cy3 (and reciprocally with dCTP-Cy5) and hybridized on two independent microarrays. Thus, every gene was represented by four intensities from two independent DNA arrays. The hybridization signal was detected by scanning with GenePix 4000B laser Scanner (Axon Instruments), and the signal quantification was transformed to numerical values using the integrated GenePix software version 3.01.

3.1.4.3 Transcript data acquisition and treatment

Both raw and statistically treated data are presented at http://biopuce.insa-toulouse.fr/jmflab/cellwallgenomics/, which provides full details for normalization and statistical regimes using our home-made **Bioplot** software. Briefly, raw intensities were corrected from the background. To take into account non-homogeneity of the noise on the array, background was measured locally around each spot. The normalisation was applied were the mean expression value of a whole genome was considered as a reference. As the spot replicates on the same array are statistically dependent first step in averaging was to calculate the mean value over spot duplicates for a given gene on the same array and then an average on arrays was calculated. Log-ratios of normalized intensities from duplicate samples were tested for statistical significance using Student's test. The differentially expressed genes (1.5-fold change in expression and p-value ≤ 0.05) were attributed to functional categories according to *Saccharomyces* Genome Database. When necessary, search for known DNA binding sites on gene promoter was performed using Yeastract software, which interrogates a

yeast database containing experimentally validated associations between genes and their transcription factors (Teixeira *et al*., 2006). Regarding dataset of Papulacandin B, GeneSpring was used to obtain hierarchical clustering of genes that were similarly expressed in three tested concentrations of this drug. GeneSpring provides sophisticated clustering methods to uncover patterns of gene expression data and the relationships between these patterns. As similarity measure Standard correlation was used which finds especially overexpressed genes (Eisen *et al*., 1998).

3.1.4.3.1 Correspondence analysis of the DNA microarray data.

We analyzed transcriptional response to the 'inhibitory' concentrations of Congo red, Calcofluor white and caffeine, after 90 min of growth, using Correspondence Analysis (Fellenberg *et al*., 2001). Signal intensities were uploaded into the M-CHIPS (multi-conditional hybridization intensity processing system) which provides a MATLAB-based tool that was used for analysis, normalization and filtering. For normalization, log-linear regression accounting for affine-linear deviations among the different hybridizations was applied. Each hybridization experiment was normalized with the respect to the gene-wise median of the control conditions (culture w/o drugs after 90 min of growth). Signal intensities of repeated hybridizations were normalized and significance levels assessed by the 'min–max separation', a stringency criterion calculated by taking the minimum distance between all data points of the two conditions. To visualize interdependencies among the high-dimensional data received in two-way contingency tables with rows representing genes and columns representing hybridizations, singular value decomposition was used for reducing dimensionality, and the chi squared (χ^2) distance among the data points was approximated. Afterwards, genes and hybridizations were plotted together in a two-dimensional space, where expression specificities are indicated by the distances from the center. The interpretation of the exemplary correspondence analysis (CA) plot is shown in Figure 57.

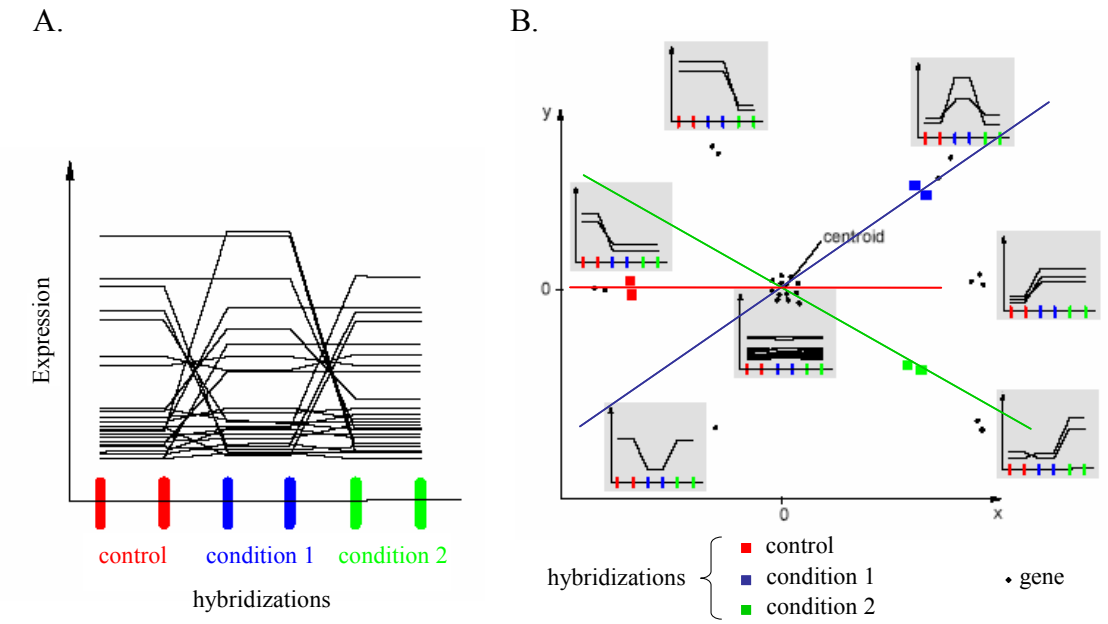


Figure 57: Interpretation of the CA plot

A. This plot shows an example of the virtual dataset comprising 24 genes. There are two perfectly reproducible hybridizations for each experimental condition. It resembles the real situation because the majority of genes does not change their expression in the tested conditions. **B**. Dataset from panel A was embedded into a plane using correspondence analysis where both axes, X and Y, are dimensionless. Gene clusters are shown together with the corresponding gene profiles. The hybridizations with similar expression profiles are the neighbours in this projection. The red, green and blue axes illustrate the major trends according to the condition medians. The genes with unchanged expression or those not expressed to a measurable amount in any tested conditions are concentrated in the center of the plot called 'centroid'. The distance to the centroid of the genes located to the outer regions of the plot reflects the significance of having a changed expression. The genes with the high expression in a given condition are placed in the direction of this condition, while the underexpressed genes are placed on the opposite side of the centroid. (adapted from a theorethical script 'Correspondence Analysis and Data Warehousing with Microarray Data' by K. Fellenberg)

3.1.4.3.2 Grouping genes in functional categories

SGD Slim Mapper and Term Finder

Slim Mapper and Term Finder are provided by SGD database that currently contains 7292 genes annotated to at least one GO term. Slim Mapper is a tool allowing grouping genes in broad biological categories e.g. 'carbohydrates metabolism', which is very useful especially for analyzing the results of microarray expression data. On the other hand, to find a particular GO term shared by genes in a given list and to calculate its significance we used Term Finder. The number of genes representing a given process in the tested transcription profile is compared with the number of genes representing this process in the whole genome, without taking into account their ratio of expression.

T-Profiler

T-profiler uses the *t*-test to score the difference between the mean expression level of predefined groups of genes and that of all other genes on the microarray (Boorsma *et al*., 2005). The main advantage of this software is that a user does not have to apply cutoffs to the expression level of individual genes. We used this software to group genes according to gene ontology (GO) nomenclature. For calculations only GO groups containing more than 6 members were used (1389 groups). Analysis of the data sets with T-profiler also allows identification of regulatory motifs in the promoter of responsive genes (region -600 to -1 bp). It is important to note that T-Profiler does not take into account how often a particular motif occurs in a promoter region and it does not allow overlapping with the neighboring ORFs. Currently, there are 153 motif groups in use. Groups of genes with a P-value < 0.05 are accepted as significantly changed.

3.1.5 Determination of the changes in the cell wall structure

3.1.5.1 Zymolyase sensitivity test

Zymolyase sensitivity test is a simple method used to verify the changes in the cell wall structure. This method is based on the rate of the cell lysis, which is fast or slow depending mainly on the level of β-1,3-glucan. As the cell wall degrading enzyme we used Zymolyase which acts mainly at the glucose polymers at the β-1,3-glucan linkages but it has also a protease activity. This additional protease ativity causes that increased resistance to Zymolyase cannot be simply explained by the increased level of β-1,3-glucan. In order to perform the test, cells collected by centrifugation were resuspended in a buffer (10 mM Tris/HCl pH 7.4, 40 mM β-merkaptoethanol) to reach OD_{600} 0.9 and incubated for 30 min. Then 50μg/ml Zymolyase 20-T (ICN Biomedicals Inc.) were added and the lysis of the cells was followed by measurements of decreasing OD_{600} at RT. The OD_{600} at time 0 was taken as 100%. Presented results are averages of three experiments.

It has been shown that a treatment with the cell wall disturbing agents results in the increased resistance to the cell wall degrading enzymes (Boorsma *et al*., 2004). We have used

this fact to verify if the used concentrations of CR, CFW and caffeine were eliciting changes in the cell wall. Yeast were cultivated like it was described for DNA microarrays (3.1.4.1, pg 118). Samples were taken after 30, 60, 90 min and 5 h of growth.

In addition, we used Zymolyase sensitivity test to examine involvement of several transcription factors in the cell wall remodeling induced by CR, CFW and caffeine. In these experiments each mutant and its parental strain were grown with the 'inhibitory' concentrations of drugs. There was no significant difference regarding Zymolyase sensitivity between the used wild-type strains (BY4741 and W303). The OD_{600} was measured each 15 min for 105 min. The increase of resistance after treatment of yeast with the tested drug in the wild-type strain was taken as 100% and the resistance of mutant strains treated with the same drug was compared to this value.

Finally, we tested sensitivity of various mutants to Zymolyase treatment to asses whether the particular deletion could influence the cell wall biosynthesis. The fraction of wild-type cells that resisted lysis after 90 min of digestion with Zymolyase 20-T (50 µg/ml) was taken as 100% and compared to mutant cells.

3.1.5.2 Determination of glucose, mannose and glucosamine levels in the cell wall

After 20 hours of growth in the presence or without drugs, cells were collected by centrifugation at 4°C, and frozen with liquid nitrogen. Cells were broken mechanically with glass beads and obtained cell walls were washed consequently with cold 10 mM Tris/HCl pH 8.0 buffer until the supernatant became clear. Glucose, mannose and glucosamine were released from the cell wall polysaccharides during sulfuric acid hydrolysis. To isolated cell walls suspended in 100 µl of water 50 µl of 72% H_2SO_4 was added and incubated in room temperature for 3h. Than 650µl of water was added to obtain 2 N H_2SO_4 concentration and samples were incubated at 100°C for 4 h. Finaly, to each sample 6 ml of water was added and the pH was brought to 7.0 with solution of 40 g/l of $Ba(OH)_2$. Precipitated $BaSO_4$ was removed by centrifugation and remaining in the supernatant carbohydrates were quantified using DIONEX column (SunnyVale, USA) by high-performance anionic exchange chromatography (HPAEC) as described by Dallies *et al*. (Dallies *et al*., 1998). This method was used to measure glucose that corresponds to the level of β-1,3- and β-1,6-glucans, mannose and acetyloglucosamine representing mannans and chitin, respectively.

3.1.6 Cellular localization of proteins

3.1.6.1 Subcellular fractionation

An overnight culture of yeast was diluted to OD 0.1 and grown to reach an OD 0.5. 160 OD units of cells were collected by centrifugation washed with water and resuspended in 20 ml in **buffer A**. After 45 min at 30ºC, cells were transferred to 10 ml of **buffer B** and in order to obtain spheroplasts Zymolyase (10 U/1 OD) was added. After 30min of incubation at 30 ºC, spheroplasts were washed with the **buffer C** and resuspended in 2 ml of cold **buffer TEA**. Next, spheroplasts were homogenized (Potler Homogenizator) at 4 ºC and centrifuged. After each round of centrifugation the pellet was kept and the supernatant was submitted for a following centrifugation. Finally, to separate organelles, six centrifugations were performed:

1) 500×*g*, 5 min
2) 3 000×*g*, 15 min
3) 10 000×*g*, 30 min
4) 25 000×*g*, 30 min
5) 50 000×*g*, 60 min
6) 100 000×*g*, 90 min

To decrease the level of contamination between fractions, three first pellets (1-3) were resuspended in TEA buffer and centrifuged again. Other pellets were washed without resuspending. All pellets and the last supernatant were transferred to the sample buffer, proteins were denaturated (4 min, 100ºC) and stored in -80ºC.

Buffer A: 100 mM Tris/HCl pH 9.5, 100 mM β-mercaptoethanol, 10 mM NaN_3, 40 mM EDTA
Buffer B: 1.5 M sorbitol, 10 mM Tris/HCl pH 7.5
Buffer C: 1 M sorbitol, 150 mM KAc, 5 mM MgAc, 20 mM HEPES pH 6.8
Buffer TEA: 10 mM triethanolamine pH 7.5, 10 mM KF, 10 mM NaN_3, 1 mM EDTA, 0.8 M sorbitol

Sample buffer: 31 µl Tris/HCl pH 6.8, 100 µl 10% SDS, 50 µl glycerol, 25 µl β-mercaptoethanol, 294 µl H_2O

3.1.6.2 Immunofluorescence microscopy

An overnight culture of yeast was diluted to OD 0.1 and grown to reach an OD 0.5. For fixation of the cells, to 10 ml culture 1.2 ml of 37% formaldehyde was added. After 15 min cells were collected by centrifugation, resuspended in the **buffer A** with additional 0.6 ml of 37% formaldehyde and incubated 30 min. Then cells were centrifuged and washed 4 times with **buffer B**. To obtain protoplasts cells were incubated 1h at 37 ºC in the 0.5 ml of buffer B with 5 µl β-mercaptoethanol, 20 µl Zymolyase and 27 µl Glusulase added. After centrifugation (2000 rpm, 5min), protoplasts were washed twice with buffer B and resuspended for 1 h in **buffer F**. Then protoplasts were centrifuged (2000 rpm, 5min), washed 7 times with buffer F, primary antibody diluted in the same buffer was added and suspension was incubated overnight. Next, protoplasts were washed (7 times with buffer F) the reaction with the secondary antibody conjugated with Texas Red lasted 2 h in the dark and was followed by analogous washing. Finally, protoplasts were resuspended in the buffer B and spread on the polylysine-coated slides (Sigma). After 45 min protoplast were attached to the slides and buffer could be delicately removed with the pipette. The slides were allowed to dry and than a rapid (1min) staining with water solution of DAPI was performed after which protoplasts were washed twice with water, covered with MOUNT and a glass slide.

Buffer A: 160 ml 50 mM KPO_4 pH 6.5, 1ml 0.1 M $MgCl_2$, up to 200 ml H_2O
Buffer B: 160 ml 50 mM KPO_4 pH 6.5, 1ml 0.1 M $MgCl_2$, 43.73 g sorbitol, up to 200 ml H_2O
Buffer F: 0.01 g KH_2PO_4, 0.85 g NaCl, 0.1 g BSA, 0.1 g NaN_3, up to 100 ml H_2O (pH 7.4)
MOUNT: 50 mg p-phenylenediamine, 5 ml 1×TBS, 45 ml glycerol (pH 9.0)

Subsequently, prepared yeast were observed using Nikon Eclipse E800 microscope (magnification × 100, oil immersion).

3.1.7 Activity of the IP pathway enzymes

3.1.7.1 Cis-*prenyltransferase activity assay*

Activity assay. Yeast cultures were grown overnight in YPD medium, then they were diluted to OD 0.1 and (were indicated 25 μg/ml lovastatin was added) grown to reach OD 0.5 when the cells were collected. The cells were subjected to the isolation of the membrane fraction (method described in chapter 3.1.8.5, pg 130) which served as the source of the enzyme. The enzyme assay was carried out according to Szkopinska *et al*. (Szkopinska *et al*., 1997). The reaction mixture in a final volume of 250 μl contained 50 mM phosphate buffer pH 7.5, 0.5 mM $MgCl_2$, 20 mM β-mercaptoethanol, 10 mM KF (to inhibit isoprenoid phosphatase), 3×10^5 cpm $\{^{14}C\}$ IPP (specific activity 52 mCi/mM) and 46 nM FPP and 450 μg of proteins. The reaction lasted 1h at 30 ºC and was terminated by addition of 2 ml of Chloroform : Methanol (3:2).

Lipids extraction. After addition of 2 ml of Chloroform : Methanol (3:2) samples were incubated 30 min in -20ºC. This mixture was washed three times with 0.5 ml of 10 mM EDTA pH 8.0 in 0.9% NaCl. After each washing and centrifugation (1 min, 4000×rpm) the upper phase and the proteins were removed. Clear organic phase phase was concentrated under stream of nitrogen, resuspended in 30 μl of Chloroform : Methanol (3:2), subjected to thin-layer chromatography on HPTLC RP-18 plates and developed in 50 mM H_3PO_4 in acetone. Then, autoradiograph which can be seen in Figure 42A was obtained (14 days of exposure). The zones containing radiolabeled polyprenols were scraped and the radioactivity was measured with a scintillation counter (LKB Wallac 1209 RockBeta Counter).

3.1.7.2 Farnesyl diphosphate (FPP) synthase activity

Yeast cultures were grown overnight in YPD medium, then they were diluted to OD 0.1 and (were indicated 25 μg/ml lovastatin was added) grown to reach OD 0.5 when the cells were collected. Yeast extract, which served later as the source of FPP synthase, was prepared in 50 mM phosphate buffer pH 7.5 supplemented with 5 mM iodoacetamide. The enzyme assay was carried out according to Chambon *et al*. (Chambon *et al*., 1991). The reaction mixture in the final volume of 100 μl contained 50 mM phosphate buffer pH 7.5, 1 mM

$MgCl_2$, 5 mM iodoacetamide, 1×10^5 cpm $\{^{14}C\}$ IPP (specific activity 52 mCi/mM), 60 μM IPP, 120 μM DMAPP and 100 μg of protein. The reaction lasted 5 min at 37 °C and was terminated by addition of 0.5 ml of H_2O, 1 ml of hexane and 0.2 ml of 1 N HCl (to dephosphorylate the products). After 30 min of additional incubation at 37°C, samples were vigorously mixed, the upper phase was separated and washed 3 times with water. The radioactivity of the organic phase was subjected to liquid scintillation counting (LKB Wallac 1209 RockBeta Counter).

3.1.7.3 Measurement of dolichol level

Yeast cultures were grown overnight in YPD medium, then they were diluted to OD 0.1 and (were indicated 25 μg/ml lovastatin was added) grown to reach OD 0.5 when the cells were collected. The cells were subjected to the isolation of the membrane fraction (method described in chapter 3.1.8.5, pg 130) which served as the source of dolichol. Lipids were extracted from the membrane fraction corresponding to 450 μg of protein, as described in *cis*-prenyltransferase assay (3.1.7.1, pg 127), and subjected to thin-layer chromatography on HPTLC RP-18 plates that were developed in 50 mM H_3PO_4 in acetone. After 15 min incubation of the plate in iodine vapor (Figure 42C), intensity of the bands corresponding to the major form of dolichol (Dol_{16}) were quantified using ImageQuant software (Molecular Dynamics). Intensity of the band of non-treated wild type cells were taken 100% of dolichol level. The result of this experiment is an average of values from three independent yeast cultures and each sample was measured in duplicate.

3.1.8 Other procedures

3.1.8.1 Mpk1 kinase phosphorylation assay

Yeast extracts for assays of the amount of Mpk1p phosphorylation were prepared in 150 μl of cold lysis buffer (50 mM Tris/HCl pH 7.5, 10% glycerol, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM KF, 1 mM sodium orthovanadate, 50 mM β-glycerol phosphate,

5 mM sodium pyrophosphate, 5 mM EDTA, 1 mM PMSF) supplemented with protease inhibitors (Protease Inhibitors Cocktail, Sigma), as described in Martin *et al.* (Martin *et al*., 2000). Total protein concentration in the supernatants was measured using Bradford reagent (Sigma). Denaturated protein samples (60 μg) were subjected to SDS-PAGE electrophoresis and transferred to Immobilon-P membrane (Millipore). To detect the phosphorylated form of Mpk1p kinase, the membranes were probed overnight at RT with anti-phospho-p44/42 MAPK antibody (New England Biolabs) at 1:1000 dilution in TBS buffer and 5% non-fat milk. To quantify total Mpk1p abundance polyclonal anti-Mpk1 antibodies were used (Martin *et al*., 1993). Primary antibodies were detected using alkaline phosphatase-conjugated anti-rabbit antibody (DakoCytomation, 1:2000, 2h, RT) and BCIP/NBT solution (Sigma). As a negative control, protein extract from the *mpk1*Δ mutant was used. The protein extract from the *gas1*Δ mutant served as a positive control. All experiments were repeated at least twice with consistent results.

3.1.8.2 Determination of cAMP level

Cells were grown with caffeine as described for DNA microarrays (3.1.4.1, pg 118) and samples were collected by rapid filtration just before caffeine addition and after 5, 15, 90 minutes and 20 hours of growht. To extract cAMP cells for 3 min were boiled in ethanol buffered with 70 mM HEPES pH 7.5 as described previously by Gonzales *et al.* (Gonzalez *et al*., 1997). Ethanol was then evaporated using the Buchi rotary evaporator (water bath 40°C, 70 mbar – 3 min, 40 mbar – 9 min). Dry residue was resuspended in 0.5 ml of water and the level of cAMP was determined with an enzyme immunoassay system (R&D Systems, DE0450) as indicated by manufacturer.

3.1.8.3 Spot assays of yeast sensitivity to various compounds

To test growth capacity of the yeast cells on various solid media, they were grown overnight in appropriate liquid medium, in the morning they were transferred to the fresh medium, diluted (OD 0.1) and allow to grow for 6 h. Then cells were washed in sterile water

and diluted to reach OD 0.5. This yeast suspension served to prepare 10-fold dilutions which were spotted onto agar plates that could be supplemented with one of the listed compounds:

100 µg/ml Congo red

50 µg/ml Calcofluor white

2, 5 or 20 mM Caffeine

1 M Sorbitol

20 mM Adenine

500 µg/ml lovastatin

50 µg/ml Hygromycin B

500 µg/ml chloramphenicol

0.15 M LiCl

0.1 M KCl

0.5 M NaCl

cyclohexymide - 10 µl of a 10 mg/ml solution in ethanol was poured into the well in the solid YPD. Approximatelly 10^7 cells were spread as a lawn on medium surface.

3.1.8.4 Measurement of the cell size and bud counting

Cells were cultivated for 24h in YPG medium without or with addition of 20 mM caffeine. Cells were visualized with Nikon Eclipse E800 microscope (magnification × 100, oil immersion) and radius of the cells was calculated using LuciaG software (Screen Measurement System, Nikon). Since yeast cells have an oval shape, a radius can only give an estimation of the actual size. Simultaneously the numbers of budding and non-budding cells were calculated.

3.1.8.5 Isolation of the membrane fraction from yeast

Collected yeast cells were suspended in Tris I solution (50 mM Tris/HCl pH 7.4, 15 mM $MgCl_2$, 9 mM β-mercaptoethanol) and broken with the glass beads (vortexing 8× 30 sec with a 30 sec-intervals on ice). After first centrifugation (4ºC, 5 min, 2000×*g*) supernatant was centrifuged for 1.5 h, 50 000×*g* at 4 ºC. Obtained pellet was resuspended in Tris II solution

(50 mM Tris/HCl pH 7.4, 3.5 mM $MgCl_2$, 6 mM β-mercaptoethanol) and homogenized (Potler Homogenizator). The obtained membrane fraction was stored at -80°C until use.

3.1.8.6 Membranes solubilization

For the membrane solubilization we used K314 strain expressing TAP-tagged *YTA7* (detailed description of the K314 strain in chapter 3.1.2, pg 114). Yeast were grown to reach OD_{600} 0.5 then yeast extract was prepared in a TEG buffer (50 mM Tris/HCl pH 7.5, 1 mM EDTA, 5% v/v glycerol) supplemented with Cocktail of Protease Inhibitors (Sigma). Yeast extract was divided on equal volumes, and to each part one of the following reagents was added to a final concentration: 1 M NaCl, 0.1 M Na_2CO_3 pH 11.5, 2 M urea or 2% Triton X-100. Additionally each sample was supplemented with 1 mM PMSF. After 1 h of incubation at 4 ºC samples were centrifuged 1.5h at 100 000×*g*. Then, proteins from the pellet and supernatant fraction served for western analysis. Yta7-TAP protein (ProtA epitope) was visualized with rabit developed primary antibodies, then secondary antibodies conjugated with alkaline phosphatases were used (Dako, Cytomation) and the activity of this protein was detected with BCIP/NBT solution (Sigma). As it can be seen in Figure 47 (pg 101), Triton X-100 was not concentrated enough to solubilize totally the membrane, but our control with an integral membrane protein Dpm1 proved that 2% concentration of Triton X-100 was enough to wash away a significant amount of this protein (not shown) as well as Yta7p.

3.1.8.7 Measurement of the K^+ content in yeast

Yeast were grown overnight in the YPD medium then they were washed with MQ water and resuspend (OD 0.1) in the fresh media YPD, SC or SC supplemented with 0.1 M KCl. When cultures reached OD 1.0 cells were collected by centrifugation and washed 3 times with cold 20 mM $MgCl_2$ solution then they were frozen in liquid nitrogen and lyophilized. Content of potassium was measured using flame atomic absorbtion spectrometry (FAAS). The results shown are averages of two experiments.

3.1.8.8 CPY glycosylation assay

CPY glycosylation assay was performed as described in Schenk *et al*. (Schenk *et al*., 2001). Briefly, yeas cells growing in YPD to OD_{600} 0.5 and extracts were prepared in a buffer containing 50 mM Tris/HCl pH 7.5, 5 mM EDTA, 1 mM PMSF supplemented with protease inhibitors (Protease Inhibitors Cocktail, Sigma). Proteins were separated by 8% SDS-PAGE electrophoresis and Carboxipeptidase Y was detected with the anti-CPY mouse monoclonal antibody (1:4000, 2h, RT). This antibody was a kind gift from Prof. Ludwig Lehle (Universitaet Regensburg, Germany). Primary antibodies were detected using alkaline phosphatase-conjugated anti-mouse antibody (DakoCytomation, 1:4000, 2h, RT) and BCIP/NBT solution (Sigma).

3.1.9 Software and databases used in this study

Databases:

Yeast databases:

- Proteom BioKnowledge Library. http://www.proteome.com/
- **SGD** - *Saccharomyces* Genome Database. http://www.yeastgenome.org/
- **CYGD** - the MIPS Comprehensive Yeast Genome Database http://mips.gsf.de/genre/proj/yeast/
- **SCMD** - Saccharomyces cerevisiae Morphological Database. http://scmd.gi.k.u-tokyo.ac.jp/datamine/

Other databases:

- **KEGG** - Kyoto Encyclopedia of Genes and Genomes. http://www.genome.ad.jp/kegg/
- **Entrez** - The Life Sciences Search Engine. http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi

Software:

DNA microarray data mining:

- **BioPlot** - web service. http://biopuce.insa-toulouse.fr/
- **M-CHiPS** - Multi-Conditional Hybridization Intensity Processing System. http://mchips.org/

- **GeneSpring** (Silicon Genetics)
- **Gene Pix Pro** (Axon Instruments) software for slide image analysis.

Detection of regulatory signals in non-coding sequences:

- **RSAT** - Regulatory Sequence Analysis Tools. http://rsat.ulb.ac.be/rsat/
- **T-profiler**: Scoring the activity of pre-defined groups of genes using gene expression data http://studentst.bio.columbia.edu/t-profiler/index.html
- **YEASTRACT** -(Yeast Search for Transcriptional Regulators And Consensus Tracking) http://www.yeastract.com/
- **SCPD** - The Promoter Database of *Saccharomyces cerevisiae*. http://rulai.cshl.edu/SCPD/

Others:

- ImageQuant (Molecular Dynamics)
- LuciaG/F (Screen Measurement System, Nikon)
- VectorNTI (Invitrogen)
- CloneManager (Sci Ed Software)

SUMMARY and CONCLUSIONS

Nowadays, while fungal infections are an increasing threat to public health we have to face the problem of the deficit of antifungal drugs. Moreover, fungi have a potential to become resistant to some drugs, thus there is a demand for the elaboration of novel compounds with differentiated modes of action. In order to design an antifungal drug, scientists have to know details of the mechanisms that allow the survival of the microorganisms in an unwelcoming environment. Therefore, we focused our research on the fungal response to the chemical compounds which, similarly to antifungal drugs, impair the cell wall or ergosterol biosynthesis. Both pathways are essential for survival of fungi. The cell wall protects them from the harmful environment whereas ergosterol ensures correct functioning of the plasma membrane. For investigation of those mechanisms, we have chosen the genetically tractable fungus, the yeast *Saccharomyces cerevisiae*.

The first part of this work was devoted to a comprehensive investigation of fungal the response to cell wall damaging agents. We have chosen four chemical compounds that are known to perform this action. First, we characterized the transcriptional response of yeast cells to Papulacandin B, an inhibitor of glucan synthase that is one of the main enzymes responsible for the biosynthesis of the cell wall. Then we investigated the defensive mechanisms elicited by Congo red and Calcofluor white, drugs that weaken the tight cell wall structure by binding to its components. Finally, we examined the action of a putative cell wall drug, caffeine. This drug has been implicated in the cell wall assembly since, similarly to Congo red and Calcofluor white, it activates the cell wall integrity pathway, which could be observed as phosphorylation of the Mpk1 kinase. In an attempt to verify the effect of caffeine on the cell wall structure, we performed suitable experiments to compare the qualitative and quantitative effects of this drug with the effects of Congo red and Calcofluor white. The obtained results led us to the conclusion that caffeine indeed induced strengthening of the cell wall but, in contrast to other drugs, this effect was not exerted through a direct interaction with the cell wall structure. Moreover, the caffeine-induced remodeling of the cell wall did not occur through the usual Rlm1p-mediated way but it required some other transcription factors like Crz1p, Swi4p and Msn2/4p. Interestingly, a genome-wide expression analysis revealed a striking resemblance of the caffeine-induced transcription profile to the profile elicited by rapamycin, a potent inhibitor of the Tor1 and Tor2 kinases. Consistently, we found that the *tor1*Δ mutant was hypersensitive to caffeine and unable to induce phosphorylation of

Mpk1p when treated with this drug. Another effect of caffeine was a rapid but transient decrease in the intracellular level of cAMP that was followed by changes in the expression of genes belonging to the cAMP signaling cascade. Finally, we have identified Rom2p, a component of the cell wall integrity pathway, as a pivotal protein mediating caffeine-derived signaling to Mpk1 kinase as well as to the cAMP signaling pathway. Altogether, we report here a number of results that bear on the relationship between the cell wall integrity, the TOR pathway and the cAMP signaling cascade that together control the growth of the fungal cell in response to stressing conditions.

In the second part of this work, we investigated the regulation of the isoprenoid pathway that biosynthesizes ergosterol in eukaryotic cells. We have applied a novel approach to identify genes whose expression could be regulated by availability of the farnesyl diphosphate, a molecule that is crucial not only for the biosynthesis of ergosterol but also for other isoprenoid compounds and the feedback regulation of the isoprenoid pathway. Thus, we treated yeast cells with two drugs, lovastatin and zaragozic acid, both known to have the same reducing effect on the amount of ergosterol with opposite effects on the cellular availability of farnesyl diphosphate. We have analyzed changes in the genome-wide expression in response to these agents and we identified the 26S proteasome-regulated protein degradation and tRNA biosynthesis as primarily affected processes. Further, we identified 59 genes whose expression was presumably regulated by the changing availability of farnesyl diphosphate. Among these genes, we have chosen *YTA7*, a gene encoding an AAA ATPase of unknown function and investigated its functional link to the isoprenoid pathway. We have characterized Yta7p as a membrane-associated protein localized to the nucleus but also to the endoplasmic reticulum. We observed that depletion of Yta7p affected the cellular levels of some isoprenoid compounds. Moreover, we presented a genetic evidence of an interaction between *YTA7* and *HMG1* coding for 3-hydroxy-3-methylglutaryl coenzyme A reductase, a tightly regulated enzyme of the early steps of the isoprenoid pathway. To conclude, we propose that Yta7p is a novel protein that affects the isoprenoid pathway.

Altogether, these results bring a new insight into the fungal response to the interruption of the cell wall biosynthesis and the isoprenoid pathway by various drugs. We believe that in the future, these data can be useful in identifying novel targets for antifungal drugs.

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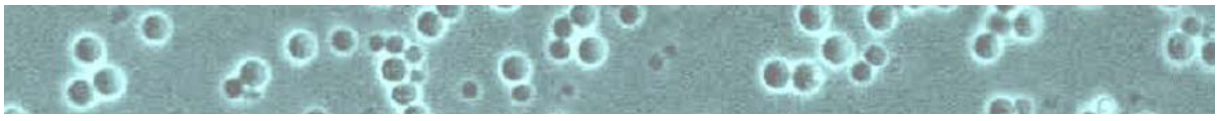

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APPENDIX

Appendix Contents:

List of Figures

List of Tables

Common Abbreviations:

CAF - caffeine

cAMP – cyclic 3',5' adenosine monophosphate

CFW – Calcofluor white

***cis*-PT** – *cis*-prenyltransferase

CR – Congo red

CWI – cell wall integrity

CWP – cell wall protein

DMAPP – dimethylallyl diphosphate

ER – endoplasmic reticulum

FPP – farnesyl diphosphate

FPPS – farnesyl diphosphate synthase

GAP – GTPase activating proteins

GEF - guanosine nucleotide exchange factor

HMG-CoA – 3-hydroxy-3-methylglutaryl coenzyme A

HMGR - HMG-CoA reductase

IC_{50} – concentration of the drug that causes a 50 % reduction of the growth rate

'non-inhibitory' concentration – concentration that exerts less than 10% of the IC_{50} value

'inhibitory' concentration – concentration that exerts twofold of IC_{50} value

IP – isoprenoid

IPP – isopentenyl diphosphate

MAP – mitogen-activated protein

MIC – minimal inhibitory concentration

Lo – lovastatin

OD_{600} – optical density measured at 600nm

PKA – cAMP-dependent protein kinase A

SQ – squalene

SQS – squalene synthase

TOR – target of rapamycin

ZA – zaragozic acid

Other abbreviations are defined in the text.

Table A 1: The genes implicated in cell wall remodeling, whose expression changed in response to CR, CFW or CAF - comparision with other published conditions

ORF	Gene	Ratio of expression						Fingerprint of cell wall stress			Description
		CFW1	CFW2	CR1	CR2	CAF1	CAF2	[1]*CW*	[2]*CR*	[3]*CFW*	
YKL096W	*CWP1*	3.63	3.88	4.27	5.68	-	-	*	*	*	Cell wall mannoprotein
YFL014W	*HSP12*	3.92	4.12	1.6	1.76	-	11.1	*	*	*	Response to stress
YKR091W	*SRL3*	2.26	1.88	1.75	2.52	-	-	*	*	*	Function unknown
YAL053W		1.89	2.27	1.44	1.88	-	2.24	*	*	*	Function unknown
YLR414C		1.58	2.47	-	-	-	-	*	*	*	Function unknown
YMR315W		1.79	1.99	1.48	1.74	-	-	*	*	*	Function unknown
YLR194C		-	3.07	2.05	2.31	-	-	*	*	*	Function unknown
YBR071W		1.67	2.02	1.59	1.49	-	-	*	*	*	Function unknown
YJL108C	*PRM10*	-	-	1.5	1.62	-	-	*	*	*	Pheromone-regulated protein
YNL058C		1.8	1.78	2.18	2.4	-	-	*	*		Function unknown
YHR097C		1.46	1.86	1.66	1.9	-	-	*	*		Function unknown
YLR121C	*YPS3*	-	1.82	1.63	1.62	-	-	*	*		GPI-anchored aspartic protease
YKL164C	*PIR1*	2.1	2.04	2.37	2.25	-	-		*	*	Structural constituent of cell wall
YJL159W	*PIR2*	2.55	3.8	2.84	2.24	-	-		*	*	Structural constituent of cell wall
YKL163W	*PIR3*	5.44	5.9	4.6	5.56	-	-		*	*	Structural constituent of cell wall
YCL040W	*GLK1*	1.99	1.8	1.52		-	-	*	*		Glucokinase
YHR142W	*CHS7*	-	1.72	1.55	1.68	-	2.9	*	*		Involved in chitin synthesis
YKR013W	*PRY2*	-	1.64	-	1.6	-	-		*		Function unknown
YHR209W		3.23	2.97	2.28	3.24	-	-		*		S-adenosylmethionine-dependent methyltransferase
YDL072C	*YET3*	1.71	1.85	1.52	1.65	-	-	*			Function unknown
YOL052CA	*DDR2*	-	1.56	-	-	-	-			*	Response to stress
YPR030W	*CSR2*	1.51	-	-	-	-	-				Chs5 Spa2 Rescue
YDR483W	*KRE2*	-	-	-	1.67	-	1.5				α-1.2-mannosyltransferase
YBL061C	*SKT5*	-	-	-	1.61	-	1.95				Activator of Chs3p
YPR159W	KRE6	-	-	-	-	-	2.1		*		1,6-β-glucan synthase
YIL146C	*ECM37*	-	-	-	-	-	2.0				Function unknown
YMR062C	*ECM40*	-	-	-	-	-	1.5				acetylornithine acetyltransferase
YGR032W	*GSC2*	-	-	-	-	-	3.3	*	*		1.3-β-glucan synthase
YDR528W	*HLR1*	-	-	-	-	-	0.59				Function unknown
YGR059W	*SPR3*	-	-	-	-	-	0.54				septin
YAL059W	*ECM1*	-	-	-	-	-	0.57				Function unknown

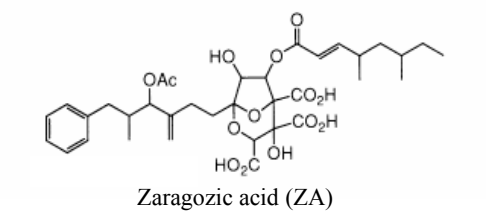
Values correspond to the ratio of expression after 90 minutes of incubation with Congo red (CR1, 20 µg/ml; CR2, 100 µg/ml), Calcofluor white (CFW1, 20 µg/ml; CFW2, 100 µg/ml) and caffeine (CAF1, 2 mM; CAF2, 20 mM). '-' indicates no significant changes in expression. An asterisk indicates activation of these genes in the

independent studies of the yeast cell wall. [1]*CW* - genes upregulated in the following cell wall mutants *fks1*Δ, *kre6*Δ, *mnn9*Δ, *gas1*Δ *and knr4*Δ (Martin-Yken *et al*., 2003); [2]*CR* -genes induced after 2, 4 and 6 hours of treatment with 30 µg/ml CR (Garcia *et al*., 2004); [3]*CFW* -genes induced after 2 hours of treatment with 10 µg/ml CFW (Boorsma *et al*., 2004).

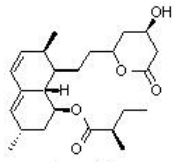
Table A 2: Pathways potentially triggered by CR, CFW, CAF and rapamycin as indicated by the induction of genes regulated by a given transcription factors (TF)

Pathway regulated by a given transcription factor (TF)	*Congo red*		*Calcofluor white*		*Caffeine*		*Rapamycin**	
	%G	%F	%G	%F	%G	%F	%G	%F
Ehrlich pathway (Aro80p)	0	0	1.96	3.3	2.9	16.7	2.4	10.0
Arginine metabolism (Arg80p)	0	0	0	0	1.7	15.8	2.4	15.8
TCA cycle and retrograde pathway (Rtg1/3p)	0	0	0	0	4.1	15.6	6.4	17.8
Nitrogen catabolite repression pathway (Gat1p & Gln3p)	0	0	4.0	7.4	5.8	29.7	26.8	98.4
General stress response (Msn2p & Msn4p)	7.7	1.1	9.8	2.6	14.1	12.9	23.4	15.6
Cell wall remodeling (Rlm1p)	23	10.4	15.7	13.8	1.7	5.2	8.1	17.2
Calcium-calcineurin pathway (Crz1p)	15.3	2.7	13.7	4.8	8.2	9.5	12.9	10.9

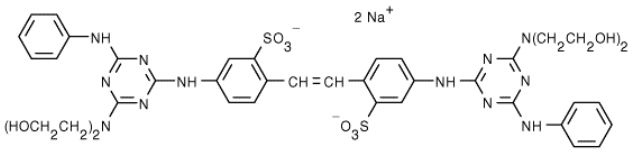
The %G value corresponded to the percentage of genes regulated by a given transcription factor (TF) relative to the number of all upregulated genes in the given conditions. The %F value corresponded to the percentage of genes regulated by a given transcription factor (TF) relative to the number of genes in the whole yeast genome that are regulated by this transcription factor. Only documented (published) regulations were taken into account of this analysis. *Data used for YEASTRACT analysis were from Hardwick *et al.* (Hardwick *et al*., 1999).



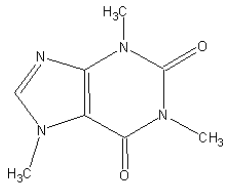
Zaragozic acid (ZA)



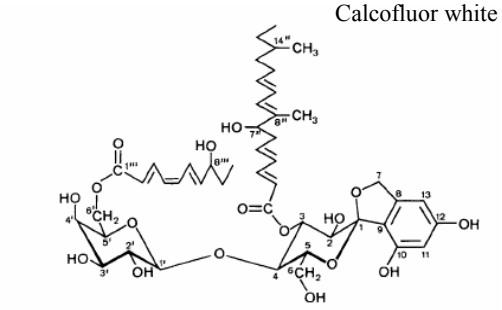
Lovastatin (Lo)



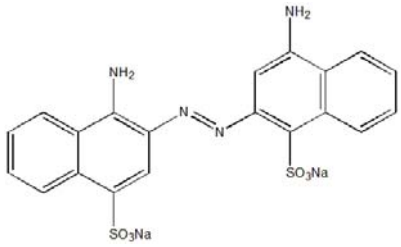
Calcofluor white (CFW)



Caffeine (CAF)



Papulacandin B (PAP)



Congo red (CR)

Figure A 1: 2D structures of the chemical compounds tested in this study. Abbreviations of their names, which were used in this study, are given in parentheses.