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Microcompartmentalization of Cell Wall Integrity Signaling in *Kluyveromyces lactis*

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It always seems impossible

until it´s done

Nelson Mandela

Summary

The yeast cell wall provides a first barrier to the environment, confers shape and stability to the cells, and serves as a model for fungal cell wall biosynthesis and function in general. During normal growth, during mating and upon cell surface stress, new wall synthesis is induced by a conserved signaling cascade, the cell wall integrity (CWI) pathway. A signal is initiated by plasma membrane-spanning sensors and transduced through a mitogen-activated protein kinase (MAPK) cascade, which ultimately activates a transcriptional activator, Rlm1.

The first part of this thesis analyses the role of this MADS-box transcription factor in the milk yeast *Kluyveromyces lactis*, which has not been investigated, until now. With respect to the distribution of the upstream CWI sensors, evidence for the existence of a special plasma membrane microcompartment, generally referred to as eisosomes, in the milk yeast is provided in the second part of the thesis.

Regarding the transcription factor KlRlm1, its impact on the physiology of *K. lactis* seems to be different from its homolog in *Saccharomyces cerevisiae,* ScRlm1, although it clearly acts in CWI signaling, too. Thus, in contrast to the *Scrlm1* mutant, a *Klrlm1* deletion is sensitive, rather than hyper-resistant, towards Congo red and Calcofluor white, typical stress agents used in cell wall research. Data on cross-complementation of the two genes in the respective heterologous yeast indicate that KlRlm1 and ScRlm1 each perform their optimal function only in the native host.

To investigate the impact of a *Klrlm1* deletion on the transcriptional profile of *K.* lactis, data from total mRNA sequencing were analyzed in comparison to a wild-type strain. Many of the genes identified did not correspond to known Rlm1 target genes in *S. cerevisiae*, but many relate to other stress responses (e.g. *KlGRE1*, *KlFMP16*, *KLLA0C05324g*, *KLLA0F18766g, KlUGX2)* and to chitin synthesis (*KlCHS1*, *KlSKT5* and *KlYEA1*), both probably connected to cell wall composition. The functions of a large group of KlRlm1 dependent genes identified here are yet uncharacterized or lack homologs in *S. cerevisiae*.

The plasma membrane of fungi is a specialized organelle, which is ordered into several lateral domains, which we define as microcompartments, since each is composed of a special combination of proteins in their lipid environment. Such microcompartments are believed to control a variety of signaling (and transport) processes in all sorts of eukaryotic cells. Microcompartmentalization is also observed in the yeast plasma membrane, e.g. displayed by the CWI sensors in *K. lactis*, as shown in this thesis. Since distribution of the latter sensors is reminiscent of that of eisosomes, it was also investigated by live-cell fluorescence microscopy, how KlPil1, KlLsp1 and KlSur7 (all homologs of eisosomal proteins in *S. cerevisiae*) are distributed. Since they form the typical membrane patches, which are not present in deletion mutants of *KlPIL1*, the major structural component of eisosomes, one can conclude, that eisosomal microcompartments form in *K. lactis* and are composed similar to their counterparts in *S. cerevisiae.* The CWI sensors are excluded from these structures and form their separate microcompartments. The exact physiological function of eisosomes in fungi is still a matter of debate and future studies in *K. lactis* may help to address this role.

Resumen

La pared celular de levaduras constituye una primera barrera frente al medio, confiere forma y da estabilidad a las células y sirve como modelo para el estudio de la biosíntesis y función de la pared celular de hongos en general. Durante el crecimiento normal, durante la conjugación y en respuesta al estrés celular, se induce la síntesis de nueva pared celular mediante una cascada de señalización conocida como ruta de señalización de integridad de la pared celular (ruta CWI). La señal es recogida por sensores, proteínas que atraviesan la membrana plasmática, y se transduce a través de una cascada de proteínas quinasas activadas por mitógenos (cascada MAPK) que finalmente activan al factor transcripcional Rlm1.

La primera parte de la tesis analiza el papel que desempeña el factor de transcripción MADS-box Rlm1 en la levadura, no caracterizado hasta el momento. En la segunda parte, se estudia la distribución de los sensores de la ruta CWI y se encontraron evidencias de la existencia de un microcompartimento de la membrana citoplasmática, especial conocido como eisosomas, en la levadura de la leche.

En relación al factor de transcripción KlRlm1, su impacto en la fisiología de *K. lactis* parece ser diferente al de su homólogo en *Saccharomyces cerevisiae* ScRlm1, aunque se ha demostrado que también actúa en la ruta de integridad de la pared celular. Así, en contraste al mutante *Scrlm1*, una cepa que tiene el gen *KRLM1* delecionado es sensible, y no hiperresistente, frente a rojo de congo y blanco de caucofluor, dos agente típicos utilizados en investigaciones sobre la pared celular. Los resultados sobre la complementación cruzada de los genes en las respectivas levaduras heterólogas indican que KlRlm1 y ScRlm1 solo pueden realizar su función propiamente en su huésped nativo.

Para investigar el impacto que tiene la deleción del gen *KlRLM1* sobre el perfil transcripcional en *K. lactis*, se secuenció el mRNA total y se compararon los datos con los obtenidos para una cepa silvestre. Muchos de los genes identificados no correspondieron a dianas conocidas para el factor Rlm1 en *S. cerevisiae*, pero están implicados en respuestas a diferentes situaciones de estrés celular (e.g. *KlGRE1*, *KlFMP16*, *KLLA0C05324g*, *KLLA0F18766g, KlUGX2)* y en la síntesis de quitina (*KlCHS1*, *KlSKT5* and *KlYEA1*), ambos grupos relacionados con la estructura de la pared celular. Un amplio grupo de genes dependientes de KlRlm1 identificados en este estudio no tienen homólogo en *S. cerevisiae* o su función aún no ha sido caracterizada.

La membrana plasmática de hongos es un organelo especializado que está ordenado en varios dominios laterales, que nosotros definimos como microcompartimentos, cada uno de ellos formado por una combinación especial de proteínas en el medio lipídico. Se cree que estos microcompartimentos controlan una gran variedad de procesos de señalización y transporte en todo tipo de células eucarióticas. La microcompartimentalización también se observa en la membrana plasmática de las levaduras, por ejemplo la mostrada por los sensores de la ruta CWI en *K. lactis*, según se demuestra en esta tesis. Puesto que la distribución de los sensores CWI recuerda a la de los eisosomes, también se investigó mediante microscopía de fluorescencia en células vivas como se encuentran distribuidas KlPil1, KlLsp1 y KlSur7, homólogas de proteínas eisosomales en *S. cerevisiae*. Se encontró que todas forman gránulos típicos en la membrana, y que estos gránulos no están presentes en mutantes que carecen del gen *KlPIL1* que codifica el componente estructural mayoritario de los eisosomas. Así, se puede concluir que los microcompartimentos formados en *K. lactis* tienen una composición similar a la de los presentes en *S. cerevisiae.* Los sensores CWI no están presentes en estas estructuras sino que forman su propio microcompartimento separado. La función fisiológica que desempeñan los eisosomas en hongos es todavía objeto de debate por lo cual los estudios en *K. lactis* pueden ayudar a dilucidar esta función.

Table of contents

1 Introduction 1

1.1 Microcompartments in the yeast plasma membrane 1

1.1.1 The plasma membrane microcompartment of eisosomes 2

1.1.2 Eisosmomes function in stress response 4

1.2 Regulation of cell wall integrity 4

1.2.1 Composition of the yeast cell wall 4

1.2.2 Cell wall integrity signaling 5

1.2.3 The CWI sensors 8

1.2.4 Rlm1 mediates the expression of cell wall biosynthetic genes 9

1.2.5 CWI cross-talk with other signaling pathways as a stress response 13

1.2.6 *K. lactis* as a model organism in molecular research 15

1.2.7 The cell wall and cell wall integrity signaling of *K. lactis* 16

1.3 Aims of the thesis 18

2 Material and methods 20

2.1 Material 20

2.1.1 Strains used in this work 20

2.1.2 Strain construction in yeast 22

2.1.3 Cultivation and media 24

2.1.4 Vectors and plasmids 25

2.1.5 Oligonucleotides 34

2.2 Methods 39

2.2.1 Mating, sporulation and tetrad analysis 39

2.2.2 Growth analysis by serial drop dilution assay 39

2.2.3 Transformation of *E. coli* cells 40

2.2.4 Transformation of yeast cells using the “freeze” method 40

2.2.5 Transformation of yeast cells with lithium acetate 41

2.2.6 Isolation of DNA 41

2.2.7 Measurement of DNA concentrations 43
2.2.8 Restriction of DNA 43
2.2.9 Dephosphorylation of DNA fragments 43
2.2.10 Agarose gel electrophoresis 43
2.2.11 Extraction of DNA from agarose gels 43
2.2.12 Ligation 44
2.2.13 Sequencing 44
2.2.14 Polymerase chain reaction (PCR) 44
2.2.15 Precipitation of DNA 44
2.2.16 Isolation of RNA 44
2.2.17 Quality control of the RNA sample 45
2.2.18 mRNA sequencing and analysis 45
2.2.19 Yeast cell extract preparation 46
2.2.20 Measuring protein concentrations 46
2.2.21 β-galactosidase activity assay 47
2.2.22 Separation of proteins 47
2.2.23 Western blot analysis 47
2.2.24 Fluorescence microscopy and cell staining 47

3 Results 50
3.1 Characterization of the transcription factor Rlm1 in *K. lactis* 50
3.1.1 KlRlm1 is important to maintain cellular integrity in the milk yeast 50
3.1.2 Cross-complementation analysis of Rlm1 52
3.1.3 Localization studies on KlRlm1 55
3.1.4 Identification of domains conferring species specificity to Rlm1 56
3.1.5 Target genes of Rlm1 in the milk yeast 60
3.2 Distribution of CWI sensors in plasma membrane compartments of *K. lactis* 76
3.2.1 Identification and characterization of the eisosomal microcompartment 76
3.2.2 The CWI sensors are excluded from the eisosomal microcompartment 83

4 Discussion 86
4.1 Characterization of the transcription factor Rlm1 in *K. lactis* 86
4.1.1 Differences in Rlm1 function between *K. lactis* and *S. cerevisiae* 86
4.1.2 Target genes of the transcription factor KlRlm1 89
4.2 Characterization of eisosomes in *K. lactis* 99
4.2.1 Imaging of eisosomes in life cells of *K. lactis* 100
4.2.2 The role of eisosomes in maintenance of cellular integrity 101
4.2.3 The function of eisosomes in plasma membrane microcompartmentalization ..102

5 Conclusions 106
6 References I
7 List of abbreviations XVIII
8 Acknowledgements XX
9 Curriculum vitae XXI
10 Statutory declaration XXIII

1 Introduction

Eukaryotic cells are characterized by the existence of a nucleus and other membrane enclosed organelles, e.g. the endoplasmic reticulum, the Golgi apparatus and mitochondria. This compartmentalization is essential for functional specification and the key to important physiological processes. It also allows for regulatory mechanisms, which are not available to prokaryotes, e.g. the nuclear/cytoplasmic shuffling of transcription factors and other regulatory proteins.

However, the membranes surrounding an organelle do not consist of homogenously distributed proteins and lipids, but are themselves organized into specific microcompartments. Microcompartments, such as respiratory supercomplexes in the inner mitochondria membrane (Vartak *et al.*, 2013), thus are defined as not constituting an entire organelle, but as specific associations of proteins and lipids, which form highly dynamic functional structures that readily change in their spatiotemporal composition.

1.1 Microcompartments in the yeast plasma membrane

The plasma membrane of yeast cells can be divided into different microcompartments, which are defined by the presence of specific proteins and lipids. It has been speculated that this membrane compartmentalization allows the functional segregation of proteins and to be involved in signal transduction, endocytosis or protein and lipid turnover (Ziolkowska *et al.*, 2012). These general distinctions were first made according to the dot-like distribution of the fluorescently labeled arginine transporter Can1 (microcompartment containing Can1 = MCC) and the network-like appearance of the plasma membrane ATPase Pma1 (MPP; (Malinska *et al.*, 2003)). However, a more recent and more detailed analysis revealed the presence of many more microcompartments in the yeast plasma membrane, with proteins showing different degrees of dot-like or network-like distributions (Spira *et al.*, 2012).

These domains vary strongly in size, structure and lifespan. Some microdomains form discrete patches, while some seem to build up network-like structures. Some domains are only found for a few milliseconds, whereas some are highly stable. In this patchwork the

various domains coexist, interact and partially overlap (Olivera-Couto and Aguilar, 2012; Spira *et al.*, 2012). Currently, at least five mechanisms are discussed, which lead to the formation of these microcompartments in eukaryotic plasma membranes: i) preferential association of lipids, so called lipid rafts; ii) lateral compartmentalization by picket fences; iii) homo-, and heterotypic protein-protein interactions; iv) protein-lipid interactions; and v) protein scaffolding (Kusumi *et al.*, 2005; Simons and Sampaio, 2011; Olivera-Couto and Aguilar, 2012).

1.1.1 The plasma membrane microcompartment of eisosomes

Eisosomes constitute one of the first identified microdomains and are suspected to trigger microcompartmentalization of the yeast plasma membrane by providing a scaffold for other proteins. Two similar and highly abundant proteins, Lsp1 and Pil1, that serve a non-redundant function none the less, are mainly responsible for eisosome assembly (Walther *et al.*, 2006). Both are characterized by a BAR domain (Ziolkowska *et al.*, 2012), which was shown to bend the plasma membrane at sides containing high concentrations of PI(4,5)P2 (Kamble *et al.*, 2011; Karotki *et al.*, 2011). By that, specific, immobile structures of uniform size form: furrow-like plasma membrane invaginations that are about 300 nm long, 150-250 nm deep and 50 nm wide (Stradalova *et al.*, 2009). The curvature of the membrane is thought to promote the clustering of proteins and lipids, thus forming specialized microcompartments within the plasma membrane (Malinska *et al.*, 2004; Olivera-Couto *et al.*, 2011). Eisosomes are now thought to trigger the formation of the MCC (for **m**embrane **c**ompartment occupied by the arginine H^+-symporter **C**an1 (Malinska *et al.*, 2003; Karotki *et al.*, 2011). Proteins that have been identified in eisosomes are listed in Table 1.

Although structure, assembly and composition of eisosomes and the MCC were studied intensively, the functional significance for the cells is still dubious. Eisosomes were suspected to mark the sides of endocytosis (Walther *et al.*, 2006) and Pil1 has been proclaimed to improve endocytosis by recruiting endocytic proteins (Murphy *et al.*, 2011). Moreover, defects in endocytosis were described in mutants lacking eisosomes (Moreira *et al.*, 2009). In contrast, recent studies show that the MCC precludes endocytosis and

rather constitutes a protective microcompartment within the plasma membrane. In this area turnover of some proteins (e.g. of the permeases Can1 and Tat2) is in fact inhibited by eisosomal membrane curvature (Grossmann *et al.*, 2008). And yet others argue that microcompartmentalization does not influence vesicular trafficking in yeast at all (Brach *et al.*, 2011; Moreira *et al.*, 2012). The authors found that the selection of endocytic sites occurred independent of the MCC and endocytosis of the permeases Can1 and Tat2 was not reduced by targeting the proteins to the MCC.

Table 1: Eisosomal proteins of *S. cerevisiae*. The proteins were identified by affinity chromatography, mass spectrometry and colocalization (Grossmann *et al.*, 2008). *K. lactis* homologs are listed if homologs could be identified in the genome.

Name	Function	Homolog in *K. lactis*
Fmp45	Sporulation; sphingolipid maintenaince	*KLLA0F20900g*
Ynl194c	Paralog of Fmp45	No
Sur7	Sporulation; sphingolipid maintenaince	*KLLA0A08184g*
Nce102	Protein secretion	*KLLA0D16280g*
Fhn1	Paralog of Nce102	No
Can1	Plasma membrane arginine permease	*KLLA0C02343g*
Tat2	Tryptophan and tyrosine permease	*KLLA0A10813g*
Fur4	Uracil permease	*KLLA0D03454g*
Pun1	Stress response	No
Pil1	Primary protein component of eisosomes	*KLLA0F08162g*
Lsp1	Primary component of eisosomes	*KLLA0E14411g*
Eis1	Proper eisosome assembly	*KLLA0C12573g*
Seg2	Paralog of Eis1	*KLLA0F13618g*
Seg1	Eisosome assembly and shape	No
Mdg1	Pheromone signaling	*KLLA0D08184g*
Pkh1	Serine/threonine protein kinase	*KLLA0E03587g*
Pkh2	Serine/threonine protein kinase	No
Pst2	Stress response	No
Rfs1	Paralog of Pst2	*KLLA0F04323g*
Ycp4	Unknown	*KLLA0F12782g*
Slm1	Phosphoinositide PI4,5P(2) binding protein, stress response	*KLLA0F03839g*
Slm2	Paralog of Slm1, predicted similar function	No
Ygr130c	Unknown	*KLLA0D16236g*

1.1.2 Eisosmomes function in stress response

A putative function for eisosomes is the response to environmental changes and stress. Thus, several eisosomal proteins have been identified to play a role in stress response before (see Table 1). The amount and distribution of eisosomes, and consequently of the MCC, change in response to alterations of the medium, e. g. in the presence of glycerol or fatty acids (Jung *et al.*, 2013).

Strains carrying deletions of *PIL1* and *LSP1* were found to be hyperresistant towards heat stress and the MAP kinase Mpk1, which mediates cell wall integrity signaling, is hyperphosphorylated in these mutants (Luo *et al.*, 2008). Furthermore, Pil1 is a target of the protein kinases Pkh1 and Pkh2 (Zhang *et al.*, 2004), which were previously reported to fulfil a function in endocytosis (Friant *et al.*, 2001) and cell wall integrity signaling. It remains unclear though, if they act in parallel to the CWI pathway or upstream of it (Roelants *et al.*, 2002). A possible direct link of Pil1 phosphorylation by these two kinases, which is important to control eisosome assembly and shape (Walther *et al.*, 2007), to cell wall maintenance has not yet been investigated.

1.2 Regulation of cell wall integrity

The cell wall of fungi is essential to determine cell shape and to ensure cellular integrity during growth, morphogenesis and under stress conditions. It is a unique fungal structure that is essential for the survival of the cells and therefore provides a prominent antifungal target (Heinisch, 2005).

1.2.1 Composition of the yeast cell wall

The cell wall of *Saccharomyces cerevisiae* is a layered structure that is composed of β-1,3-glucan, β-1,6-glucan, chitin and mannoproteins (Cabib *et al.*, 1982; Lipke and Ovalle, 1998; Molina *et al.*, 2000; Klis *et al.*, 2002). All components are cross-linked in various ways (Kollar *et al.*, 1997; Orlean, 2012) and the nature and amount of cross-linking was shown to be altered upon cell wall stress. Glucans make up the largest part of the inner cell wall layer, whereas chitin constitutes only 1-2 % of the cell wall polysaccharides under

normal growth conditions. Chitin accumulates in the bud scars and at the bud neck, but some polymer is present within the lateral wall as well (Molano *et al.*, 1980). Interestingly, the overall chitin level was shown to increase to about 20% under some stress conditions (Valdivieso *et al.*, 2000). The long β-1,3-glucan chains (approximately 6000 sugar residues) are primary responsible for elasticity and strength of the cell wall (Lesage and Bussey, 2006). In contrast, the rather short β-1,6-glucans (200-500 residues) act as a flexible linker, being connected to β-1,3-glucan, chitin and the CWPs (Kollar *et al.*, 1997).

Mannoproteins, which are formed by highly glycosylated polypeptides (Lipke and Ovalle, 1998), form the outer layer of the cell wall. There are generally two classes of cell wall proteins (CWPs): GPI-dependent cell wall proteins and PIR proteins, which are directly attached to β-1,3-glucan (Toh-e *et al.*, 1993; Kapteyn *et al.*, 1999).

A thickness of about 100 to 115 nm has been determined for the *S. cerevisiae* cell wall under standard growth conditions (rich medium; logarithmically grown cells) (Backhaus *et al.*, 2010; Dupres *et al.*, 2010). The general thickness, the relative thicknesses of the two layers, the organization and the composition of the cell wall can differ depending on the growth conditions and in cell wall mutants. These characteristics also depend on the cell cycle and the growth phase and can be altered upon cell wall stress by hypoosmolarity, heat or drug treatment (Free, 2013).

1.2.2 Cell wall integrity signaling

The adaption to different growth conditions and a proper response to cell wall stress are mediated by the cell wall integrity (CWI) signaling pathway (Figure 1). Activation of this pathway starts a compensatory mechanism that ultimately leads to an alteration in gene expression and remodeling with concurrent strengthening of the cell wall.

A set of plasma membrane spanning sensors detects cell surface stress, which affects either the cell wall or the plasma membrane. *S. cerevisiae* disposes of five plasma membrane-spanning sensors: Wsc1, Wsc2, Wsc3, Mid2 and Mtl1 (Ketela *et al.*, 1999; Lodder *et al.*, 1999; Rajavel *et al.*, 1999; Zu *et al.*, 2001). These CWI sensors are thought to activate the small GTPase Rho1 (for **R**as **ho**molog) by stimulating it´s GDP to GTP nucleotide exchange via guanine nucleotide exchange factors (GEFs). Rom2 was described

as the major GEF for Rho1 activation in the context of cell wall integrity (Ozaki *et al.*, 1996; Lorberg *et al.*, 2001a; Philip and Levin, 2001), while the Rom2 paralog Rom1 only plays a minor role. The Rho1-GEF Tus1 seems to be not involved in CWI signaling (Krause *et al.*, 2012; Wittland, 2012). The GPTase activating proteins (GAPs) Bem2, Sac7 and Lrg1 act as Rom2 antagonists in the process, returning Rho1 to the inactive, GDP-bound, state (Lorberg *et al.*, 2001b; Schmidt *et al.*, 2002).

Rho1 is a member of the family of Ras-like GTPases (Yamochi *et al.*, 1994). Activation of the small GTPase has various effects. It can lead to the reorganization of the actin cytoskeleton (Madden and Snyder, 1998) by regulating the formin proteins Bni1 and Bnr1 (Evangelista *et al.*, 2003). Rho1 is also involved directly in establishing cell polarity by recruiting Sec3 to the exocytic sites during polarized growth (Zhang *et al.*, 2008).

The GTPase Rho1 can also control cell wall synthesis as a regulatory subunit of the β-1,3-glucan synthase Fks1 (Mazur and Baginsky, 1996) and may be critical for β-1,6-glucan synthesis (Levin, 2011). Furthermore, Rho1 physically interacts with the protein kinase C (Pkc1) in cell wall integrity signaling to trigger activation of the Map kinase cascade (Nonaka *et al.*, 1995; Schmitz *et al.*, 2002b). Depending on these various functions, Rho1 localizes to different sites during the cell cycle (e. g. to the bud neck) (Abe *et al.*, 2003; Yoshida *et al.*, 2009). Owing to this localization and to the presence of different regulators described above, presumably only a subset of all Rho1 targets is activated at each phase.

The Rho1 effector Pkc1 controls the expression of cell cycle-dependent genes (Darieva *et al.*, 2012), is involved in ribosomal biogenesis (Mitjana *et al.*, 2011), mediates the response to oxidative stress (Vilella *et al.*, 2005) and regulates actin dynamics (Schmitz *et al.*, 2002a).

In response to cell wall stress, the protein kinase C activates signaling through a highly conserved MAPK (**m**itogen-**a**ctivated **p**rotein **k**inase) cascade that comprises the MAPKKK Bck1, the two redundant MAPKKs Mkk1 and Mkk2 and the MAPK Mpk1 (Lee and Levin, 1992; Irie *et al.*, 1993; Lee *et al.*, 1993; Mazzoni and Mann, 1993; Kamada *et al.*, 1995; Heinisch *et al.*, 1999; Jimenez-Sanchez *et al.*, 2007). Mpk1 is also called Slt2 according to its first isolation as a suppressor mutant of a lytic phenotype (Torres *et al.*, 1991), but the

former designation will be used throughout this thesis. Phosphorylated Mpk1 is transported to the nucleus by the karyopherins Kap60 and Kap95. This leads to the nuclear accumulation of this CWI pathway member (Martinez-Bono *et al.*, 2010) and triggers the transcriptional response to cell wall stress, mainly via the transcription factor Rlm1 (Jung *et al.*, 2002).

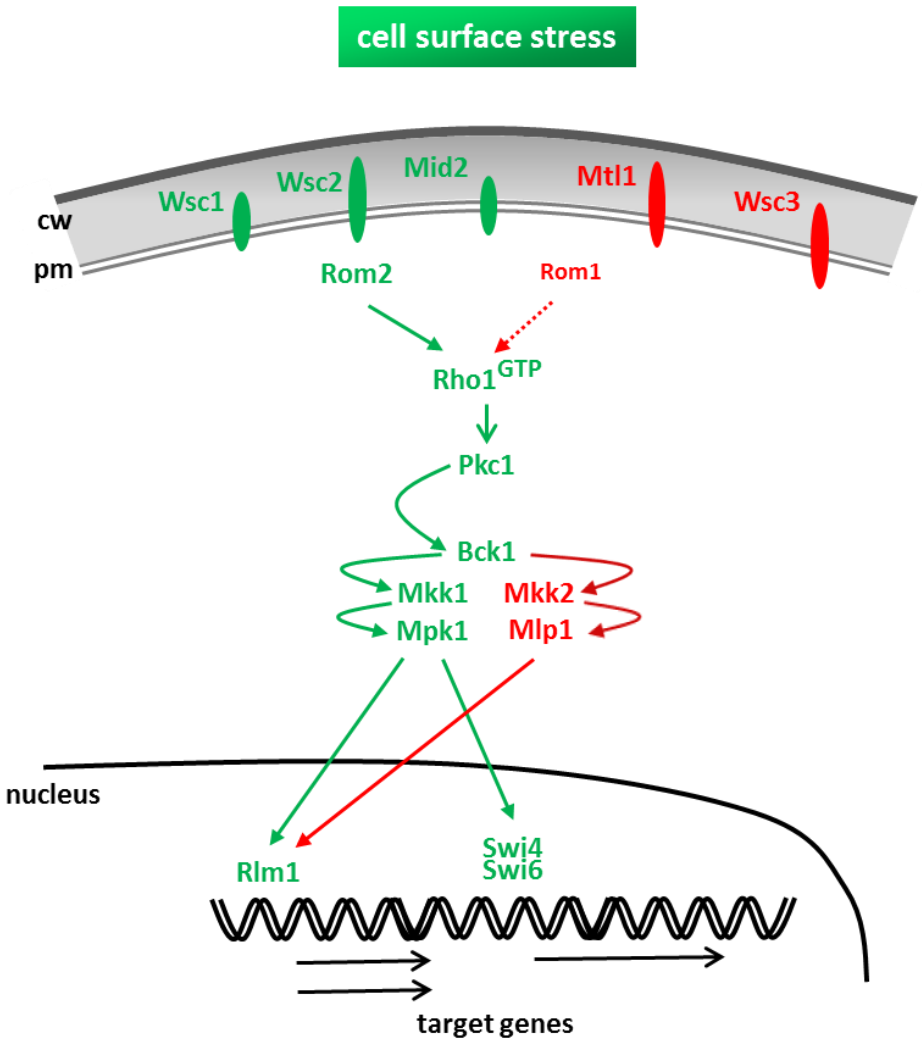


Figure 1: Schematic overview of the cell wall integrity signaling pathway of *K. lactis* and *S. cerevisiae*. Components of the cell wall integrity pathway of *K. lactis* are shown in green. The proteins of the signaling cascade shown in red are not present in *K. lactis*, but are involved in CWI signaling in *S. cerevisiae*. Arrows indicate activation of components. See text for details. cw≙ cell wall, pm≙ plasma membrane.

Deletion mutants of any of the cell wall integrity pathway components, with the exception of Rlm1, show cell lysis at least at elevated temperatures (37°C). Some, like the *pkc1*

deletion mutant, are unable to grow without osmotic stabilization, e.g. by addition of 1M sorbitol to the medium (Jacoby *et al.*, 1997). Moreover, the mutants display an increased sensitivity to cell wall perturbing agents like Calcofluor white, Congo red and caspofungin. Calcofluor white and Congo red bind to chitin in the cell wall (Elorza *et al.*, 1983; Imai *et al.*, 2005), which affects its composition. Consequently, an increased rate of chitin polymerization was measured in the presence of either cell wall stressor (Roncero and Duran, 1985). In contrast, caspofungin is a noncompetitive inhibitor of both β-1,3-glucan synthases (Fks1 and Fks2) (Reinoso-Martin *et al.*, 2003), and thus dramatically impairs glucan synthesis (Marco *et al.*, 1998). Walls of cells treated with caspofungin were recently shown to contain increased chitin and decreased β-glucan levels (Formosa *et al.*, 2013). The proper transcriptional response to all three stressors is largely dependent on the transcription factor Rlm1.

1.2.3 The CWI sensors

Five sensors were described to be involved in detection of cell wall stress in the budding yeast. Comparing their primary sequences, all sensors contain a signal peptide for secretion, a single transmembrane domain, a relatively short cytoplasmic tail and an extracellular region, which makes up the largest part of the protein (Jendretzki *et al.*, 2011). This part comprises serine and threonine rich regions which are highly O-mannosylated (Philip and Levin, 2001; Lommel *et al.*, 2004). The extracellular region is presumably connected to other cell wall components (Lodder *et al.*, 1999; Hutzler *et al.*, 2008). Despite this overall structural similarity, the sensors divide into two subgroups: Wsc-type and Mid-type (Rodicio and Heinisch, 2010). These two subfamilies are discriminated by a cysteine-rich domain near the N-terminal end of the Wsc-type sensors and a N-glycosylated asparagine residue near the N-terminal end of the Mid-type sensors. Among the five sensors, Wsc1 and Mid2 serve the most prominent function in cell wall integrity signaling, since the corresponding deletion mutants display the strongest phenotypes upon cell wall stress (Rodicio and Heinisch, 2010). Because of their anchorage in the cell wall as well as in the plasma membrane, the sensors are thought to work as a mechanosensing device (Heinisch *et al.*, 2010b; Jendretzki *et al.*, 2011). Current research

from our lab indicates that the sensor Wsc1 of the cell wall integrity signaling pathway forms its own microcompartment (Kock, personal communication). Previously, fluorescence microscopy already revealed that the sensor Wsc1 forms discrete patches within the plasma membrane (Verna *et al.*, 1997; Straede and Heinisch, 2007). Furthermore, by atomic force microscopy of single sensor molecules the Wsc1 sensor was shown to cluster into discrete microcompartments within the plasma membrane under stress conditions (Dupres *et al.*, 2009; Heinisch and Dufrene, 2010; Merzendorfer and Heinisch, 2013). Altering the cysteine rich domain of Wsc1, destroyed the capacity of the sensor for clustering, as well as its signaling function (Heinisch *et al.*, 2010b). Thus, microcompartmentalization appears to be fundamental for CWI signaling.

1.2.4 Rlm1 mediates the expression of cell wall biosynthetic genes

Activation of the cell wall integrity pathway finally results in the activation of the MADS-box transcription factor Rlm1.

Rlm1 stands for **r**esistance to **l**ethality of **M**KK1^{P386} over expression and was first described in a screen for suppressors of a growth defect caused by a constitutively active Mkk1 mutant (Watanabe *et al.*, 1995). Rlm1 was found to be a member of the group of MADS-box (**M**CM1, **A**gamous, **D**eficiens, **S**erum response factor) transcription factors. This family includes several hundred proteins from various eukaryotic species, where they are involved in signal transduction and developmental control (West *et al.*, 1997; Gramzow *et al.*, 2010). MADS-box proteins are characterized by a conserved sequence of ~60 amino acids, the so called MADS-domain. The N-terminal and central parts of the domain trigger the DNA binding; the C-terminus contributes to protein dimerization (Pellegrini *et al.*, 1995; Huang *et al.*, 2000). Despite numerous studies on MADS-box transcription factors, the exact mode of recognizing the DNA-target site, $(CC(A/T)_6GG$, also referred to as CArG-box (Sharrocks *et al.*, 1993)) and the mechanisms that ensure DNA-binding specificity, are not well understood.

In plant cells more than one hundred different MADS-box transcription factors are known alongside the name giving Agamous (Schwarz-Sommer *et al.*, 1990) and Deficiens (Sommer *et al.*, 1990). They play prominent roles in the floral organ specification and in

the morphogenesis of almost all organs throughout the plant life cycle (Smaczniak *et al.*, 2012; Muino *et al.*, 2013). In animals, this family of transcription factors is also involved in the regulation of cell-differentiation processes. One member, the serum response factor, plays a role in cell adhesion and migration (Schratt *et al.*, 2002). It also has been shown to be required for terminal differentiation of skeletal, cardiac and smooth muscle cells (Miano, 2010) and to regulate the expression of actin-cytoskeleton-related genes (Miano *et al.*, 2007).

In the baker´s yeast *S. cerevisiae* four MADS-box transcription factors are known: Mcm1, Arg80, Smp1 and Rlm1. Mcm1 participates in the regulation of pheromone-induced gene expression, metabolism (Treisman and Ammerer, 1992) and DNA replication (Chang *et al.*, 2004), whereas Arg80 is involved in regulating the expression of arginine-responsive genes (Dubois *et al.*, 1987). Smp1 regulates the response to high osmolarity in *S. cerevisiae* and is a paralog of Rlm1 that probably arose from the whole genome duplication de (Nadal *et al.*, 2003).

Rlm1 is phosphorylated upon activation of the CWI pathway (Dodou and Treisman, 1997). The MAP kinase Mpk1 localizes to the nucleus (Huh *et al.*, 2003) and phosphorylates Rlm1 at three specific sites (S374, Ser427 and Thr439), with phosphorylation at S374 apparently lacking functional consequences (Jung *et al.*, 2002). Phosphorylated Rlm1 is believed to be active and to regulate the transcription of several target genes, by binding their promoter regions at the consensus sequence $TA(A/T)_4TAG$ (Jung and Levin, 1999). There, Rlm1 shows a complex interaction with the SWI/SNF chromatin-remodeling machinery: binding of Rlm1 at one promoter site recruits the SWI/SNF complex, whereas binding of the later then recruits phosphorylated Rlm1 to another binding site, altogether facilitating transcription of the downstream genes (Sanz *et al.*, 2012). The role of the SAGA histone-modifying complex in this process is currently being investigated (Sanz, personal communication).

Among the genes regulated by Rlm1 are several genes related to cell wall biosynthesis and composition, which include those encoding PIR proteins and GPI-anchor proteins, as well as proteins involved in chitin synthesis and the glucan synthase encoding genes. Both PIR proteins and GPI-anchor proteins are components of the outer cell wall layer of

mannoproteins and crucial for cell wall integrity. Apart from that, many genes related to the metabolism of carbohydrates, amino acids and sphingolipids were identified, as well as genes required for the generation of energy (Jung and Levin, 1999; Boorsma *et al.*, 2004; Garcia, 2004). Some contradictory results were obtained, since different groups applied different stresses to activate the CWI pathway and used different approaches to monitor the cellular response. It is therefore difficult to compare these data, especially since different cross-talks between signaling pathways may occur and the concept of a linear signaling chain may be misleading in connecting a specific stressor to a given response (Garcia *et al.*, 2009).

In 1999, Jung and Levin first identified 23 genes that were regulated by Rlm1 upon activation through a constitutively active $MKK1^{S386P}$ allele (Jung and Levin, 1999). In later studies expression of several of these genes was confirmed to be controlled by Rlm1. Thus, Boorsma and colleagues presented the transcription profiles of cells treated with both Calcofluor white and Zymolyase, a lytic enzyme mix, that hydrolyzes the β-1,3 glucan network of the cell wall (1). They found 52 genes with upregulated transcription under stress conditions. The genes were clustered into five groups: stress response, cell wall maintenance, osmosensing, carbohydrate utilization and cell growth. While these genes were detected after Calcofluor white dependent stress, only half of them respond to Zymolyase treatment. Generally, transcriptional activation of genes after Zymolyase stress appeared to be less powerful. Interestingly, the results point to the involvement of the high osmolarity glyerol (HOG) pathway, in response to Zymolyase treatment. (Boorsma *et al.*, 2004). In contrast, cell wall perturbations triggered by Congo red resulted in a transcriptional response that exclusively depends on the MAP Kinase Mpk1 and Rlm1 (Garcia, 2004).

The involvement of the HOG pathway in response to cell wall stress caused by Zymolyase was also confirmed by the group of Arroyo, demonstrating that Zymolyase activates both MAP kinases: Hog1 and Mpk1. This response was shown to require the Sho-branch of the HOG pathway (Bermejo *et al.*, 2008). The connection between the HOG- and the CWI pathway as a response to Zymolyase stress was also confirmed by transcriptional profiling,

in which the bulk of transcriptional regulation was still Rlm1 dependent (Garcia *et al.*, 2009).

Interestingly, unlike other mutants of the CWI pathway, *S. cerevisiae* cells deleted for *RLM1* grow normally, even at elevated temperatures (Watanabe *et al.*, 1995) and are less sensitive to caspofungin stress. On the other hand, *rlm1* deletion mutants show an increased resistance to Calcofluor white, Congo red and towards treatment with Zymolyase (Watanabe *et al.*, 1995; Lopez-Garcia *et al.*, 2010), in contrast to the expected hypersensitive phenotype. This indicates that Rlm1 does not exclusively act on cellular integrity, or, alternatively, the presence of a protein with overlapping function. Thus, additional transcription factors may be involved. The paralog Smp1, which displays 76 % amino acid identity to Rlm1 (within the MADS-box even 89 % identity, theoretically would to be a good candidate. However, a *smp1* deletion mutant is neither sensitive to Calcofluor white, caffeine or Zymolyase nor does it enhance the phenotypes of the *rlm1* null mutant in this respect (Dodou and Treisman, 1997).

Another well-known downstream target of the cell wall integrity pathway is the SBF complex, composed of the proteins Swi4 and Swi6 (Andrews and Moore, 1992). The SBF complex is a regulator of cell cycle progression and activates the transcription of genes specific for the late G1 phase (Andrews and Herskowitz, 1989; Breeden, 2003), The SBF complex has been described to be involved in CWI signaling as a direct target of Mpk1 as well (Madden *et al.*, 1997; Baetz *et al.*, 2001). It induces the transcription of four cell wall specific genes: *FKS2*, *CHA1, YLR042c* and *YKR013w* (Kim and Levin, 2010). However, deletion mutants of any of these genes do not exhibit sensitivity to cell wall stress. Only two out of the four genes have been characterized in detail; the transcriptional regulation of the *FKS2* gene is quite complex, depending on the calcineurin pathway and on the Mig1 transcription factor in glucose signaling (Zhao *et al.*, 1998). Moreover, the glucan synthase Fks2 has a functional homolog in Fks1, which has been described to be activated by Rho1 (Mazur and Baginsky, 1996). More target genes for Swi4-Swi6-Mpk1 have not yet been identified. The promoter binding sites for the SBF complex in response to cell wall stress vary from those recognized during cell cycle regulation, though the core motif of seven nucleotides is conserved in both cases (Kim *et al.*, 2008). Furthermore, the transcriptional

response mediated by the cell wall integrity pathway upon caspofungin treatment required Rlm1- but not Swi4 (Reinoso-Martin *et al.*, 2003), indicating, that the SBF complex plays only a minor role in cell wall stress response.

In summary, neither the data on Smp1 nor those on the SBF complex provide an explanation for the observed lack of a strong phenotype of the *rlm1* deletion in *S. cerevisiae*.

1.2.5 CWI cross-talk with other signaling pathways as a stress response

A broad variety of different stresses leads to the activation of the cell wall integrity pathway: changes of temperature, pH and osmolarity, oxidative stress, nutrient limitations and chemical agents. To adequately respond to all these kinds of different stimuli, the CWI pathway clearly has to receive lateral inputss and cannot function in a downright linear manner (Harrison *et al.*, 2004). Cross-talk with other signaling pathways helps in fine tuning the proper cellular response and amplifies the signaling capabilities of the CWI pathway.

A prominent example for this is the previously mentioned cross-talk of the, in principal opposing, HOG and CWI pathways. Zymolyase treatment of cells resulted in a transcriptional response that depended to large extend on both pathways (Garcia *et al.*, 2009). Further it was shown, that transcription of the *MPK1/SLT2* gene under hyperosmotic conditions was controlled by the Rlm1 transcription factor and the Hog1 kinase (Hahn and Thiele, 2002) and osmotic solutes like sorbitol, sodium chloride, or glucose lead to the phosphorylation of Mpk1 (Davenport *et al.*, 1995). A third stress that has been described to activate both pathways is heat shock. The response to elevated temperatures is mainly mediated by the CWI pathway, but the Hog1 MAPK was also reported to be activated by heat stress (Winkler *et al.*, 2002). The phosphatases Sgp1, Msg5, Ptp2, Ptp3, which can inactivate both MAP kinases (WurglerMurphy *et al.*, 1997; Mattison *et al.*, 1999), have been suggested to be the key players in control of the cross-talk. Recently it was reported, that the two protein kinases Rck1 and Rck2 are involved in this process as well (Chang *et al.*, 2013). Yet the specific roles of the phosphatases remain

unclear, especially how they mediate the cross-talk while maintaining specificity of each MAPK signaling pathway at the same time (Winkler *et al.*, 2002).

Oxidative stress has been claimed to activate the protein kinases Hog1, Mpk1 and Fus1 (Staleva *et al.*, 2004). Activation and cross-talk between the three pathways is thought to provide additional defense (Fuchs and Mylonakis, 2009). In contrast, another work suggested that the cellular response to oxidative stress does only require the sensors Wsc1 and Mid2 as well as Rom2 and Pkc1, but not the other CWI pathway components (Vilella *et al.*, 2005). Mtl1 has also been described to play a role (Petkova *et al.*, 2010), so only the upper part of the CWI pathway seems to be required in this model, too.

The CWI pathway also enables tolerance to changes in the pH value. Sensing of alkaline conditions depends on Wsc1, but did not require Rlm1 or the HOG pathway (Serrano *et al.*, 2006). On the other hand, Mid2 mediates the response to acidic conditions, a process resulting in activation of Rlm1 and enhanced by the phosphatase Rgd1 (Claret *et al.*, 2005). Consequently, *RGD1* expression upon acidic growth conditions is Hog1 dependent (Gatti *et al.*, 2005). Again, this indicates putative cross-talk of the HOG and CWI pathway.

Regarding the cross-talk with other signaling pathways, Mpk1 activation has been shown to stimulate the influx of calcium through the plasma membrane via a Cch1-Mid1 channel, which triggers activation of the calcineurin pathway (Garrettengele *et al.*, 1995; Chen and Thorner, 2007). Moreover, the CWI sensor Wsc1 mediates the cross-talk of the cAMP/PKA-pathway and the CWI pathway as a heat stock response (Thevelein and de Winde, 1999; Fuchs and Mylonakis, 2009). Additionally Wsc1 is thought to control the GPTases Rho3 and Rho4 via Rgd1 (Fernandes *et al.*, 2006) under some stress conditions. An indirect connection between the central carbohydrate metabolism and cell wall biosynthesis is also indicated by the sensitivity of mutants towards cell wall stress agents, which are defective in the SNF1 complex, a trimeric AMP kinase complex, commonly thought to mediate glucose signaling (Backhaus *et al.*, 2013).

Connections of the CWI pathway and the TOR signaling pathway were found to be dependent on Rom2 (Schmidt *et al.*, 1997; Torres *et al.*, 2002). Whether this is a direct or indirect influence is still matter of debate. Caffeine, a drug that also induces the CWI pathway (Martin *et al.*, 2000; Levin, 2011) and is frequently used on CWI pathway

mutants, has been confirmed to act in a TOR signaling dependent manner (Kuranda *et al.*, 2006). In contrast to treatment with Calcofluor white and Congo red, cell wall remodeling of caffeine treated cells was independent of the CWI pathway and Rlm1, suggesting that CWI activation is just a side-effect of caffeine stress and not important for survival of the cells (Kuranda *et al.*, 2006).

In summary, the cell wall integrity signaling pathway is not activated in a linear, uniform manner, but is embedded in a complicated signaling network. This provides the necessary flexibility to react to diverse stimuli and challenges. Consequently, the cellular and finally the transcriptional response vary depending on the stress applied.

1.2.6 *K. lactis* as a model organism in molecular research

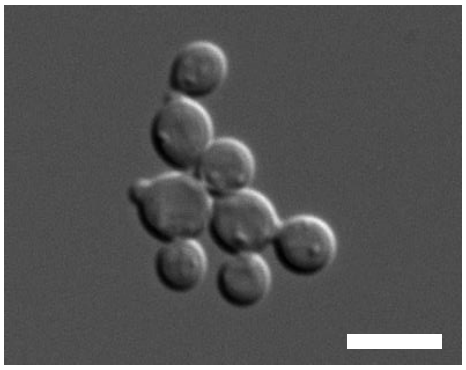


Figure 2: Bight field microscopy image of *K. lactis* wild typ cells. Scale bar 10nm.

The milk yeast *K. lactis* belongs to the ascomycetous yeasts. Like it´s well studied relative *S. cerevisiae, K. lactis* is a popular tool in industrial applications and in current research. The milk yeast is commercially used for heterologous protein production (Morlino *et al.*, 1999; van Ooyen *et al.*, 2006), for example to obtain pure chymosin in a cost-effective manner. The protease chymosin is largely used in cheese making (2). Because of its ability to utilize lactose (Webster and Dickson, 1988), can be employed to remove lactose from milk for the production of lactose free foods (Rodicio and Heinisch, 2013).

Several characteristics make *K. lactis* an useful asset in research as well (Schaffrath and Breunig, 2000): A varity of molecular techniques are available and in most cases the handling is similar to the well-known of *S. cerevisiae*. The genome has been sequenced (Dujon *et al.*, 2004; Sherman *et al.*, 2004) and a congenic strain series was constructed in our laboratory, which improves the comparison of results with less genetic heterogeneities as opposed to the use of different laboratory strains (Heinisch *et al.*, 2010a).

Unlike the baker´s yeast, *K. lactis* is Crabtree-negative (Tarrio *et al.*, 2006) and is not able to grow under anaerobic conditions, though it has the ability to ferment (Snoek and Steensma, 2006). The biggest advantage of *K. lactis* over *S. cerevisiae* is the lower gene redundancy, since it did not undergo the whole genome duplication, in contrast to *S. cerevisiae* (Wolfe and Shields, 1997). That makes *K. lactis* especially attractive in science, because physiological and genetic research becomes simplified and especially in CWI signaling cross-talk with other pathways may be easier to understand.

1.2.7 The cell wall and cell wall integrity signaling of *K. lactis*

The cell wall of *K. lactis* is about 40% thinner under normal growth conditions, compared to the one of *S. cerevisiae*. Yet, the overall structure does not show remarkable differences as the typical inner and outer layer can be observed in transmission electron micrographs (Uccelletti *et al.*, 2000; Backhaus *et al.*, 2010). The general composition of the *K. lactis* cell wall also appeared to be similar to the one of *S. cerevisiae* in a first study (Nguyen *et al.*, 1998). Later, detailed analyses of the polysaccharide content of the *K. lactis* cell wall under different growth conditions confirmed that there are no major differences to *S. cerevisiae*. The glucan to mannan ratio was a little higher in *K. lactis* when the cells reached stationary phase or when they were grown on ethanol, but the chitin levels were alike in both organisms under all tested conditions (Backhaus *et al.*, 2010). The proteome of the *K. lactis* cell wall showed no drastic differences between the two organisms either. Several characteristic proteins for the cell wall with homologs in *S. cerevisiae* were found. Among those, GPI proteins form the largest group.

Like in *S. cerevisiae* the composition and thickness of the *K. lactis* cell wall is altered upon changes of the growth conditions (Backhaus *et al.*, 2010). The control of those changes in *K. lactis* also seems to rely on a cell wall integrity signaling pathway. Elementary function and components of the CWI pathway are generally very similar in *K. lactis* and *S. cerevisiae* (Figure 1). Homologs for most of the CWI pathway components have been identified, but there are various species-specific differences and some yet largely unknown features (Backhaus *et al.*, 2011; Rodicio and Heinisch, 2013).

As mentioned before, *K. lactis* is characterized by a smaller redundancy of proteins since it did not duplicate its genome during evolution. Therefore, only three CWI integrity sensors could be identified in *K. lactis*, as compared to five in *S. cerevisiae* (Rodicio *et al.*, 2008). They were named after their corresponding *S. cerevisiae* homologs: KlMid2 (41% identity in the amino acid sequence), KlWsc1 (39% amino acid identity), and KlWSC2/3, which shows similarity to both ScWsc2 (35% identity) and ScWsc3 (32% identity). Localization studies by fluorescence microscopy of these cell wall integrity revealed microcompartmentalization of the sensors within the plasma membrane of *K. lactis* (Rodicio *et al.*, 2008).

KlRom2 has been identified as the essential GEF for the GTPase KlRho1, a deletion of either *KlROM2* or *KlRHO1* is lethal (Lorberg *et al.*, 2003). Cross-complementation analyses showed that KlRom2 is functionally equivalent to its *S. cerevisiae* homolog. This is also true for the KlRho1 and KlPkc1 proteins (Rodicio *et al.*, 2006). The functionality of the MAPKKK KlBck1 is conserved among the two species as well and proteins of both organisms display a high degree of similarity. However, cells deleted for *KlBCK1* do not show strong phenotypes upon treatment with cell wall stress agents (Jacoby *et al.*, 1999). Consequently, the MAPKKK seems to play a less important role in *K. lactis*. Surprisingly, deletion of the genes encoding the sole CWI MAPKK of *K. lactis* Kl*MKK1* (unpublished results from this laboratory; Maria Diaz Garcia, 2011) or the MAPK Kl*MPK1* (Kirchrath *et al.*, 2000) display both a stronger phenotype then the deletion of the gene for the upstream kinase Kl*BCK1.* Kl*mkk1* as well as *Klmpk1* deletions are sensitive to various cell wall stresses. All functional domains of KlMpk1 are highly conserved and an increased KlMpk1 phosphorylation was demonstrated after heat shock (Kirchrath *et al.*, 2000). The Mpk1-like protein kinase Mlp1 that acts in parallel to Mpk1 in *S. cerevisiae* apparently lacks a homolog in the *K. lactis* genome. The characterization of the presumed target of KlMpk1, the transcription factor KlRlm1 is the subject of this thesis.

Although the overall organization of the cell wall integrity pathway has been proven to be conserved in *K. lactis*, a variety of differences between *S cerevisiae* and *K lactis* are present, especially regarding the higher simplicity and linearity in case of *K. lactis*. Even though some phenotypes of deletion mutants of CWI pathway components remain

contradictory, further investigation on the function of this signaling cascade in *K. lactis* should be rewarding, especially in terms of putative target genes and the cross-talk with other signaling pathways.

Besides these studies, the MAPK module of the pheromone pathway of *K. lactis* has been investigated in some detail (Kawasaki *et al*., 2008). Moreover, the high osmolarity glycerol (HOG) pathway also seems to serve similar functions in *K. lactis* as it does in *S. cerevisiae*, although the experimental data are still relatively scarce (Siderius *et al*., 2000).

1.3 Aims of the thesis

The hypothesis followed in this thesis is that microcompartmentalization of the fungal plasma membrane into a patchwork of microdomains allows for the proper segregation of proteins and is important for cellular signaling and the regulation of many biological functions.

Clustering of the cell wall integrity sensors into microcompartments within the plasma membrane may thus be crucial for accurate sensor function. The cell wall is also involved in plasma membrane domain organization, as degradation of the cell wall leads to large changes in microcompartment distribution (Spira *et al.*, 2012). The transcriptional response for cell wall maintenance is mediated by the cell wall integrity signaling pathway and the transcription factor Rlm1. The role of Rlm1 and the better understanding of cell integrity signaling is of particular interest in this thesis. In light of lower genetic and thereby reduced functional redundancy, the objectives of this thesis were carried out basically in the milk yeast *K. lactis*. This thesis explores:

- the overall function of Rlm1 in *K. lacti*s
- differences, similarities and species-specific features of Rlm1 function
- target genes of the transcription factor in *K. lacti*s

The localization pattern of the CWI sensors resembled the punctual distribution of eisosomes. Both microcompartments appear as highly stable and immobile patches of similar size and shape, so the question of a possible connection and a resulting functional

significance for cell wall integrity signaling arises. Since eisosomes have not been described in *K. lactis* yet, questions concerning the structure, composition and physiological function of eisosomes in the milk yeast were investigated.

The key questions in this thesis are:

- Do eisosomes exist in *K. lactis*?
- Do sensors and eisosomes belong to the same microcompartment or do they constitute a microdomain on their own?
- Is the distribution of the sensors influenced by eisosomes or vice versa?
- Do eisosomes function in cell wall integrity maintenance?

2 Material and methods

2.1 Material

2.1.1 Strains used in this work

2.1.1.1 Yeast strains

The *Saccharomyces cerevisiae* strains employed in this thesis are listed in Table 2.

Table 2: *S. cerevisiae* strains that were employed in this thesis

Name	Genotype	Reference
BY4741	*MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0*	Euroscarf
BY4742	*MATα his3Δ 1 leu2Δ 0 lys2Δ 0 ura3Δ 0*	Euroscarf
BY4743	*MATa/α his3Δ 0/his3Δ 0 leu2Δ/leu2Δ 0 met15Δ 0/MET15 LYS2/lys2Δ 0 ura3Δ 0/ura3Δ 0*	Euroscarf
HSK13-2D	*MATa ura3-52 leu2-3,112 his3-11,15*	Koch, 2007
HD56-5A	*MATα ura3-52 leu2-3,112 his3-11,15*	Arvanitidis, 1993
DHD5	*MAT a/α ura 3-52/ura3-52 leu2-3,122/leu2-3,122 his3-11,15/his3-11,15*	Kirchrath, 2000
BYΔrlm1-a	*MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0 rlm1::KanMX*	Euroscarf
BYΔrlm1-α	*MATα his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0 rlm1::KanMX*	Euroscarf
HMZ13-A	*MATa ura3-52 his3-11,15 leu2-3,112 rlm1::SkHIS3*	Zuckermann, 2011
HMZ13-C	*MATα ura3-52 his3-11,15 leu2-3,112 rlm1::SkHIS3*	Zuckermann, 2011

The *K. lactis* strains employed are listed in Table 2. A ll *K. lactis* strains are based on the congenic strain series (Heinisch *et al.*, 2010a).

Table 3: *K. lactis* strains employed in this thesis.

Name	Genotype	Stock no.	Reference
KDR1-1D	*MATa ura3 leu2 his3::loxP lac4::loxP snf1::kanMX*		Rippert
KDR1-6A	*MATα ura3 leu2 his3::loxP lac4::loxP KU80*	39	Rippert
KHO139-1C	*MATα ade2::loxP*	51	Jürgen Heinisch

Name	Genotype	Stock no.	Reference
KHO139-2B	*MATa ade2::loxP*	52	Jürgen Heinisch
KHO151-1A	*MATα ura3 leu2 lac4::loxP bck1::kanMX rlm1::loxP*		Jürgen Heinisch
KHO187-4A	*MATα ura3 leu2 his3::loxP Kllsp1::ScLEU2*		Jürgen Heinisch
KHO187-9A	*MATα ura3 leu2 his3::loxP Kllsp1::ScLEU2*		Jürgen Heinisch
KHO191-2C	*MATa his3 leu2 ura3 lsp1::LEU2*	60	this study
KHO46-3D	*MATa ura3 ade2::loxP*	21	Jürgen Heinisch
KHO62-3D	*MATα ura3 leu2 ade2::loxP lac4::loxP*	23	Jürgen Heinisch
KHO69-14A	*MATa ura3 leu2 ade2::loxP ku80::loxP*	1	Heinisch *et al.*, 2010
KHO69-8C	*MATα ura3 leu2 his3::loxP ku80::loxP*	2	Heinisch *et al.*, 2010
KHO70	*MATa/alpha ura3/ura3 leu2/leu2 his3::loxP/HIS3 ade2::loxP/ADE2 ku80/ku80*	46	Jürgen Heinisch
KLSMO1	*MATα leu2 his3::loxP ku80::loxP rlm1::ScURA3*	3	this study
KLSMO2-4B	*MATα leu2 rlm1::ScURA4*	14	this study
KLSMO2-5D	*MATα rlm1::ScURA3*	8	this study
KLSMO3-3	*MATα ura3 leu2 his3 rlm1::loxP*	18	this study
KLSMO4-9C	*MATa leu2 his3 rlm1::URA3 lac4::loxP*	24	this study
KLSMO5-4B	*MATa ura3 leu2 his3 lac4::loxP rlm1::loxP*	25	this study
KLSMO5-5A	*MATa ura3 leu2 his3 rlm1::loxP*	26	this study
KLSMO9-4	*MATα ura3 leu2 ku80::loxP pil1::HIS3*	54	Jürgen Heinisch
KLSMO9-7	*MATα ura3 leu2 ku80::loxP pil1::HIS3*	55	Jürgen Heinisch
KLSMO10-2A	*MATα ura3 his3::loxP ku80::loxP can1::ScLEU2*	50	this study
KLSMO11-1	*MATα ura3 leu2 his3::loxP ku80::loxP KlLSP1-mCherry::SCURA3*	56	this study
KLSMO12-1D	*MATa ura3 leu2 his3 lsp1::LEU2 pil1::HIS3*	58	this study
KLSMO13-6C	*MATα ura3 leu2 his3 lsp1::LEU2 pil1::HIS3*	59	this study

2.1.1.2 *Escherichia coli* strain

The *E. coli* strain DH5α (*F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ–*) was used for amplification of plasmids and cloning.

2.1.2 Strain construction in yeast

The construction of yeast deletion strains is illustrated for one example in Figure 3. First, the *ScURA3* cassette was amplified from the plasmid pJJH955U with the oligonucleotides 05.103 and 05.104, resulting in a PCR product of 1.4 kb. The DNA fragment carries the *URA3* gene from *S. cerevisiae* with the *TEF* promoter and the *TEF* terminator from *Ashbya gossypii*, which are flanked by *loxP* sides. The PCR product also contains flanking sequences of 40 bps homologous to the target locus at each side. These flanking sequences direct the site specific homologous recombination into the genome. The PCR product was transformed into the wild type strain of *K. lactis.* Resulting transformants were selected by the auxotrophy marker *ScURA3.* The correct deletion of the *RLM1* gene was then confirmed by PCR using the oligonucleotide combinations depicted in Figure 3, resulting in three different DNA fragments of 500 bps, 627 bps and 1785 bps in size. The resulting strain was crossed to remove the deletion of *KU80*, a deletion that is necessary in *K. lactis* during strain construction to prevent random recombination events. Then the strain was transformed with the plasmid pJJH959r that encodes the Cre recombinase. By expressing the Cre recombinase from the *GAL1* promoter, the *ScURA3* cassette was erased from the genome by site specific recombination at the *loxP* sites. The removal of the *URA3* gene was checked again by PCR. The resulting strain, KLSMO3, allows the selection for uracil auxotrophy in further experiments again.

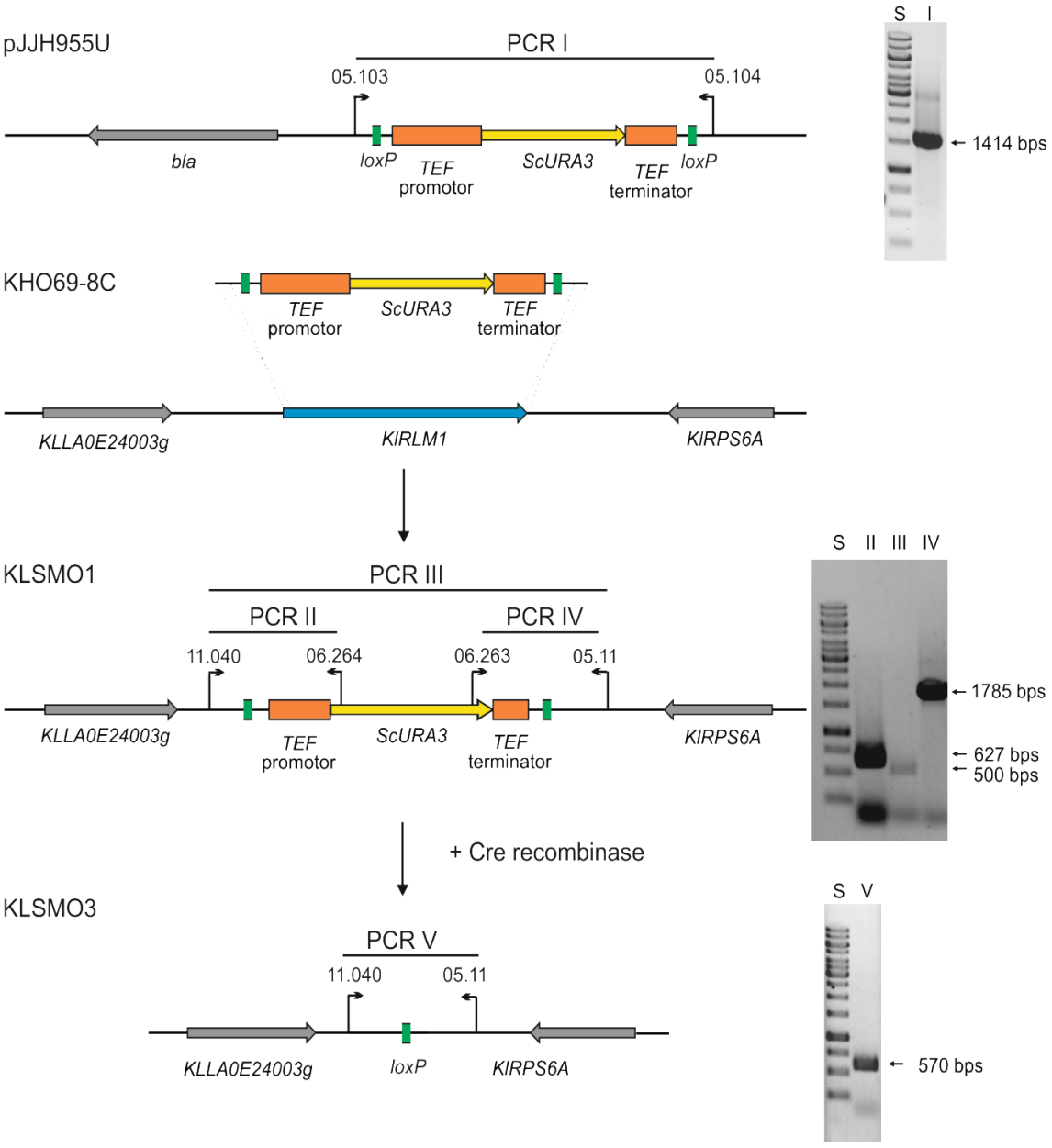


Figure 3: Construction of a yeast deletion strain by homologous recombination. The *RLM1* deletion of *K. lactis* is shown here exemplary. *ScURA3* cassette, amplified from the plasmid pJJH955U (PCR I), was transformed in the wild type strain KHO69-8C, where the gene *RLM1* was substituted by homologous recombination. Successful deletion of *KlRLM1* was verified by PCR (II-IV). Expressing the Cre recombinase from a plasmid removed the marker cassette by site specific recombination; this step was also confirmed via PCR (V). See text for details. Genes are drawn as arrows. Orange boxes show *TEF* promoter and terminator. *LoxP* sites are marked in green. S: DNA standard (1 kb ladder, Thermo Scientific).

2.1.3 Cultivation and media

2.1.3.1 Yeast media, cultivation and storage

YEP, rich medium: 1 % yeast extract; 2 % peptone; as carbon source 2 % glucose or 2 % galactose were added after sterilization. For the preparation of plates 1.5 % agar was added.

SC, synthetic medium: 0.67 % YNB w/o amino acids; 0,06 % CSM w/o histidine, leucine, tryptophane, uracil and adenine. The remaining amino acids/bases were added as required (Sherman, 2002). 2 % glucose or 2 % galactose were added after sterilization as carbon source. 1.5 % agar was added for pates and the pH was set at 6.2 before autoclaving.

Malt extract plates: 5 % malt extract; 3 % agar; pH was adjusted to 6.2.

Potassium acetate plates: 1 % potassium acetate; 3 % agar

For the selection of *kanMX* expression, 200 µg/ml G418 were added to the medium. 1 M of sorbitol was added to the media if osmotic stabilization was required. Yeast cultures were incubated at 30°C if not noted otherwise; liquid cultures were agitated.
Strains and cultures were stored at 4°C for a maximum of two months. For permanent storage of yeast strains, 500 µl of a stationary overnight culture were mixed with 1 ml of 33 % glycerol, incubated one hour and frozen at -80°C.

2.1.3.2 E. coli media and cultivation

Rich medium (LB): 1% tryptone; 0.5% yeast extract; 0.5% sodium chloride

Plates were made with 1.5 % agar. All *E. coli* cultures were grown at 37°C; liquid cultures were shaken for adequate oxygen supply. Selective media was produced by the addition of 100 µg/ml ampicillin, which was added after sterilization and chilling of the medium.

For a blue/white screening, 100 µl of a 2 % X-Gal solution (in DMF) were distributed on plates before the *E. coli* cells were struck out.

2.1.4 Vectors and plasmids

Relevant vectors and plasmids of previous studies are shown in Table 4. The plasmids that were constructed as part of this thesis are listed in

Table 5.

Table 4: Vectors and plasmids that were employed in this thesis.

Name	characteristics	application
pAJ001	*bla; SkHIS3*; mCherry; pKT-series	C-terminal mCherry fusion
pCse20	*bla; KlLEU2;KlCEN2; Klori*	*K. lactis* single copy vector
pCse24	*bla; KlLEU2; CEN/ARS; pKD1ori*	*K. lactis-S.cerevisiae* shuttle vector
pCXJ18	*bla; KlURA3; KlCEN2; Klori*	*K. lactis* single copy vector
pCXJ20	*bla; KlLEU2; KlCEN2; Klori*	*K. lactis* single copy vector
pCxs22	*bla; KlURA3; CEN/ARS; pKD1ori*	*K. lactis-S.cerevisiae* shuttle vector
pFA6a-GFP(s65t)-kanMX6	*GFP (S56T); bla; kanMX;*	C-terminal GFP fusion
pJJH955H	*ScHIS3; bla*	*ScHIS3* deletion cassette
pJJH955L	*ScLEU2; bla*	*ScLEU2* deletion cassette
pJJH955Uneu	*ScURA3; bla*	*ScURA3* deletion cassette
pJJH1524	*bla; kanMX; mCherry*; pFA6a-series	C-terminal mCherry fusion
pJJH1525	*bla; SkHIS3; mCherry*; pFA6a-series	C-terminal mCherry fusion
pJJH1564	*bla; KlURA3; lacZ; CEN/ARS; pKD1ori*	lacZ / promoter fusion
pJJH1617	*bla; SkHIS3;* codon optimised Biotinylase*;*	C-terminal BicA fusion
pJJH1618	*bla; kanMX4;* codon optimised Biotinylase*;*	C-terminal BicA fusion

Name	characteristics	application
pKT127	*bla; SkHIS3; yEGFP;* pKT-series	C-terminal GFP fusion
pKT128	*bla; kanMX4; yEGFP;* pKT-series	C-terminal GFP fusion
pUC19	*bla; lacZ'*	cloning
pUG6	*bla; kanMX4; loxP* sites	*kanMX* deletion cassette
pUG72	*bla; KlURA3; loxP* sites	*KlURA3* deletion cassette
pUG73	*bla; KlLEU2*; *loxP* sites	*KlLEU2* deletion cassette
pUK1921	*kan^R; lacZ'*	cloning
pUK78	*bla; 3yeGFP; kanMX*	GFP fusion
YCplac111	*bla; KlLEU2; CEN/ARS*; *lacZ'*	cloning
pXW3	*bla; KlURA3; CEN/ARS; pKD1ori; lacZ; lacY; lacA*	lacZ / promoter fusion
YEp13	*bla; tetR; KlLEU2; 2 micron origin*	cloning
YEp352	*bla; KlURA3; 2 micron origin; lacZ'*	cloning
pRRO71	*bla; KlURA3; KlCEN2; kanMX; KlWSC1-GFP; Klori*	Fluorescence microscopy
pRRO73	*bla; KlURA3; CEN/ARS; pKD1ori; KlWSC1-GFP*	Fluorescence microscopy
pRRO99	*bla; KlURA3; CEN/ARS; pKD1ori; KlMID2-GFP*	Fluorescence microscopy
pRRO100	*bla; KlURA3; KlCEN2; KlMID2-GFP; Klori*	Fluorescence microscopy
pULF20	*bla; KlURA3; CEN/ARS; pKD1ori; KlWSC2/3-GFP*	Fluorescence microscopy
pJJH958r	*GAL1p_CreR; bla; KlURA3; ScCEN/ARS; pKD1ori*	inducible Cre recombinase activity
pJJH959r	*GAL1p_CreR; bla; KlLEU2; ScCEN/ARS; pKD1ori*	inducible Cre recombinase activity
pYM14	*6HA,kanMX,bla*	6HA tag

Table 5: Plasmids that were constructed and used in this thesis

Name	Insert	Backbone	Construction
pJJH1594	*KlPKH1*	pCXs22	Amplification using the oligonucleotides 12.656 and 12657; *KlPKH1* was cloned with SalI; HindIII into pCXs22 SalI; HindIII
pJJH1595	*KlLAC4p_ KlPKH1*	pCXs22	*KlLAC4p* cloned from pJJH1593 into pJJH1594
pJJH1776	*KlSED1p_lacZ*	pJJH1774	The *KlSED1* promoter was amplified from chromosomal DNA using the oligonucleotides 14.067 and 14.068, cloned into pJJH1774 by BamHI, HindIII
pJJH1777	*KlCIS3p_lacZ*	pJJH1774	The *KlCIS3* promoter was amplified from chromosomal DNA using the oligonucleotides 14.069 and 14.070, cloned into pJJH1774 by BamHI, HindIII
pJJH1778	*KlHSP12p_lacZ*	pJJH1774	The *KlHSP12* promoter was amplified from chromosomal DNA using the oligonucleotides 14.071 and 14.072, cloned into pJJH1774 by BamHI, SalI
pJJH1779	*KlYNL144c p_lacZ*	pJJH1774	The *KlYNL144* promoter was amplified from chromosomal DNA using the oligonucleotides 14.073 and 14.074, cloned into pJJH1774 by BamHI, HindIII
pSMO19	*KlRLM1*	pCse24	*KlRLM1* was cut from pSMO18 and cloned into pCse24 using HindIII ans SmaI

Name	Insert	Backbone	Construction
pSMO28	*ScBGL2p_lacZ*	pXW3	The *ScBGL2* promoter was amplified from chromosomal DNA using the oligonucleotides 11.327 and 11.328, cloned into pJJH1774 by BamHI, HindIII
pSMO34	*ScRLM1p _KlRLM1/ ScRLM1*	pCse24	The 5´end of *KlRLM1* was amplified from genomic *K. lactis* DNA using the oligonucleotides 11.424 and 11.425, inserted by homologous recombination into pSMO134
pSMO38	*ScMPK1 p_lacZ*	pXW3	The *ScMPK1* promoter was amplified from chromosomal DNA using the oligonucleotides 12.060 and OVI227, cloned into pXW3 by BamHI, HindIII
pSMO39	*KlYLR194cp_ lacZ*	pXW3	The *KlYLR194c* promoter was amplified from chromosomal DNA using the oligonucleotides 12.062 and OVI228, cloned into pXW3 by BamHI, HindIII
pSMO40	*KlMpk1p_lacZ*	pXW3	The *KlMpk1* promoter was amplified from chromosomal DNA using the oligonucleotides 12.078 and OVI230, cloned into pXW3 by BamHI, SalI
pSMO41	*KlBgl2p_lacZ*	pXW3	The *KlBGL2* promoter was amplified from chromosomal DNA using the oligonucleotides 12.064 and OVI229, cloned into pXW3 by BamHI, HindIII
pSMO48	*KlPIL1*	pCXs22	*KlPIL1* was amplified from chromomal DNA using the oligonucleotides 12.284 and 12.285, cloned into pCXS22 with EcoRI and SalI

Name	Insert	Backbone	Construction
pSMO51	*KLPIL1-mCherry*	pCXs22	mCherry was amplified from pAJ001 using the oligonucleotides 12.286 and 12.291, inserted by homologous recombination into pSMO48
pSMO55	*KlPIL1*	pCse24	*KlPIL1* was isolated from pSMO48 and cloned into pCse24 with EcoRI and HindIII
pSMO56	*KlPIL1*	pCXJ18	*KlPIL1* was isolated from pSMO48 and cloned into pCXJ18 using EcoRI and HindIII
pSMO58	*KLPIL1-mCherry*	pCse20	*KlPIL1-mCherry* was isolated from pSMO51 and cloned into pCse20 with XbaI and HindIII
pSMO61	*KlPIL1-GFP*	pCXs22	*GFP* was amplified from pKT128 using the oligonucleotides 12.286 and 12.291, fused by homologous recombination to *PIL1* in pSMO48
pSMO64	*KlLSP1*	pCXs22	*KlLSP1* was amplifid from chrom. DNA using the oligonucleotides 12.543 and 12.542. The gene was cloned with ScaI and EcoRI into pCXs22 (SmaI, EcoRI)
pSMO73	*Kllsp1::LEU2*	pCXs22	*ScLEU2* deletion cassette was amplified from pJJH955L using the oligonucleotides 12.544 and 12.545. *KlLSP1* was deleted by homologous recombination from the plasmid pSMO64
pSMO75	*KlLSP1-GFP*	pCXS22	The GFP cassette was amplified from pKT128 using the oligonucleotides 12.621 and 12.622. The *KlLSP1-GFP* fusion was obtained by homologous recombination on the plasmid pSMO64

Name	Insert	Backbone	Construction
pSMO77	*KlLSP1-mCherry*	pCXS22	The *mCherry* cassette was amplified from pAJ001 using the oligonucleotides 12.621 and 12.622. The *KlLSP1-mCherry* fusion was obtained by homologous recombination on the plasmid pSMO64
pSMO79	*KlSUR7*	pCXS22	*KlSUR7* was amplified from chrom. DNA using the oligonucleotides 13.006 and 13.007, the gene was cloned into pCXs22 (EcoRI, BamHI) using EcoRI and BglII
pSMO84	*KlSUR7-mCherry*	pCXs22	*mCherry* was amplified from the plasmid pAJ001 with the oligonucleotides 13.004 and 13.005, *SUR7* fusion was obtained by homologous recombination on the plasmid pSMO79
pSMO96	*ScRLM1p_ KlRLM1/ ScRLM1*	pCXJ20	The 5´end of *KlRLM1* was amplified from genomic *K. lactis* DNA using the oligonucleotides 11.424 and 11.425, inserted by homologous recombination into pSMO135
pSMO101	*KlPIL1-GFP*	pCSe20	*KlPIL1-GFP* was isolated from pSMO61 (cut BclI and HindIII) and cloned into pCse20 (cut BamHI and HindIII)
pSMO113	*KlRLM1*	pCse24	The *KlRLM1* promoter was amplified from genomic *K. lactis* DNA using the oligonucleotides 13.179 and 13.180, inserted by homologous recombination into pSMO19
pSMO116	*KlRLM1/ ScRLM1*	pCse24	The *KlRLM1* promoter was amplified from genomic *K. lactis* DNA using the oligonucleotides 13.179 and 13.180, inserted by homologous recombination into pSMO34

Name	Insert	Backbone	Construction
pSMO118	*KlRLM1*	pCXJ20	The *KlRLM1* promoter was amplified from genomic *K. lactis* DNA using the oligonucleotides 13.179 and 13.180, inserted by homologous recombination into pSMO90
pSMO12	*ScRLM1-3yeGFP*	pCse24	*3yeGFP* was amplified from pUK78 with the oligonucleotides 10.258 and 10.257. *GFP* fusion was done by homologous recombination on the plasmid pSMO134
pSMO121	*KlRLM1p_ KlRLM1/ ScRLM1*	pCXJ20	The *KlRLM1* promoter was amplified from genomic *K. lactis* DNA using the oligonucleotides 13.179 and 13.180, inserted by homologous recombination into pSMO96
pSMO123	*KlRLM1-6HA*	pCXJ20	The *6HA* was amplified from pYM14 with the oligonucleotides 07.247 and 13.160. *6HA-KlRLM1* fusion was done by homologous recombination on the plasmid pSMO118
pSMO125	*KlRLM1-GFP*	pCXJ20	*GFP* was amplified from pKT128 with the oligonucleotides 13.029 and 13.030. *GFP* fusion was done by homologous recombination on the plasmid pSMO118
pSMO126	*ScRLM1p_ Sc100RLM1/ KlRLM1*	pCse24	A large part of the 5´end of *KlRLM1* was amplified from genomic *K. lactis* DNA using the oligonucleotides 11.419 and 13.250, inserted by homologous recombination into pSMO134

Name	Insert	Backbone	Construction
pSMO127	*ScRLM1p_ Sc100RLM1/ KlRLM1*	pCXJ20	A large part of the 3´end of *KlRLM1* was amplified from genomic *K. lactis* DNA using the oligonucleotides 11.419 and 13.250, inserted by homologous recombination into pSMO135
pSMO130	*ScRLM1p_ Kl100RLM1/ ScRLM1*	pCse24	The first 300 bases of *KlRLM1* were amplified from genomic *K. lactis* DNA using the oligonucleotides 11.424 and 13.251, inserted by homologous recombination into pSMO134
pSMO131	*ScRLM1p_ Kl100RLM1/ ScRLM1*	pCXJ20	The first 300 bases of KlRLM1 were amplified from genomic *K. lactis* DNA using the oligonucleotides 11.424 and 13.251, inserted by homologous recombination into pSMO135
pSMO132	*KlRLM1p_ Kl100RLM1/ ScRLM1*	pCse24	The *KlRLM1* promoter was amplified from genomic *K. lactis* DNA using the oligonucleotides 13.179 and 13.181, inserted by homologous recombination into pSMO130
pSMO133	*KlRLM1p_ Kl100RLM1/ ScRLM1*	pCXJ20	The first 300 bases of KlRLM1 were amplified from genomic *K. lactis* DNA using the oligonucleotides 11.424 and 13.251, inserted by homologous recombination into pSMO140
pSMO134	*ScRLM1*	pCSe24	*ScRLM1* amplified from chromosomal *S. cerevisiae* DNA using the oligonucleotides 05.197 and 10.255; cloned into pSce24 with PstI and SmaI
pSMO135	*ScRLM1*	pCXJ20	ScRLM1 was isolated from pSMO134 and cloned into pCXJ20 using PstI and SmaI

Name	Insert	Backbone	Construction
pSMO136	*ScRLM1p_ ScRLM1/ KlRLM1*	pCse24	The 3´end of *KlRLM1* was amplified from genomic *K. lactis* DNA using the oligonucleotides 11.418 and 11.419, inserted by homologous recombination into pSMO134
pSMO137	*KlLM1p_ ScRLM1*	pCse24	The *KlRLM1* promoter was amplified from genomic *K. lactis* DNA using the oligonucleotides 13.179 and 13.181, inserted by homologous recombination into pSMO134
pSMO139	*ScRLM1p_ KlRLM1*	pCse24	The *ScRLM1* promoter was amplified from genomic *S. cerevisiae* DNA using the oligonucleotides 13.293 and 13.309, inserted by homologous recombination into pSMO113
pSMO140	*KlRLM1p_ ScRLM1*	pCXJ20	The *KlRLM1* promoter was amplified from genomic *K. lactis* DNA using the oligonucleotides 13.179 and 13.181, inserted by homologous recombination into pSMO135
pSMO142	*KlRLM1-GFP*	pCse24	*GFP* was amplified from pKT128 with the oligonucleotides 13.029 and 13.030. *GFP* fusion was done by homologous recombination on the plasmid pSMO113
pSMO143	*ScRLM1p_ KlRLM1*	pCXJ20	The *ScRLM1* promoter was amplified from genomic *S. cerevisiae* DNA using the oligonucleotides 13.293 and 13.309, inserted by homologous recombination into pSMO118
pSMO144	*ScRLM1p_ ScRLM1/ KlRLM1*	pCXJ20	The 3´end of *KlRLM1* was amplified from genomic *K. lactis* DNA using the oligonucleotides 11.419 and 11.418, inserted by homologous recombination into pSMO135

Name	Insert	Backbone	Construction
pSMO145	*KlRLM1p_ ScRLM1/ KlRLM1*	pCse24	The *KlRLM1* promoter was amplified from genomic *K. lactis* DNA using the oligonucleotides 13.179 and 13.181, inserted by homologous recombination into pSMO136
pSMO146	*KlRLM1p_ ScRLM1/ KlRLM1*	pCXJ20	The 3´end of *KlRLM1* was amplified from genomic *K. lactis* DNA using the oligonucleotides 11.419 and 13.250, inserted by homologous recombination into pSMO140
pSMO147	*KlRLM1p_ Sc100RLM1/ KlRLM1*	pCXJ20	A large part of the 3´end of *KlRLM1* was amplified from genomic *K. lactis* DNA using the oligonucleotides 11.419 and 13.250, inserted by homologous recombination into pSMO140
pSMO148	*KlRLM1p_ Sc100RLM1/ KlRLM1*	pCse24	The *KlRLM1* promoter was amplified from genomic *K. lactis* DNA using the oligonucleotides 13.179 and 13.181, inserted by homologous recombination into pSMO126
pSMO153	*KlGRE1p_lacZ*	pJJH1774	The *KlGRE1* promoter was amplified from chromosomal DNA using the oligonucleotides 14.075 and 14.076, cloned into pJJH1774 by BamHI, SalI

For description of the chimeric genes a slash was used to separate the parts of each organism. Sc100RLM1/KlRLM1 encodes for the first 100 amino acids of ScRlm1 and the C-terminus of KlRlm1, respectively.

2.1.5 Oligonucleotides

The oligonucleotides that were employed in this work are registered in table Table 6. Oligonucleotides were obtained by Metabion.

Table 6: Oligonucleotides used in this thesis.

Number	Name	Sequence
00.53	leu2-3' outneu	GCCGAAGAAGTTAAGAAAATCCTTGC
05.11	KlRLM1end Xho	GGCGCTCGAGGTTTAGTGTTACAAATAAAAGCGG
05.22	KlURA3raus5	GGATTTTAATCTTCACGCAGGATG
05.23	KlURA3raus3	GGTAGAGATCCTAAGGTTGAAGGTG
05.24	KlLEU2raus5	GTCATTCAAGATTATCTCTTTTGG
05.25	KlLEU2raus3	GGTATGCTTGAAATCTCAAGG
05.30	Scrlm1del5	GGGTAGACGGAAGATTGAAATCCAGAGAATTTCTGATGACA GAAATAGGGCTTCGTACGCTGCAGGTCGAC
05.31	Scrlm1del3	GAATTTTTTTCTCCTGAAATATCAGTCGAAAAATTAAAGAATT TAGTGCCGCATAGGCCACTAGTGGATCTG
05.103	KlRLM1del5	CCCATCGCCACAAGTTTTCATAAGACCTCTTTCTTTTAGCATAC GATGGGCTTCGTACGCTGCAGGTCGAC
05.104	KlRLM1del3	GGAATAACTCCTAATCTTCCTGTCATTTATCTACAGATCCTTTA TCTGACCCGCATAGGCCACTAGTGGATCTG
06.54	KlSLA2	GTATTCAACAACAGGAAATGGAAAAGG
06.55	KlMATa1	GGAGTCATGTGCGACAATGATATGGCAG
06.56	KlMATalpha1	GTAGATAAACAGACAAGAAAGAATTGGG
06.263	ScURA35raus	GCTTGGCAGCAACAGGACTAGGATG
06.264	ScURA33raus	GGGAAGGGATGCTAAGGTAGAGGGTG
08.370	ScLEU2_5out	GTAAAAGGTAAGAAAAGTTAGAAAG
09.91	KlKU80vv	GTGTTCAAGCTGCTGCCACCATGTG
09.92	KlKU80wwv	GATTCAAGTGCATTATGGTG
09.93	KlKU80nn	GGAATAGCTTCCATGATGGCTTGG
10.316	SMP1del3	CGAATGCTTTCTTGTAGTAAAAAGTAGCTAGAAAGCGATTGC ATAGGCCACTAGTGGATCTG
10.317	SMP1del5	GATAAGCAAACACAATTATTTTCAAAAAAATTTCTGCTAATTC GTACGCTGCAGGTCGAC
11.040	Klrlm1-vor	TTCAATCCGCTAGATTTCCC
11.246	ScRlm1-3NcoI	TGGCCATGGGTAGACGGAAGATTGA
11.247	ScRlm1-5EcoRVSphI	GGGATATCGCATGCGCTTGGAATATTCATACTGGTC
11.248	ScSmp1-3 NcoI	CCGGGCCATGGGTAGAAGAAAAATTGA
11.249	ScSmp1-5 EcoRVSphI	GGGATATCGCATGCTTAATCTGGAGAGTTTGTCG
11.327	ScBGL2p-nach	GGCAAGCTTGGCATCTTTGACCGTTTTTCTTTTTGG
11.328	ScBGL2p-vor	ACGGATCCAACGCCTCCAAACTTACTGG
11.418	KlRlm1C3	ATGTACAATCTTAACCAGCCTTCATCCAGTTCATCTTCTCCTGC TACTAGTCTGAACTCAA
11.419	KlRlm1C5	TCTTATGCTTGGAATATTCATACTGGTCAAATTTTTTGGTTCAT TTATCTACAGATCC

Number	Name	Sequence
11.424	KlRlm1N3	TAAATATTAAAGTGTCGCAAAACTATACTATAGATACAACCGA TGGGTAGACGTAAAATT
11.425	KlRlm1N5	AGGAGAAGATGAACTGGATGAAGGCTGGTTAAGATTGTACA TGGAAGCAGAATGAGCTCCTG
12.060	Mpk1-prom-BamHI	TGGGATCCGGTGGTGAAAATGAAGGA
12.062	KlYLR194c-prom-BamHI	ACGGATCCGAATTGAGACCAAACTTCG
12.064	KlBgl2-prom-BamHI	ACGGATCCCATTCGATCAAACGAAAT
12.078	KlMpk1-prom-BamHI	TGGGATCCTCAGGTGGACGCAAAGCAAC
12.284	KlPIL1vorSal	GCATCACAGCAAGGCTGTCGACCGG
12.285	KlPIL1nachEco	gcgcgaattCAGGTAAGGTTAGTCGAATAG
12.286	KlPIL1mChF5	AAGCTGAAGCCGAAGCTCAAGAAGAACACCCAGTTGCTGCTG GTGACGGTGCTGGTTTA
12.290	Klpil1del5	TAATAGTTAAAAAAAAAGATAAAGACACCATATATTACAACCT TCGTACGCTGCAGGTCGAC
12.291	Klpil1del3	AAGAGATTTTTAGAAGCACCTAAGGAGTAGAAGAGTGAGAA GCATAGGCCACTAGTGGATCTG
12.292	Klpil1wvor	GCGTGAGTTCTTGAAGTCAG
12.293	Klpil1wnach	GACAATTAAGAGATAGTAGTTGG
12.542	KlLSP1vorSpe	CCATAACCTGTACCAACGCCC
12.543	KlLSP1nachEco	CACGTTGTTCCGTATCTTATCC
12.544	Kllsp1del5	ATATCAAGAAGTTAAATTAAATACCGATACTAGTCTCCAAAAC TTCGTACGCTGCAGGTCGAC
12.545	Kllsp1del3	TAGCGGCATTGGTAAAATAAACTAACGCATGTGATCGCGAGA GCATAGGCCACTAGTGGATCTG
12.621	KlLsp1-GFP-F5	TGGCCAAGAAGAAGAAGCTGTTGAGACTACTGAAGTTAAAG GTGACGGTGCTGGTTTA
12.622	KlLsp1-GFP-R3	ACATACTACCGTTTTACCTGTCACACAGTAACATTGCGAGAAT TCGAGCTCGTTTAAACT
12.656	KlPKH1forSal	GCTAGGTGTCGACATTCTCTG
12.657	KlPKH1revHind	GATGACATAACCAAGCTTAAAG
12.658	Klpkh1del5	ATATTTTAAGGTTTGATGGTTGAAGCATCTCCTTTTTTTGCATT GCATAGGCCACTAGTGGATCTG
12.659	Klpkh1del3	AACTTCTCTAGAGCATTGAGCTATGACTCTTACTAGAATTTTG ATCTTCGTACGCTGCAGGTCGAC
13.004	KlSur7-GFP-F5	CAAGATTAGAAGAACTCAAAAATCTGATGAAGAATCGATTGG TGACGGTGCTGGTTTA
13.005	KlSur7-GFP-R3	GTTGGTTTGTTCAATAGTATAAGGTTACAGGGTGCGAGACTC GAGCTCGTTTAAACT

Number	Name	Sequence
13.006	KlSur7-BglII-vor	GCGGTGGGAGTGTTCGAATT
13.007	KlSur7-EcoRI-rev	CCGGAATTCGACTAAATCACAATTGTCC
13.026	KlPIL1-BIRA-for	AGCTGAAGCCGAAGCTCAAGAAGAACACCCAGTTGCTGCTGC AGGTCGAGGCGGCCGCCAG
13.027	KlPIL1-BIRA-rev	CTTGCCAGCCAGTGGGCTACTCATTGAAAAGAATCGAAACTC GAGCTCGTTTAAACT
13.029	KlRLM1-F5	TGCAGGGTCAACAACATATGGGTCAGATAAAGGATCTGTAGA TAAAGGTGACGGTGCTGGTTTA
13.030	KlRLM1-R3	TCGTATTCCGGGGTGGTTGTTTGCATCTGAAGCGTCCTATTCG AGCTCGTTTAAACT
13.031	pKlMUC1a ForwPst	ATGCCTGCAGTGATGAAGAAACG
13.032	pKlMUC1a RevBam	gcgcggatccCATTTTGTGAGTGATTGCACG
13.033	pKlMUC1b ForwSal	gcgcgtcgACGTTCTTCCATCGCCTCG
13.034	pKlMUC1b RevBam	gcgcggatccCATGGTCTTCGCGATTGTAAAGG
13.039	pKlNHP6AforHind	gcgcaagcTTGGGCCGCTGCTCTCAGCCAG
13.040	pKlNHP6ArevBam	gcgcggatccCATATTGCCTGGTATTATTTG
13.041	pKlFKS1for Sal	GTGCGTCGACCGAGACATTTCTCGCG
13.042	pKlFKS1rev Bam	gcgcggatccCATAGCGAATGTGGGGTGAGG
13.043	pKlPST1for Sal	gcgcgtcgACAGGATCGGAGAAAGAGAGAGG
13.044	pKlPST1revBam	gcgcggatccCATTATTGTATAGCGAGAGG
13.045	pKlYIL117C forHind	gcgcaagCTTGATATATGCCTGTCATGG
13.046	pKlYIL117C revBam	gcgcggatccCATCTTCACTAATTTAGTATCC
13.047	pKlPIR1b forHind	CATAAAGCTTTAAGCTCCACTTATGG
13.048	pKlPIR1a revBam	gcgcggatccCATTGGTAATGTGTTGTATTTAGAG
13.160	KlRLM1HAfor	GTCAACAACATATGGGTCAGATAAAGGATCTGTAGATAAACT TCGTACGCTGCAGGTC
13.179	KlRLM1prom-vor	ACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTAC GCGTCATGACCGTGTCCC
13.180	KlRLM1prom-rev	CGTGCGTTATCGGCTCGATATTAATTTTACGTCTACCCATCGT ATGCTAAAAGAAAGAGG
13.181	KlRLM1prom-sc-rev	CATCAGAAATTCTCTGGATTTCAATCTTCCGTCTACCCATCGTA TGCTAAAAGAAAGAGG

Number	Name	Sequence
13.250	KlRLM1-C-neu	CGAAGTGAAAGATCCTTCCGATTATGGAGACTTTCACAAAAA GGAACGCGTTGTGCTAAG
13.251	KlRLM1-N-neu	ACGACCTGAGTAGGTCTTGATTTATGTTAACGGATGCACTTTT AACGTAGTCCCCATAAT
13.253	Klmpk1del5	ATATCAGTCCGGATCAACTGATTGTGCCCTTTTCTCCACCGAA CTTCGTACGCTGCAGGTCGAC
13.254	Klmpk1del3	TACAAAGAGAGTGGATGAAGCTATATACCAGACGGTTTCATT AGCATAGGCCACTAGTGGATCTG
13.293	ScRLM1prom-for	ACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTCG TAACAATGCTTCATAATC
13.294	ScRLM1prom-rev	ATGGGTAGACGTAAAATTAATATCGAGCCGATAACGCACGGT TGTATCTATAGTATAGTT
13.309	ScRlm1-atg-rev	CGTGCGTTATCGGCTCGATATTAATTTTACGTCTACCCATGTT GTATCTATAGTATAGTT
OVI227	ScMPK1-prom-HindIII-neu3´	GGCAAGCTTGGCATCTCCCAGAATTGTTATACAC
OVI228	KlYlr194c-prom-HindIII-neu3´	ACGGTCGACCATTTCGGTGGAGAAAAGGGCAC
OVI229	KlBgl2-prom-HindIII-neu3´	GGCAAGCTTAGTGTAGAGAAACGCATAGCT
OVI230	KlMpk1-prom-HindIII-neu3´	GGCAAGCTTGGCATTGTCAGTTTGGATTACCGCT
10.255	RLM1-3XmaI	ATCCCGGGTGGGTCTTGTCCTCTATGAC
10.256	RLM1-5SacII	TCCGCGGACTCCAGGCCAGGCGGCAAT
03.44	KanMX-3´out	GTATTGATGTTGGACGAGTCGG
03.45	KanMX-5´out	GGAATTTAATCGCGGCCTCG
14.067	KlSED1pHindfor	ggcgaagcttCGGAACACATTACAAACAGG
14.068	KlSED1pBamrev	gcgcggatccTTTTTGCAGTATTTCTTTATTGATG
14.069	KlCIS3pHindfor	ggcgaagcttGGCTACTGTAAGGATCACCGGAG
14.070	KlCIS3pBamrev	gcgcggatccCGTGATGTGTAGGAGTCAATGACG
14.071	KlHSP12pSalfor	gcgcgtcgACATATGGATATGGGGAAATG
14.072	KlHSP12p Bamrev	gcgcggatccTTTTATTATTAGTGTTTGTGTGTG
14.073	KlYNL144Cp Hindfor	GAAACAAAAAGCTTATGGAAGG
14.074	KlYNL144Cp Bamrev	gcgcggatccATATGAAGTGGGTCCGAAGG
14.075	KlGRE1p Salfor	gcgcgtcGACAAAGCAAAGCAACCTGATGG
14.076	KlGRE1 Bamrev	gcgcggatccTTTGATATTATAGATGTTCTTG

2.2 Methods

2.2.1 Mating, sporulation and tetrad analysis

The mating of haploid yeast cells was performed by mixing a *MATα* and a *MATα* strain on YEPD plates (*S. cerevisiae*) or malt extract plates (in case of *K. lactis*). The plates were incubated overnight at 30°C and cells were transferred to suitable media to select diploid cells.

For sporulation, diploid yeast cells were grown overnight in 5 ml selective medium and harvested by centrifugation (2 min; 30000 rpm). The supernatant was discarded, yeast cells resuspended in the remaining fluid and dropped on sporulation medium. After two or three days of incubation at 30°C, successful sporulation was reviewed by microscopy.

A few million of the sporulated cells were transferred to a cup and distributed in 70 µl of water, containing 1 µl of Zymolyase (12.5 mg/ml). 15 minutes incubation at room temperature destroyed the ascus wall for the following dissection of the four spores by micromanipulation. Dilution by addition of 630 µl of water and incubation on ice paused further Zymolyase activity. 25 µl of the mixture were streaked on a YEPD plate and spores were separated using the micromanipulator (MSM300; Singer instruments). Germinated spores were analyzed by replica plating onto different selective media.

2.2.2 Growth analysis by serial drop dilution assay

Precultures of the strains under investigation were freshly inoculated to an optical density of 0.2. Exponentially grown cells were harvested at an OD_{600} of approximately 1.0 and diluted to an OD_{600} of 0.3 in fresh medium. Serial dilutions of 10^{-1}; 10^{-2}; 10^{-3} and 10^{-4} were made and dropped on media containing various amounts of cell wall stressors. For the here investigated issues, Congo red concentrations of up to 4mg/ml proved to be ideal in *K. lactis* (for *S. cerevisiae* 50- 500 µg/ml), Calcofluor white concentrations varied from 10 to 100 µg/ml (20-100 µg/ml) and caspofungin was usually added in concentrations of about 15-45 ng/ml (80-160 ng/ml). After growth for two or three days documentation of the experiment was achieved using the "GelDoc-It Ultraviolet Transilluminator" (UVP, Upland, USA).

2.2.3 Transformation of *E. coli* cells

Transformation of *E. coli* with DNA was conducted using the rubidium chloride method (Hanahan, 1983).

100 ml of logarithmically grown *E. coli* cells were harvested at 3000 rpm and 4°C for 10 minutes, resuspended in 5 ml of RF1 solution (100 mM RbCl; 50 mM MgCl2; 30 mM KAc; 10 mM CaCl2; 15 % glycerol; pH 5.8) and incubated for one hour. Cells were spun down afterwards and the pellet was resuspended in 4 ml of RF2 solution (10 mM MOPS; 10 mM RbCl; 75 mM CaCl2; 15 % glycerol; pH 6.8). After incubation of 20 minutes, aliquots of 100 µl were made, which were frozen at -80°C until use.

For the transformation, DNA (usually less than 1µl of plasmid) was added to an aliquot of frozen, competent *E. coli* cells. The cells were defrosted, mixed and incubated for 20 minutes on ice. 45 seconds of heat shock at 37°C and two additional minutes on ice were followed by addition of 1 ml LB-medium and one hour of incubation on 37°C. The cells were spun down (6000 rpm, 1 min) and plated on respective media to select for successful transformation.

2.2.4 Transformation of yeast cells using the "freeze" method

For transformation of *K. lactis* with DNA the "freeze" method was used (Klebe *et al.*, 1983). 50 ml of cells were spun down at an OD_{600} of 0.6 (3000 rpm; 2 minutes), washed with buffer A (1 M sorbitol, 10 mM bicine pH 8.35, 3 % ethylene glycol) and resuspended in 2 ml of buffer A. The cells were aliquoted (200 µl) and frozen at -80°C until further use.

5 to 10 µl of DNA and 5 µl of carrier DNA (10 mg/ml) were added to the competent, sill frozen cells for transformation. During incubation (5 min; 37°C) the cells were mixed carefully a few times. Then 1 ml of buffer B (40 % PEG1000; 200 mM Bicine; pH 8.35) was added, the samples were incubated for one hour at 30°C and spun down (3000 rpm; 2 min) before the pellet was plated onto selective media. Selection with G418 required a previous incubation for about 4 hours (overnight) in rich medium.

2.2.5 Transformation of yeast cells with lithium acetate

The protocol using lithium acetate (Gietz *et al.*, 1995) was applied for transformation of *S. cerevisiae* cells. 50 ml of cells were harvested at an OD_{600} of 2.0, washed with 1 ml of 100 mM lithium acetate and resuspended in 400 µl of LiAc (100 mM). 50 µl of the cells were centrifuged (3.000 rpm; 2 min) and 240 µl of PEG4000 (50 %), 36 µl of LiAc (1 M), 10 µl of carrier DNA (10mg/ml) and 2 µl of plasmid DNA were added. After mixing thoroughly, the preparation was incubated (42°C; 20 minutes), spun down (3000 rpm; 2 minutes) and streaked out onto selective media (for G418 selection see above).

2.2.6 Isolation of DNA

2.2.6.1 Isolation of genomic DNA from yeast

To obtain genomic DNA for PCR reactions, usually the "quick and dirty" method was used. A small amount of yeast cells was incubated for 20 minutes in 20 µl NaOH (50 mM) and transferred afterwards to the microwave (3 Minutes; 600W) to further destroy the cells. 1 µl was used later in PCR as template.

For experiments that required higher purity of the DNA, chromosomal DNA was isolated from spheroblasts. Therefore, 1.5 ml of cells from an overnight culture where harvested (13000 rpm; 1 min) and resuspended in 389 µl of spheroblast-buffer (0.9 M sorbitol; 0.1 M EDTA; pH 6-8). After addition of 8 µl of DTT (1 M) and 3 µl of Zymolyase (12.5 mg/ml), the cell wall was digested for one hour a 37°C. Spheroblasts were pelleted (3000 rpm; 5 min) and lysed by addition of 500 µl of TE-buffer (10 mM Tris-HCl; 1 mM EDTA; pH 7.5) containing 3 % SDS (65°C; 15 min). After 5 minutes of cooling at room temperature, proteins were precipitated by adding 150 µl of potassium acetate (5 M) and incubation on ice (30 min), before they were pelleted by centrifugation (4°C; 13000 rpm; 15 min). 500 µl of the supernatant were then transferred to a new cup and mixed with 2.5 volumes of ethanol and 0.1 volumes of sodium acetate (3 M; pH 5.5) to precipitate the DNA. Centrifugation (13000 rpm; 15 min) pelleted the nucleic acids, which were resolved in 200 µl of RNase-buffer (200 µg/ml RNase A; 150 mM sodium acetate; pH 5.5) and incubated at 37°C (30 min) to remove the RNA. Precipitation of the DNA was performed

by the addition of 10 µl sodium acetate (3 M; pH 5.5) and 500 µl of pure ethanol, followed by a centrifugation (13000 rpm; 15 min). The DNA-pellet was dried and finally dissolved in 150 µl of sterile H_2O. The isolated DNA was stored at -20°C.

2.2.6.2 Isolation of plasmid DNA from yeast

For plasmid DNA isolation from *S. cerevisiae* an overnight culture of cells was harvested and resuspended in 400 µl of resuspension buffer ("GeneJET Plasmid Miniprep Kit"; Thermo Scientific). The cells were destroyed by the addition of 500 µl glass beads (Ø 0.25-0.5 mm; Roth) and shaking for 15 min at 4°C on a "Vibrax VXR basic" (IKA). Next, 250 µl of the solution were taken to isolate the plasmid DNA using the "GeneJET Plasmid Miniprep Kit" (Thermo Scientific) as described in the user's manual, starting with step 2. For elution of the DNA from the column 20 µl of water were used. Half of the eluate was transformed into *E. coli* to increase the gain of plasmid DNA.

2.2.6.3 Isolation of plasmid DNA from *E. coli*

For a first glimpse, plasmids were isolated by alkaline lysis of *E. coli* cells and precipitation of the DNA with ethanol. 1.5 ml of an *E. coli* overnight culture were centrifuged (6000 rpm; 1 min) and resuspended in 125 µl of solution1 (50 mM Tris; 10 mM EDTA; 100 µg/ml RNase A), then 125 µl of solution2 (200 mM NaOH; 1 % SDS) was added to lyse the cells. By addition of 175 µl potassium acetate (3M) precipitation of the proteins was induced, which were pelleted by centrifugation (13000 rpm; 5 min). The supernatant was mixed with 1 ml of ethanol; the mixture was centrifuged (13000 rpm; 5 min) and the pelleted plasmid DNA was dried, before it was solved in 50 µl of H_2O for further investigation.

For further studies and sequencing, plasmids that were confirmed to be correct, were isolated from *E. coli* using the "GeneJET Plasmid Miniprep Kit" (Thermo Scientific), according to the description.

2.2.7 Measurement of DNA concentrations

DNA concentrations were determined using the "Quant-iT dsDNA HS Assay kit" and the "Qubit fluorometer" (invitrogen).

2.2.8 Restriction of DNA

Restriction of DNA is needed for restriction analyses or cloning. "FastDigest" restriction enzymes (Thermo Scientific) were used here as recommended in the instructions.

2.2.9 Dephosphorylation of DNA fragments

To prevent religation of restricted vector DNA, thermosensitive alkaline phosphatase (1 U/µl; Thermo Fisher) was used for dephosphorylation as described in the user´s manual.

2.2.10 Agarose gel electrophoresis

To separate DNA fragments by size, DNA was mixed with 10 x "fast digest green buffer" (Thermo Scientific) and loaded onto agarose gels (0.8- 1 % agarose in TAE buffer (40 mM Tris; 20 mM acetic acid; 1 mM EDTA; pH 8.3, MP; MP Biomedicals Europe). Depending on objective, electrophoretic separation occurred for about 50 minutes (90 V; 400 mA; "Power Pack P25", Biometra). The "O'GeneRuler 1 kb DNA Ladder" (Thermo Scientific) was used as a molecular weight marker. The DNA was stained by incubation of the agarose gel in ethidium bromide (1 µg/ml; 20 min) and visualized by exposure to UV light (λ = 366 nm) "GelDoc-It Ultraviolet Transilluminator" (UVP).

2.2.11 Extraction and Purification of DNA from agarose gels

The respective DNA bands were cut out from the agarose gel and DNA was isolated from the agarose using the "GeneJET PCR Purification Kit" (Thermo Scientific) following the included instructions. The same kit was used for PCR product purification.

2.2.12 Ligation

Ligation of DNA fragments was done with the "T4 DNA ligase" (Thermo Scientific) according to the description. 10 µl of each ligation were subsequently transformed into *E. coli*.

2.2.13 Sequencing

Plasmid DNA was sent to SRD GmbH (Bad Homburg) for sequencing. Probe preparation was performed as requested.

2.2.14 Polymerase chain reaction (PCR)

Definite DNA fragments were amplified using the "Expand High Fidelity Polymerase" (Roche) in case of cloning, or the "DreamTaq DNA Polymerase" (Thermo Scientific) for verification of accurate strain construction. The PCR mix preparation was performed as noted in the corresponding user's manual in a "Personal Cycler" (Biometra). In a standard program a primary DNA denaturation step (94°C; 2 min) was followed by 35 cycles of: DNA denaturation (94°C; 40 sec), primer annealing (56°C; 40 sec) and DNA elongation (72°C; 1 min per kb of the product). To complete the synthesis of all DNA fragments the PRC program closes with a final elongation step (72°C; 5 min).

2.2.15 Precipitation of DNA

DNA was precipitated by addition of 2.5 volumes of ethanol and 0.1 volumes of sodium acetate (3 M; pH 5.5). Then the mixture was frozen (-20°C; 30 min), the DNA pelleted (13000 rpm; 15 min; 4°C), dried and resolved in the needed amount of H_2O.

2.2.16 Isolation of RNA

RNA was isolated using the "TRIzol Reagent" (Ambion) (Chomczynski and Sacchi, 1987). 50 ml of yeast culture were harvested at an OD_{600} of 1.0 (3000 rpm; 2 minutes). To gently destroy the cells, first spheroblasts were made by addition of 480 µl of spheroblast-buffer (0.9 M sorbitol; 0.1 M EDTA; pH 6-8), 10 µl of DTT (1 M) and 10 µl of Zymolyase

(12.5 mg/ml). Digestion at 30°C for 10 minutes reduced the risk of a cellular stress response. After the spheroblasts were pelleted (3000 rpm; 1 min), 1 ml of TRIzol reagent was added, everything mixed thoroughly and incubated for five minutes, to lyse the cells. After a centrifugation step (12.000 g; 10 min; 4°C), the supernatant was mixed with 200 µl of chloroform (15 seconds), then incubated (3 min, room temperature) and centrifuged again (12000 g; 10 min; 4°C). Of the three appearing phases (aqueous phase containing RNA; interphase, DNA; organic phase, proteins), the colorless, aqueous phase was mixed in a new cup with 500µl isopropyl alcohol to precipitate the RNA (10 min incubation). The RNA was pelleted (12000 g; 8 min; 4°C), washed with 1 ml of 75 % ethanol (7500 g; 10 min; 4°C) and dried, before being dissolved in 20 µl of DEPC water (60°C; 300 rpm; 10 min). After addition of 1 ml of 80 % ethanol, centrifugation (14000 rpm; 10 min) and drying for purification, the pelleted RNA was dissolved in 100 µl of RNase-free water (60°C; 300 rpm; 10 min). As a last purification step the solution was spun down (14000 rpm; 5 min) and the supernatant was transferred to a fresh cup.

2.2.17 Quality control of the RNA sample

To verify successful and clean RNA isolation, a RNase-free agarose gel electrophoresis (1.1 % agarose in TAE buffer, instant addition of ethidium bromide) was conducted. 1 µl of the isolated RNA was loaded. Clear and bright 18S- and 28S- rRNA bands indicate a satisfactory result.

The quality and amount of RNA was further checked by photometric measurements. The RNA isolate was diluted 1: 100 with DECP water and optical densities at 230, 260, 280 and 320 nm were determined. OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios of about 2 are ideal.

2.2.18 mRNA sequencing and analysis

Isolation of mRNA from the RNA samples and sequencing of the isolated RNA was done by BGI TECH SOLUTIONS (HONGKONG) CO., LIMITED. The company is also responsible for the initial data analysis and bioinformatics.

2.2.19 Yeast cell extract preparation

For the isolation of cell extracts from yeast, cells were grown to an OD_{600} of 1.0, harvested (3000 rpm; 2 min) and resuspended in 500 µl of potassium phosphate buffer (50mM; pH 7 (61.5 ml of 1M K_2HPO_4; 38.5 ml 1 M KH_2PO_4 ad 1 l)). 500 µl of glass beads were added and cells were shaken for 15 minutes on the "Vibrax VXR basic" (IKA). The supernatant was transferred to a new cup, then the glass beads were washed with another 500 µl of KP_i buffer, which were added to the new cup after short mixing. Centrifugation (13000 rpm; 10 min) pelleted cellular fragments, the supernatant was transferred to a new cup and protein concentration was measured, before the cell extract was used in enzyme assays.

2.2.20 Measuring protein concentrations

For determination of the protein concentration of cell extracts the following methods were used, depending on the following experiment and availability of the reagents.

2.2.20.1 Biuret

950 µl of H_2O were mixed with 50 µl of cell extract. Then 500 µl of biuret reagent was added and protein concentration was measured at an OD_{290} after 20 minutes of incubation.

2.2.20.2 Bradford

5 µl of cell extract were mixed with 95 µl of water and 900 µl of Bradford reagent (100 µg/ml Coomassie Brilliant Blue G-250; 5 % ethanol; 8.5 % H_3PO_4). After 15 minutes of incubation the OD_{595} was measured in the photometer and the protein concentration was calculated using the straight calibration line of different BSA concentrations.

2.2.20.3 Photometer

Protein concentrations were measured using the "Quant-iT protein Assay kit" and the "Qubit fluorometer" (invitrogen).

2.2.21 β-galactosidase activity assay

To determine β-galactosidase activity of cell extracts, 50 µl of extract were mixed with 950 µl of prewarmed (30°C) LacZ buffer (60 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM potassium chloride; 1 mM magnesium sulfate; 2 mg/ml ONPG). The enzymatic reaction was stopped after 15 minutes by addition of 500 µl sodium carbonate (1M) and the OD_{420} was measured to calculate the specific enzyme activity.

2.2.22 Separation of proteins

SDS Polyacrylamide gel electrophoresis was used to separate proteins ordered by their mass (Shapiro *et al.*, 1967). The prepared stacking gels contained a 3 % acrylamide, the separation gels 7.5 %.

2.2.23 Western blot analysis

Proteins separated by SDS-PAGE were transferred from the gel to nitrocellulose membranes with the "Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell" (Bio-Rad) following the instructions. Membranes were blocked with TBST (50 mM Tris-HCl; 150 mM NaCl; 0.05 % Tween 20, pH 8) containing 3% BSA. Binding of the antibodies (in 3 % BSA) occurred at room temperature (1h) or at 4°C (overnight). Remaining free antibodies were removed by washing (5 times; TBST). The fluorescent secondary antibodies were visualized with the "Odyssey Infrared Imaging System" (Li-Cor).

2.2.24 Fluorescence microscopy and cell staining

2.2.24.1 Microscopy

For GFP/m-Cherry fluorescence microscopy, yeast cells were grown in synthetic complete (SC) medium, collected by centrifugation and immediately analyzed by fluorescence microscopy. For image acquisition two microscopes were used, depending on the given problem: an "Axioplan2" microscope (Carl Zeiss AG) or a "DeltaVision Elite" fluorescence microscope (Applied Precision). Both were equipped with a "CoolSNAP HQ" camera (Roper Scientific) and appropriate filter sets.

In case of the "Axioplan2" microscope a 100x/1.45 NA Oil DIC" objective (Carl Zeiss AG) and filter sets obtained by Chroma were used. Light was provided by the LED based "SPECTRA light engine" (Lumencor).

Images acquired with the "DeltaVision Elite" microscope were deconvolved using SoftWorx; the "Axioplan2" images were deconvolved with "Huygens Essential" software (Scientific Volume Imaging B.V.). The obtained pictures were processed using "ImageJ" and/or "MetaMorph v6.2" software (Universal Imaging Corporation).

2.2.24.2 Actin staining

For the visualization of actin structures, 5 ml of an early logarithmic culture were harvested, resuspended in 100 µl PBS buffer (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4 pH 7,4) and fixed by the addition of 4% (v/v) formaldehyde (37 % stock). The sample was incubated (1h), washed twice in buffer and eluted in 50 µl. 2.5 µl Rhodamine phalloidin (stock 200 U/ml; Life Technologies) and 2.5 µl Triton X-100 (1%) were added to the fixed cells. After incubation on ice (30 min) the cells were washed three times and then stored until further use (4°C) or used in fluorescence microscopy (dsRed filer, excitation/emission 540/565 nm).

2.2.24.3 DAPI staining

50 µl of formaldehyde fixed cells (see section above) were mixed with 1 µl of DAPI (1mg/ml; Roth), incubated on ice (20 min, darkness) and washed three times with PBS buffer. Staining of DNA was visualized by fluorescence microscopy (DAPI filter, 358/461 nm) (Kapuscinski, 1995). To detect an additional GFP signal in the same cells, paraformaldehyde was used for fixation to not destroy the GFP fluorescence.

2.2.24.4 FM4-64 staining

The FM4-64 dye (*N*-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide; Life Technologies) stains yeast vacuolar membranes

selectively. Since prior fixation of cells is not necessary, logarithmic grown cells were harvested, solved in 100 µl of medium and mixed with 1µl FM4-64 (1mM). After incubation (30 min) the cells were washed three times with PBS buffer and afterwards examined by fluorescence microscopy (dsRed filter, 515/640 nm).

2.2.24.5 Calcofluor white staining

To visualize chitin in the cell wall, cells were harvested, resuspended in 100µl water and 1µl of Calcofluor white (10mg/ml, fluorescence brightener (ROTH)) was added. After incubation (5 min) cells were washed, solved in 100µl of water and stained chitin was detected by fluorescence microscopy (DAPI filter, 360/460 nm).

3 Results

3.1 Characterization of the transcription factor Rlm1 in *K. lactis*

Although a number of upstream components in CWI signaling have been described in *K. lactis* (Backhaus *et al.*, 2011), the major transcription factor KlRlm1 has not yet been characterized in detail. This thesis therefore was initiated by investigating the phenotypes of null mutants lacking the encoding *KlRLM1* gene and followed up by homologous and heterologous complementation studies.

3.1.1 KlRlm1 is important to maintain cellular integrity in the milk yeast

To explore the function of the transcription factor Rlm1 in *K. lactis*, a *rlm1* deletion mutant was constructed in a congenic *K. lactis* series (Heinisch *et al.*, 2010a) as described and illustrated before (Figure 3, Material and methods). First, the growth related phenotypes of the *Klrlm1* mutant in response to cell wall stress were analyzed. As evident from the drop dilution assays depicted in Figure 4A, deletion of *KlRLM1* led to an increased sensitivity towards the cell wall stressors Congo red, Calcofluor white and caspofungin. This phenotype is characteristic for mutants lacking components of the cell wall integrity pathway and is thus consistent with a function of KlRlm1 in this signaling pathway.

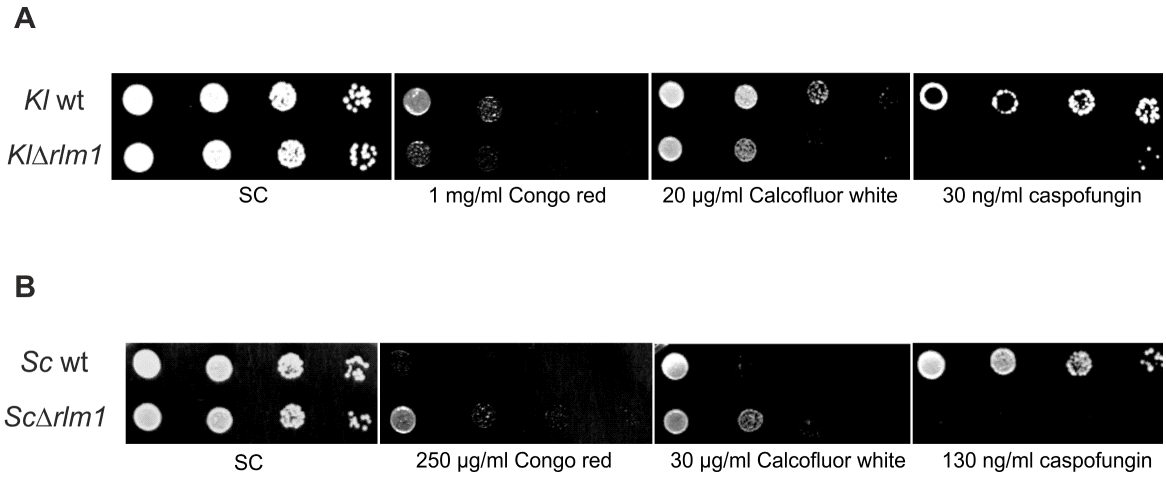


Figure 4: Deletion of *RLM1* in *K. lactis* and *S. cerevisiae* leads to cell wall defects. Serial drop dilution assay to analyze the growth of **(A)**: The *K. lactis* wild type (KHO69-8C) and the *Klrlm1* deletion mutant (KLSMO3-3). And **(B)**: The *S. cerevisiae* wild type (HD56-5A) and the *Scrlm1* deletion mutant (HMZ13-C).

To further confirm these results, growth of the wild type and the *Klrlm1* null mutant were also tested in liquid culture in the presence of the glucan synthase inhibitor caspofungin (Figure 5). Clearly, the *Klrlm1* deletion mutant displays an increased sensitivity to caspofungin in this assay as well, further substantiating the role of KlRlm1 in cell wall integrity signaling in *K. lactis.*

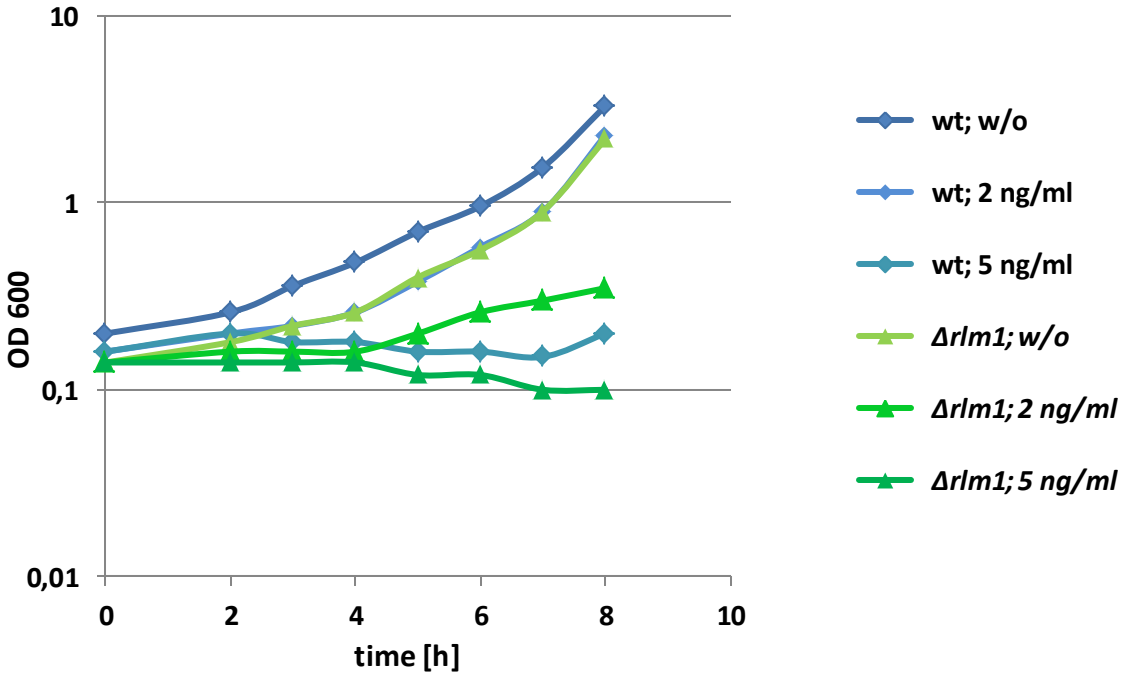


Figure 5: Deletion of *KlRLM1* results in increased caspofungin sensitivity. A *K. lactis* wild type strain (wt; strain KHO69-8C) and a *Klrlm1* deletion strain (*Δrlm1;* strain KLSMO3-3) were grown in SC medium in the presence of different caspofungin concentrations. Optical density of the cultures was determined in a photometer.

In contrast to this behavior, previous studies on Rlm1 in *S. cerevisiae* showed that a deletion of the *ScRLM1* gene results in increased resistance to Calcofluor white, Congo red and Zymolyase (Lopez-Garcia *et al.*, 2010) and cells deleted for *ScRLM1* are not sensitive to higher temperatures, e.g. 37°C (Watanabe *et al.*, 1995). To confirm these results for the genetic background of the strain used herein, serial drop dilution assays were performed with the *Scrlm1* deletion mutant, to test its response to cell wall stress. The mutant was sensitive to caspofungin, but hyperresistant towards Congo red and Calcofluor white as compared to the wild type (Figure 4B). Thus, the *ScΔrlm1* mutant shows a contradictory

growth behavior on Calcofluor white and Congo red compared to the deletion mutants of upstream ScRlm1 effectors, as well as to the phenotypes of the *Klrlm1* deletion strain.

3.1.2 Cross-complementation analysis of Rlm1

To investigate possible differences of Rlm1 function between the two yeast species, which would explain the partially different phenotypes, heterologous complementation studies were performed. For that, *KlRLM1* (as a control) and *ScRLM1* were expressed in *the Klrlm1* deletion mutant from single copy vectors. Either the native or the heterologous *RLM1* promoter was employed to drive the expression of the gene. As expected, introduction of the wild-type *KlRLM1* gene under the control of its native promoter complemented the

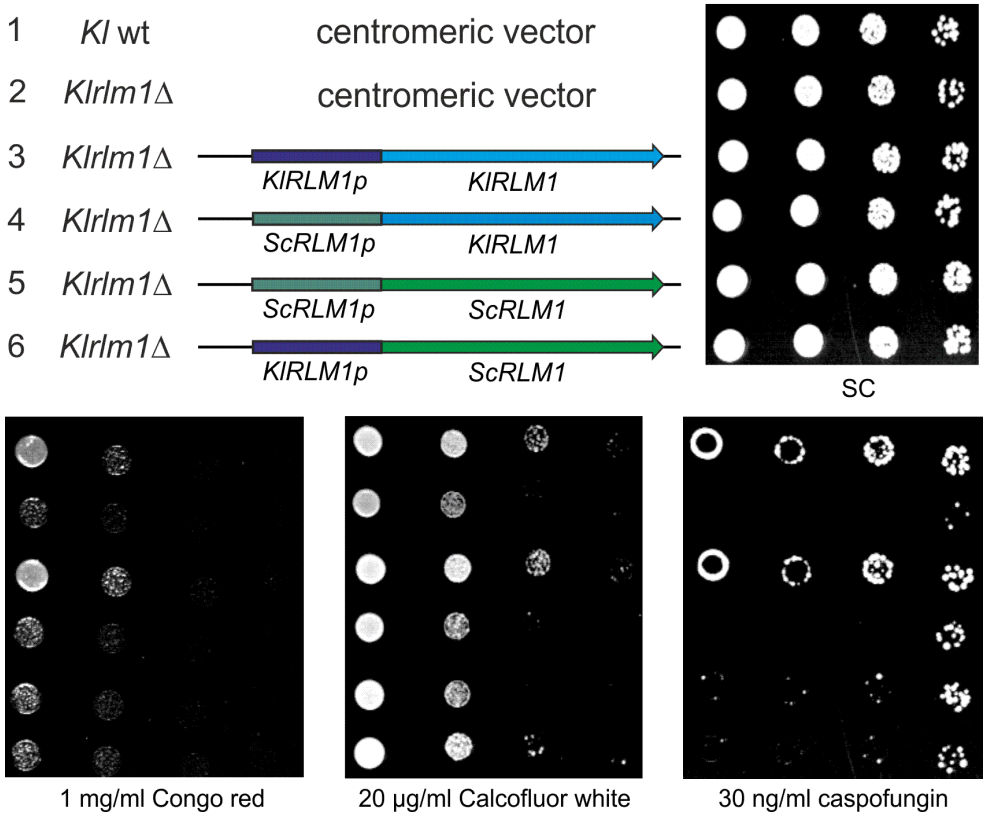


Figure 6: *ScRLM1* cannot complement the *Klrlm1* deletion. Serial drop dilution assays on SC-LEU medium of a *K. lactis* wild type (**1**, Kl wt, KHO69-8C) and a *Klrlm1* deletion strain (**2-6**, *Klrlm1Δ*, KLSMO3-3), which harbors different centromeric plasmids. Plasmids employed (from top to bottom): pCX20 (**1+2**, vector); pSMO118 (**3**, *KlRLM1p-KlRLM1*); pSMO143 (**4**, *ScRLM1p-KlRLM1*); pSMO135 (5, *ScRLM1p-ScRLM1*) and pSMO140 (**6**, *KlRLM1p-ScRLM1*).

growth defects of the null mutant (Figure 6, third row from top). On the other hand, neither the *KlRLM1* gene when placed under the control of the *ScRLM1* promoter, nor the *ScRLM1* gene, expressed from its native or the *K. lactis* promoter, complemented the phenotypes of the *Klrlm1* deletion. (Figure 6, lower three rows). This demonstrates that ScRlm1 does not function in *K. lactis,* indicating a species-specific action of the transcription factor. Moreover, transcription driven by the *ScRLM1* promoter is also not sufficiently strong in *K. lactis* to support proper expression of the native *KlRLM1* gene.

Vice versa, the two genes were expressed under the control of the native *ScRLM1* promoter in the *Scrlm1* deletion mutant. As expected, drop dilution assays showed that expression of *ScRLM1* complemented the phenotypes of the deletion mutant, i.e. it restored caspofungin resistance and reversed the hyperresistance towards Congo red and Calcofluor white. In contrast, expression of KlRLM1 carried on a single copy *CEN/ARS* vector did not (Figure 7A, last row from top).

Interestingly, expression of *ScRLM1* from the heterologous *K. lactis RLM1* promoter in the *ScΔrlm1* mutant resulted in a partial complementation of the growth defect on caspofungin (Figure 7B). Thus, the *K. lactis RLM1* promoter drives gene expression also in *S. cerevisiae*. Intriguingly, the *KlRLM1p-ScRLM1* construct also enhanced the hyperresistance of the Scrlm1 deletion towards Congo red and Calcofluor white. Since expression of *ScRLM1* in the deletion should result in a restoration of sensitivity towards these drugs, this observation currently lacks an explanation.

In summary, ScRlm1 and the *ScRLM1* promoter are not functional in *K. lactis;* and neither is KlRlm1 in *S. cerevisiae*. However, the *K. lactis RLM1* promoter can at least partially be used to drive gene expression in *S. cerevisiae,* but may lead to a certain degree of misregulation of cell wall integrity signaling upon stress applied by Congo red and Calcofluor white, but not in response towards caspofungin.

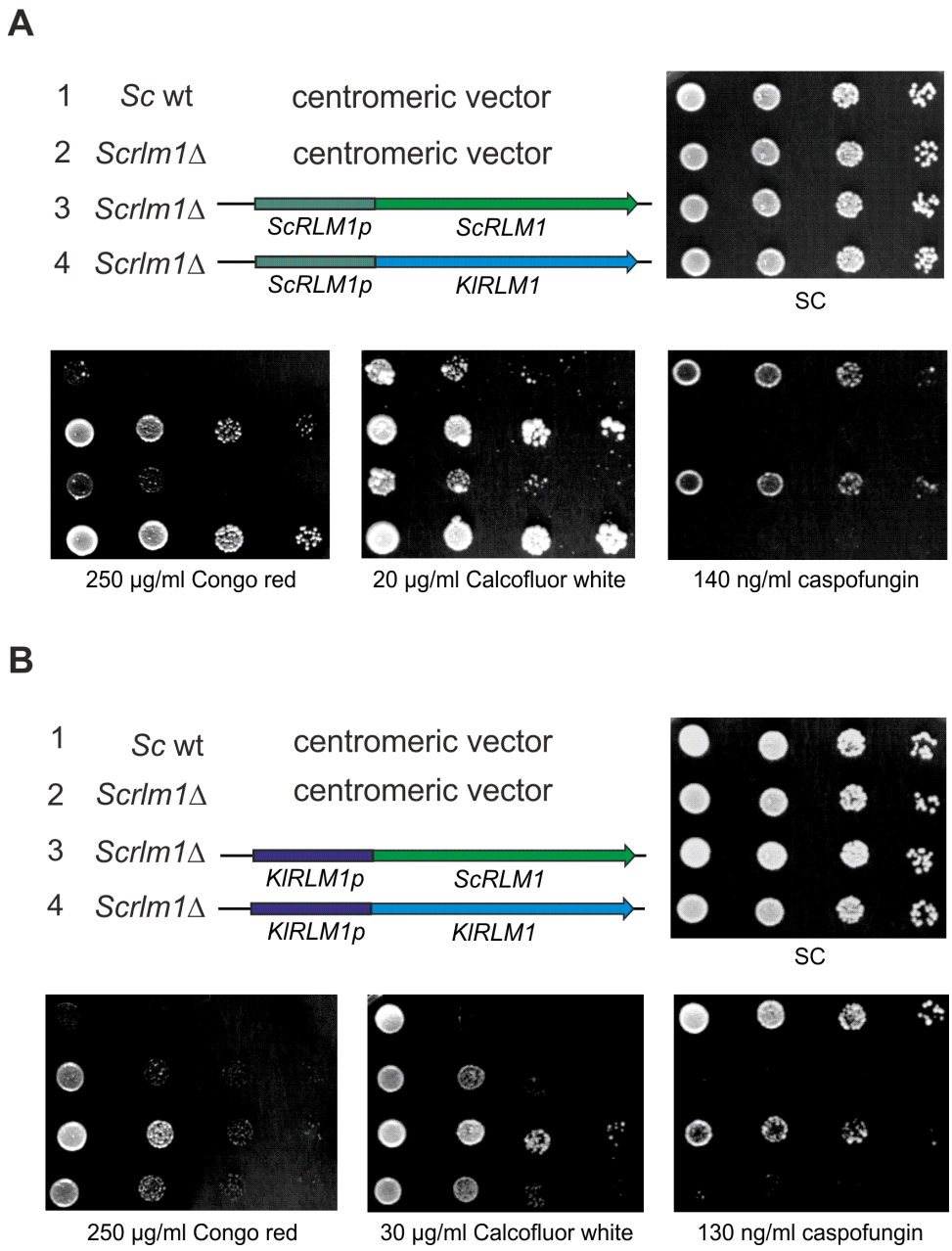


Figure 7: *KlRLM1* cannot complement the *Scrlm1* deletion. Serial drop dilution assays of the *S. cerevisiae* wild type strain (**1**, Sc wt; HD56-5A) and the *Scrlm1* deletion mutant (**2-4**, *Scrlm1Δ*; HMZ13-C) on SC-LEU medium. Centromeric plasmids employed as follows: pCSe24 (**1+2**, centromeric vector); pSMO134 (*ScRLM1p-ScRLM1*, row **3A**), pSMO139 (*ScRLM1p-KlRLM1,* row **4A**) pSMO137 (*KlRLM1p-ScRLM1*, row **3B**) and pSMO113 (*KlRLM1p-KlRLM1*, row **4B**).

3.1.3 Localization studies on KlRlm1

Since the Rlm1 homologs seem to function only in their native species, *KlRLM1* was fused to *GFP* to determine the localization of the fusion protein in *K. lactis* and *S. cerevisiae*. Wild type cells of both organisms were fixed and treated with the fluorescent dye DAPI, which specifically binds to nucleic acids, for nuclear staining. KlRlm1-GFP was expressed from single copy plasmids in both strains. Fluorescence microscopy revealed that the

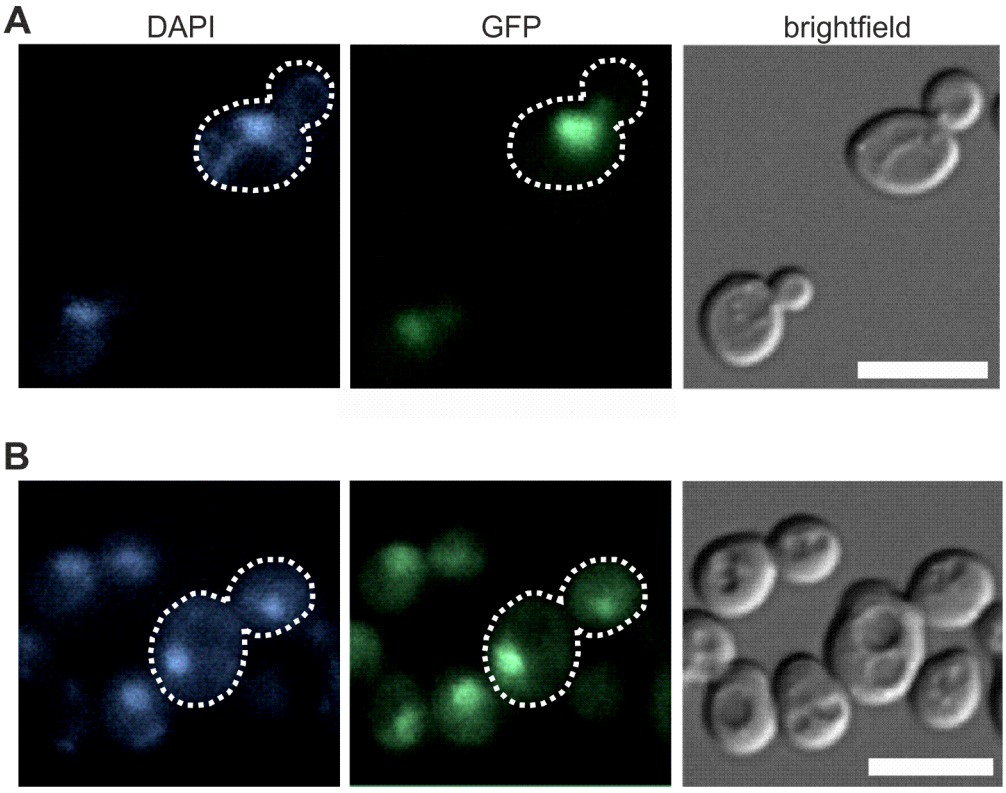


Figure 8: KlRlm1 localizes to the nucleus in *K. lactis* and *S. cerevisiae.* Wild type cells of **(A)** *K. lactis* (KHO69-8C) and **(B)** *S. cerevisiae* (HD56-5A) expressing the *KlRLM1* gene fused to *GFP* from centromeric plasmids (pSMO125 and pSMO142, respectively). Cells were fixed and nuclei were stained with the fluorescent dye DAPI. Left panels: Fluorescence pictures obtained with the DAPI filter. Middle: Fluorescence images of KlRlm1-GFP obtained with the FITC/GFP filter. Right: Corresponding brightfield images. Scale bar: 5µm.

transcription factor localized to the nuclei in both yeasts (Figure 8) as expected and previously shown for ScRlm1 (Huh *et al.*, 2003). Thus, the lack of heterologous complementation cannot simply be attributed to a mislocalization of KlRlm1 in *S. cerevisiae*. In addition, these experiments demonstrate that KlRlm1 localizes properly within the nucleus in *K. lactis*, as would be expected from its proposed function as a transcriptional activator.

3.1.4 Identification of domains conferring species specificity to Rlm1

To further investigate why Rlm1 failed to complement the deletion phenotypes in the heterologous yeast, chimeric genes were constructed in order to identify the species-specific domains. All chimeras were expressed under the control of respective native promoters in *K. lactis* and in *S. cerevisiae rlm1* deletions. Two constructs encode one half of each Rlm1 protein. In two additional constructs the first 300 bases were exchanged, so that they encode the MADS-box of the heterologous protein, which is located at the N-terminus and presumed to mediate DNA binding. Thus, the question was if the N-terminal MADS-box is responsible for the previously observed differences, or if it is the transcriptional activation function triggered by Mpk1 phosphorylation within the C-terminal part of the protein.

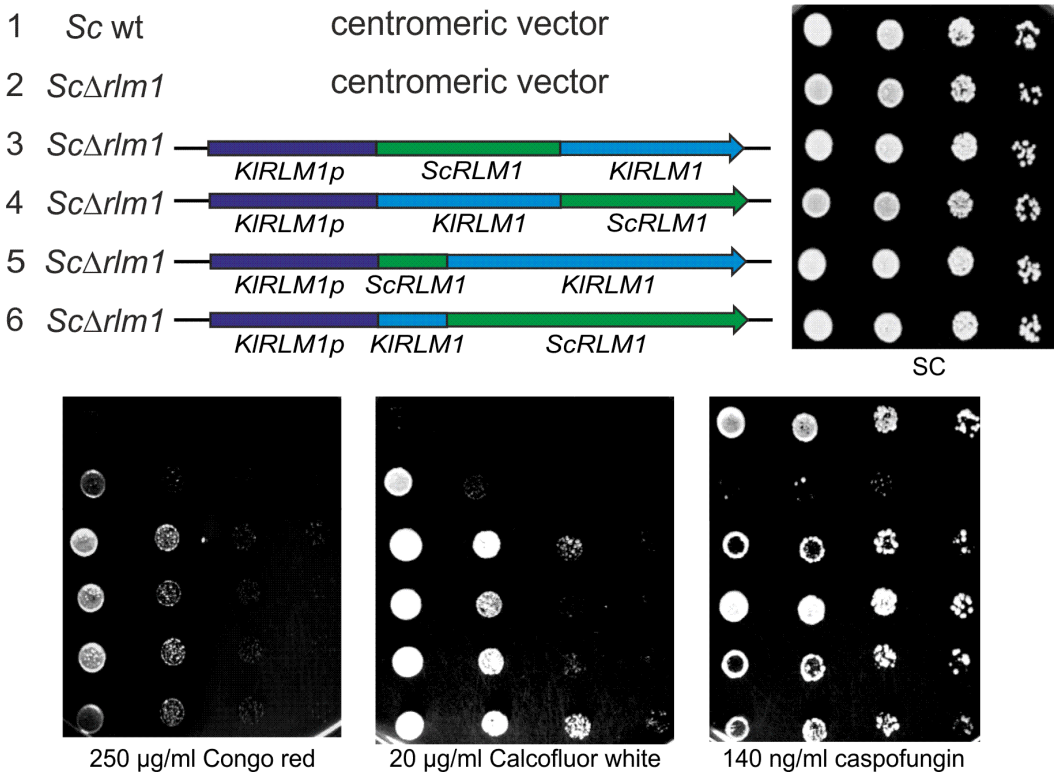


Figure 9: Expression of chimeric *RLM1* genes from the heterologous *KlRLM1* promoter results in hyperresistance towards cell wall stress in *S. cerevisiae*. Serial drop dilution assays of the *S. cerevisiae* wild type strain (**1**, Sc wt; HD56-5A) and the *Scrlm1* deletion mutant (**2-6**, Sc*rlm1Δ;* HMZ13-C) on SC-LEU medium. The four chimeras are expressed from centromeric plasmids and illustrated in the upper right corner The *ScRLM1* part is shown in green, the *KlRLM1* part in blue. Plasmids used from top to bottom: pCXJ20 (vector), pSMO145 (*KlRLM1p-ScRLM1/KlRLM1,* row **3**), pSMO116 (*KlRLM1p-KlRLM1/ScRLM1,* row **4**), pSMO148 (*KlRLM1p-Sc100RLM1/KlRLM1,* row **5**) and pSMO132 (*KlRLM1p-Kl100RLM1/ScRLM1,* row **6**).

Expression of the four chimeric *RLM1* genes from the *K. lactis* promoter in a *Scrlm1* deletion mutant displayed similar results as obtained for the complete *ScRLM1* gene from this promoter. All four constructs complemented the *rlm1* deletion phenotype under caspofungin stress and led to hyperresistance towards Congo red and Calcofluor white; so all chimeras enhanced the *rlm1* deletion phenotype under these stress conditions (Figure 9, see also Figure 7B). Using the *K. lactis* promoter also confirms that all tested chimeric proteins are generally functional, as they resemble the phenotype observed for expression of the full-length *ScRLM1* gene (Figure 9).

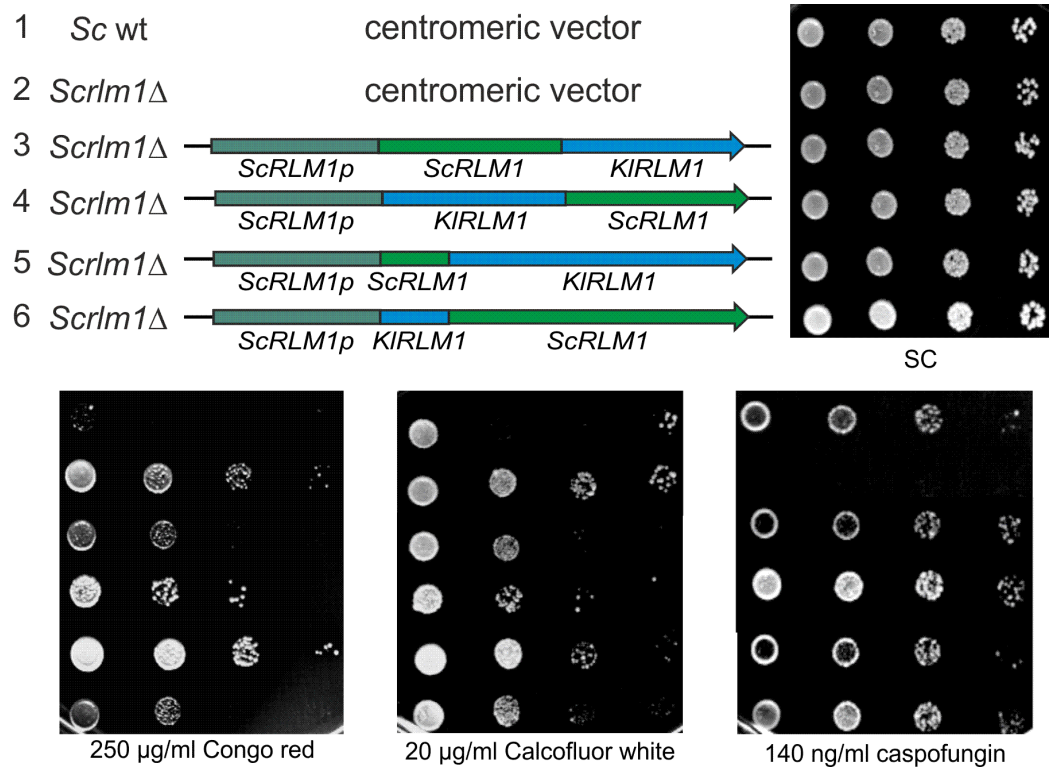


Figure 10: Expression of chimeric *RLM1* genes from the *ScRLM1* promoter restores wild type growth of the *Scrlm1* deletion mutant. Serial drop dilution assays of the *S. cerevisiae* wild type strain (**1**, Sc wt; HD56-5A) and the *Scrlm1* deletion mutant (**2-6**, Sc*rlm1Δ;* HMZ13-C) on SC-LEU medium. The four chimeras are expressed from centromeric plasmids under control of the *ScRLM1* promoter in the mutant and illustrated in the upper right corner of the figure. The *ScRLM1* part is shown in green, the *KlRLM1* part in blue. The plasmids used here were: pCse24 (**1+2**, centromeric vector); pSMO136 (*ScRLM1/KlRLM1,* row **3**), pSMO34 (*KlRLM1/ScRLM1,* row **4**), pSMO126 (*Sc100RLM1/KlRlm1,* row **5**) and pSMO130 (*Kl100RLM1/ScRlm1,* row **6**), respectively.

The expression of the four chimeric constructs from the *ScRLM1* promoter in a *Scrlm1* null mutant and the respective drop dilution assays are shown in Figure 10. On plates containing Congo red or Calcofluor white only one out of four constructs, the one carrying the ScRlm1 MADS-box, did not complement the *Scrlm1* deletion phenotype and caused a mild hyperresistance. In contrast, the same construct did perfectly complement the *Scrlm1* deletion phenotype under caspofungin stress, whereas expressing the two constructs that contain the *K. lactis* Rlm1 MADS-box led to hyperresistance. The fourth chimera, encoding the KlRlm1 C-Terminus, complemented the *rlm1* deletion phenotype partially under all stress conditions.

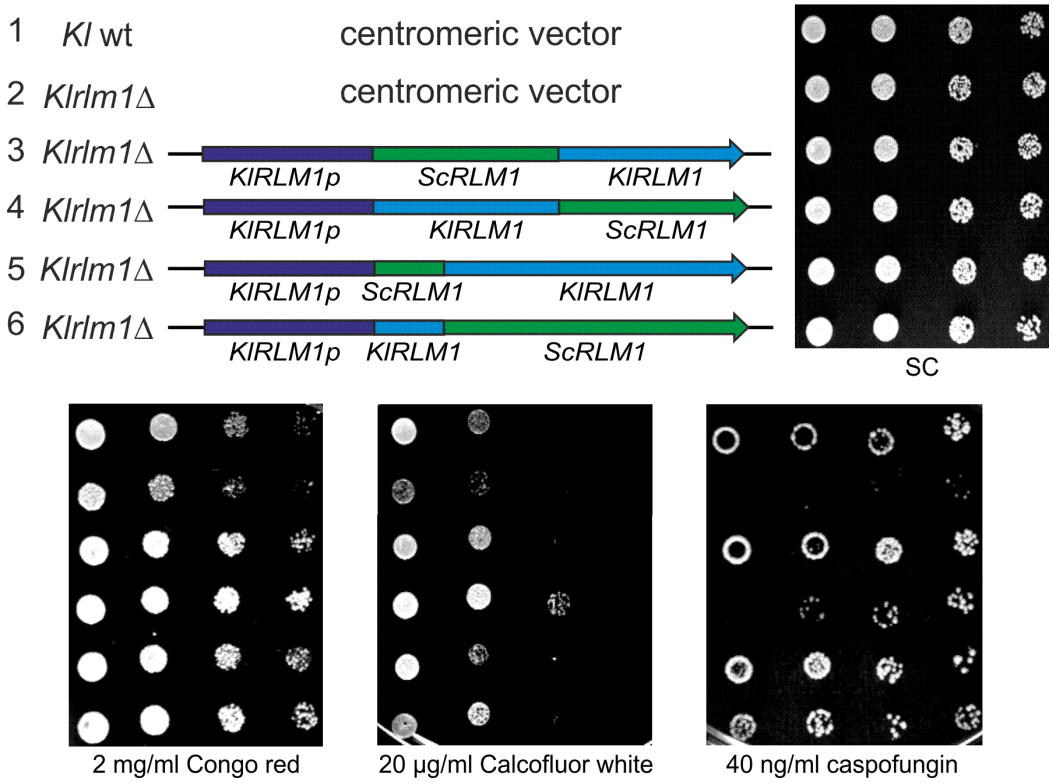


Figure 11: Expression of chimeric *RLM1* genes from the *KlRLM1* promoter restores wild type growth of the *Klrlm1* deletion mutant. Serial drop dilution assays of the *K. lactis* wild type strain (*Kl* wt; KHO69-8C) and the *Klrlm1* deletion mutant (*Klrlm1Δ;* KLSOM3-3) on SC-LEU medium. The four chimeras were expressed from centromeric plasmids under control of the *KlRLM1* promoter in the mutant and are illustrated in the right corner of the figure. The *ScRLM1* part is shown in green, the *KlRLM1* part in blue. The plasmids used here were: pCXJ20 (vector), pSMO146 (*ScRLM1/KlRLM1,* row 3), pSMO121 (*KlRLM1/ScRLM1,* row 4), pSMO147 (*Sc100RLM1/KlRlm1,* row 5) and pSMO133 (*Kl100RLM1/ScRlm1,* bottom row), respectively.

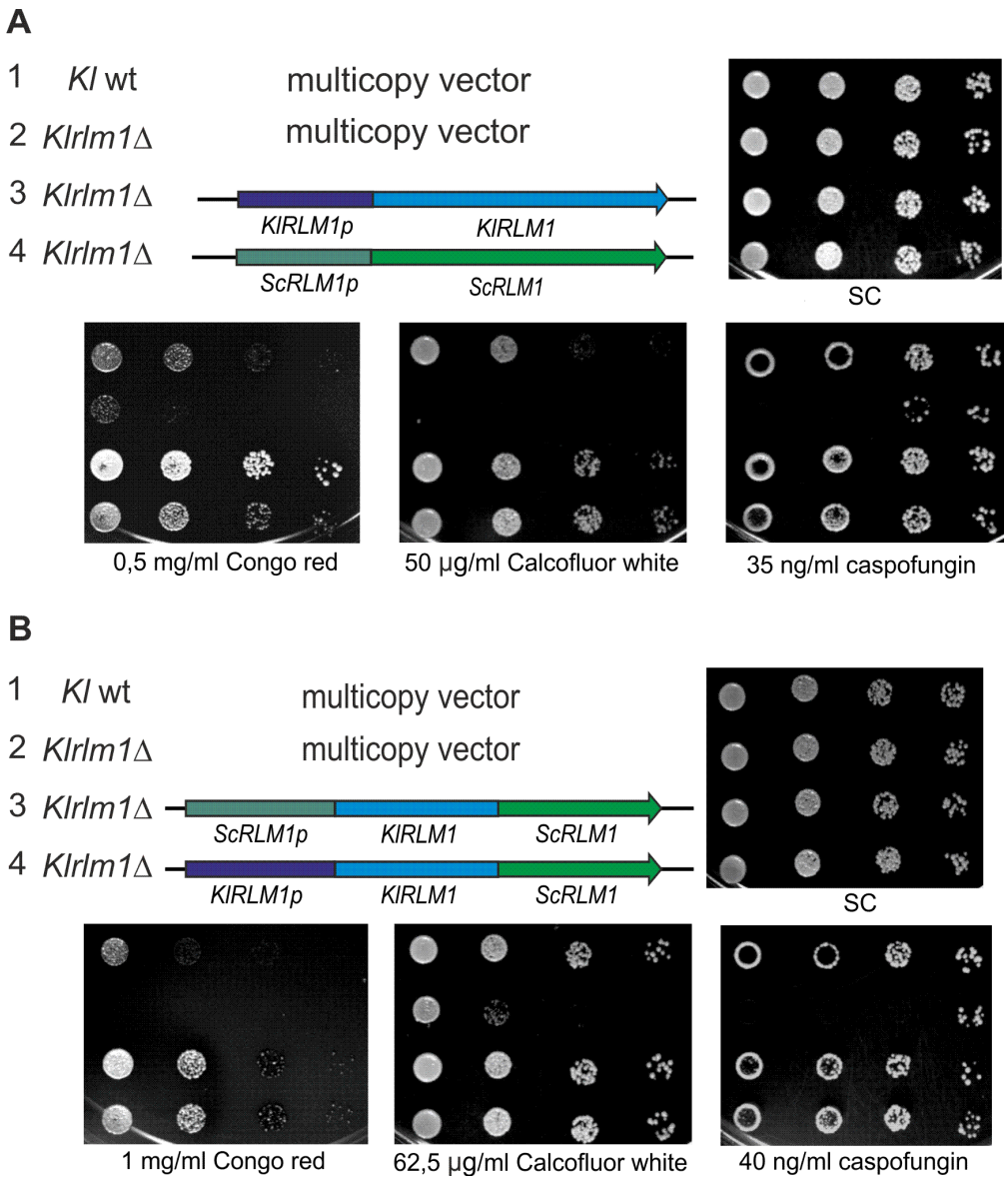


Figure 12: Expression of the chimeric genes from multicopy vectors *Klrlm1* phenotype. Serial drop dilution assays to analyze the growth of the *K. lactis* wild type (**1**, *Kl* wt; KHO69-8C) and the *Klrlm1* deletion mutant (**2-4**, Kl*rlm1Δ;* KLSMO3-3). Wild type and chimeric genes are expressed from multi copy plasmids. **(A)** Expression of pSMO113 (*KlRLM1p-KlRLM1,* row **3**) and pSMO134 (*ScRLM1p-ScRLM1,* row **4**). **(B)** Expression of chimeras, pSMO34 (*ScRLM1p-KlRLM1/ScRLM1,* row **3**) and pSMO116 (*KlRLM1p-KlRLM1/ScRLM1,* row **4**).

Expression from the native promoter showed that all the chimeric constructs are functional in *K. lactis* (Figure 11). All chimeras led to hyperresistance towards Congo red exposure. A similar phenotype can also be observed for Calcofluor white stress, with the

construct that encodes the *S. cerevisiae* Rlm1 MADS-box being less resistant than the others. Upon treatment with caspofungin both chimeras encoding the *S. cerevisiae* N-terminus show strong growth, as well as the construct containing the *K. lactis* MADS-box. In contrast, the chimera encoding the N-terminal half of *K. lactis* Rlm1 and the C-terminal half of ScRlm1 shows only a slight growth and is not able to complement the *KlRLM1* deletion. Interestingly, this construct was the best adapted to caspofungin stress when expressed in *S. cerevisiae* under the control of both the homologous and the heterologous promoter. Since all chimeric Rlm1 constructs are functional in *K. lactis* but not the full length *S. cerevisiae* wild type gene, we investigated whether *ScRLM1* can complement the *Klrlm1Δ* defect when expressed from multicopy vectors.

Expression of the transcription factor from multicopy pKD1 plasmids in *K. lactis* clearly proved the importance and sensitivity of regulatory processes. High expression of both full-length genes, *KlRLM1* and *ScRLM1*, under control of their native promoters suppressed the phenotype of the deletion entirely and even induced mild hyperresistance towards Congo red and Calcofluor white treatment (Figure 12A). This result prompted us to investigate whether the chimera encoding the N-terminal half of *K. lactis* Rlm1 was also able to complement the *Klrlm1* deletion when introduced on multicopy vectors. As evident from Figure 12B it complemented the phenotypes of the Klrlm1 deletion and even conferred hyperresistance towards Congo red stress. Interestingly, these phenotypes did not dependent on the promoter used in this case. Apparently, the genes are sufficiently well expressed from the *ScRLM1*, when the gene dosage was increased by plasmid copy number.

3.1.5 Target genes of Rlm1 in the milk yeast

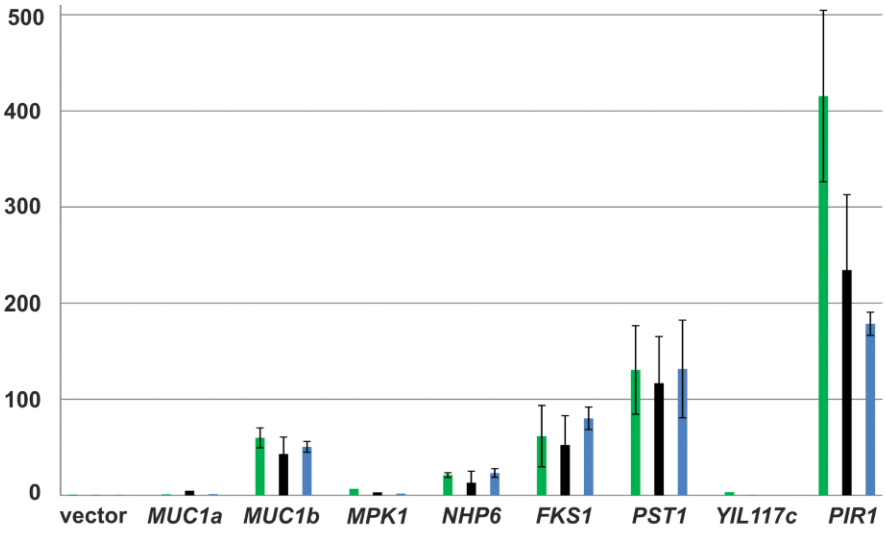
Since the *KlRLM1* deletion mutant is sensitive to caspofungin, Congo red and Calcofluor white as compared to the wild type and this phenotype is characteristic for mutants affected in the cell wall integrity pathway (see Figure 4 and Figure 5). In contrast a deletion of the *ScRLM1* gene results in increased resistance to Calcofluor white, Congo red but sensitive to caspofungin (see Figure 4; Lopez-García et al., 2010). In addition, the native transcription factor only functions properly in the native yeast species. These

results suggest that different target genes may be activated by Rlm1 in *K. lactis* and in *S. cerevisiae*. Therefore two approaches were followed to determine such target genes:
i) The promoter regions of *K. lactis* homologs of known *S. cerevisiae* target genes were tested for their expression in a *Klrlm1* deletion mutant versus a wild type background. ii) The entire transcriptomes of a *Klrlm1* deletion and a wild type strain were compared.

3.1.5.1 Detection of KlRlm1 responsive genes

The targets of ScRlm1 in the baker's yeast have been intensively studied by a variety of different approaches, such as Northern blot analyses, bioinformatics on the recognition sequence in the promoters and transcriptome analysis using DNA chips (Jung and Levin, 1999; Boorsma *et al.*, 2004; Garcia *et al.*, 2009). In *K. lactis* such studies have not yet been performed in relation to CWI signaling. Hopefully, identification of the KlRlm1 target genes will therefore increase the understanding of the complex response to different stresses in both yeast species and further allude to the question of species-specific functions.
In a first approach to identify KlRlm1 target genes, a set of *K. lactis* genes was chosen, whose *S. cerevisiae* homologs are known to be transcriptionally upregulated by activation of the CWI pathway: *MUC1a, MUC1b, MPK1, NHP6A, FKS1, PST1, YIL117c, PIR1, BGL2, YLR194c* (Jung and Levin, 1999). Plasmids were constructed, which contain the promoter regions of these genes fused to the bacterial *lacZ* reporter gene. Thus, they should lead to an increased ß-galactosidase activity in strains where the CWI pathway is activated and to a decreased activity in strains where components are defective (e.g. in a *Klrlm1* deletion). The map of one construct, plasmid pJJH1630, which carries the *PIR1* promoter fused to the *lacZ* gene, is shown as an example in Figure 13C. In order to analyze putative transcriptional regulation of the genes by Rlm1, a *Klrlm1* deletion mutant and a wild-type strain were transformed with the set of different reporter plasmids. The specific β-galactosidase activities were tested in these transformants and are shown in Figure 13.

A

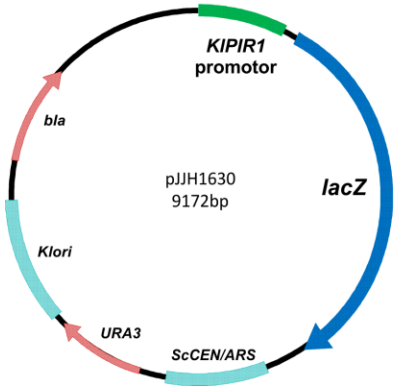


B

	vector	*KlMUC1a*	*KlMUC1b*	*KlMPK1*
wt	0,9	1,2	59,9	6,9
Δbck1	0,8	4,8	43,1	3,2
Δrlm1	0,7	1,3	50,6	1,9

	KlNHP6a	*KlFKS1*	*KlPST1*	*KlYIL117c*	*KlPIR1*
wt	21,1	61,6	130,5	3,5	415,4
Δbck1	13,2	52,4	116,8	0,6	234,4
Δrlm1	23,3	80,2	131,5	0,2	178,6

C



D

Tested promotor	*lacZ* expression altered upon *KlRLM1* deletion
MUC1a	X
MUC1b	X
MPK1	X
NHP6	X
FKS1	X
PST1	X
YIL117c	X
PIR1	YES
BGL2	X
YLR194c	X

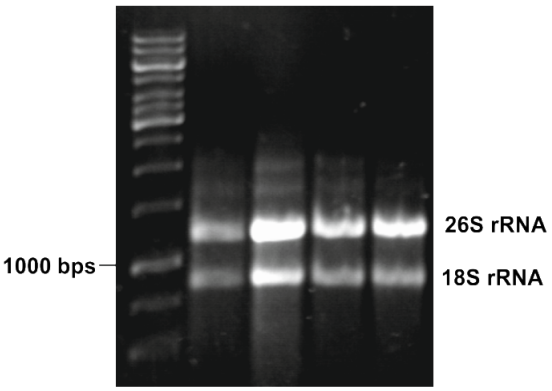
Figure 13 *PIR1* is a target of Rlm1 in *K. lactis*. Promoter fragments were cloned into the vector pJJH1564A. Transformants were grown in selective medium and yeast extract were prepared for enzymes and protein determinations. **(A+B)** Measurement of the specific β-galactosidase activity of crude extracts of transformants of the the *K. lactis* wild type (wt, KDR1-6A, shown in green) the *bck1* deletion (*Δbck1*, KOH90-6A, black) and the *rlm1* deletion (*Δrlm1*, KLSMO5-4B, blue) employing different plasmids. The data were obtained in three independent experiments. The activities are given in mU/mg protein and were determined from pooled transformants grown in SC-URA medium + 1M sorbitol at 30°C to an OD_{600} of about 1 **(C)** Schematic illustration of the plasmid pJJH1630. **(D)** List of promoters that were tested for Rlm1 dependence. Only expression from the *KlPIR1* promoter is regulated by Rlm1.

Clearly, only the *KlPIR1* promoter showed a reduced expression in a *Klrlm1* deletion mutant as compared to a wild-type strain, which was also confirmed by the reduction observed in a *Klbck1* deletion (Figure 13 A+B). Consequently *PIR1* is a target of Rlm1 in *K. lactis* as it is in *S. cerevisiae* and gene expression of *PIR1* is upregulated by the transcription factor, which encodes for a highly glycosylated cell wall protein (Toh-e *et al.*, 1993). In contrast, expression of the *lacZ*-constructs was either not changed in the *Klrlm1* deletion as compared to the wild-type cells, or it could not be detected at all as judged from the ß-galactosidase activities. This indicates that transcription of the respective genes is not Rlm1 dependent in *K. lactis* and that these genes are not regulated in response to cell wall stress in the milk yeast.

3.1.5.2 Comparative transcriptome analyses

The lacZ reporter assays described above do not permit the identification of specific KlRlm1 target genes. Therefore the total mRNA pool was sequenced to quantify expression levels on a whole genome basis and to identify genes which are regulated by KlRlm1 in the milk yeast.

For this purpose, RNA was isolated from two independent cultures of a *K. lactis* wild-type strain and of a *Klrlm1* deletion mutant, respectively. One culture of each was treated for three hours with 1 ng/ml caspofungin to induce cell wall integrity signaling. This concentration of the stressor was chosen after prior determination of growth of the *Klrlm1* deletion, which was not yet inhibited at 1 ng/ml caspofungin (also compare Figure 5). The quality and quantity of the isolated total RNA was checked by agarose gel electrophoresis and photometric determinations (Figure 14). Further quality controls, purification of the polyadenylated mRNA and sequencing were performed by BGI Tech/Hong Kong China (all data in the attachment).



	WT	WT + caspofungin	*Δrlm1*	*Δrlm1* + caspofungin
230nm	0,113	0,192	0,123	0,287
260nm	0,275	0,489	0,319	0,717
280nm	0,158	0,283	0,184	0,411
320nm	-0,003	-0,002	0,002	0
260/230 ratio	2,4	2,53	2,58	2,5
260/280 ratio	1,72	1,72	1,73	1,74
concentration [ng/ul]	1111	1967	1284	2868

Figure 14: Evaluation of the mRNA sample qualities. 1 µl of the isolated RNA was separated on an agarose gel (0.7 %). The 26S and 18S rRNA bands are indicated. The table shows the results of spectrophotometric determinations of the RNA probes, diluted 1:1000 in DEPC water. The quantity of RNA in the sample is high (OD_{260}), contamination with proteins (OD_{280}) or phenol (OD_{230}) is below the critical level, which is also indicated by the OD260/230 ratio > 1.7 and the OD260/280 ratio 1.8-2.0 respectively.

Bioinformatics of the sequenced mRNA revealed only one gene that was upregulated in the *Klrlm1* deletion mutant after three hours of caspofungin treatment, with the generic name *KLLA0F26818g* from the *K. lactis* genome database (http://www.genolevures.org). This ORF in the *K. lactis* genome encodes a small, yet uncharacterized protein. No homolog was identified so far in any other fungal species yet.

Caspofungin treatment altered the expression of only five genes in the wild type, as compared to untreated cells. Four of them, namely *KlFLO1*, *KlGPI15*, *KLLA0C11517g* and *KLLA0C00374g* were upregulated under caspofungin stress and one was downregulated, namely *KLLA0E16853g*.

Three of the four genes that are upregulated upon caspofungin treatment actually encode homologs of cell wall proteins of *S. cerevisiae*, whereas *KLLA0C11517g* is related to mating

type determination. Although these genes fall into the expected target range, caspofungin treatment had only a weak effect, suggesting that the concentration employed was too low to trigger expression of all genes responsive to this drug.

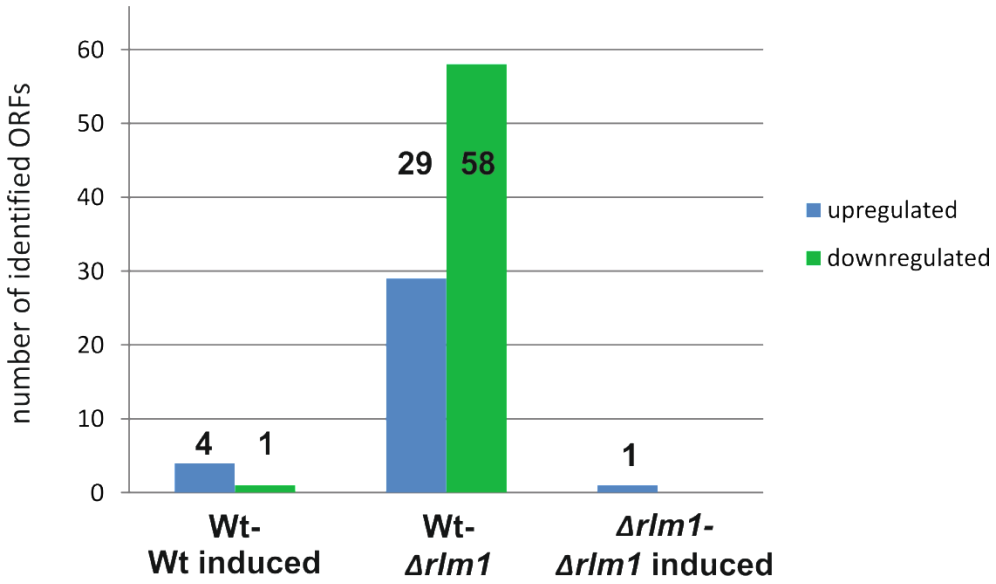


Figure 15: Classification of the genes identified by mRNA sequencing. ORFs whose transcripts are repressed/activated at least 2-fold were counted.

A much larger number of differentially regulated genes appeared in the comparison of the transcriptome of the wild type versus that of the *Klrlm1* deletion. 29 genes were significantly upregulated in the absence of Rlm1, whereas 58 were downregulated. The genes whose expression is upregulated are listed in Table 7; those which are downregulated are listed in Table 8.

Since ScRlm1 has been described as a transcriptional activator, one can assume that its lack in the *Klrlm1* deletion affects expression of these target genes in an indirect manner, i.e. the lack of expression of primary target genes may lead to perturbations of the cell wall integrity, which in turn are compensated by the proteins encoded by the upregulated genes. Interestingly, the largest group of such upregulated genes comprises of the *FLO* genes, which all encode different lectin-like proteins, the so called flocculins. As stated above, the *FLO1* gene was also found to by upregulated in response to caspofungin stress, thus further pointing to a compensatory function of the encoded flocculin to cell wall perturbances. A second group of genes with an increased mRNA level in the *RLM1*

deletion mutant are the *PHO* genes, which encode homologs of *S. cerevisiae* acid phosphatases. Three different genes with homology to the highly similar *S. cerevisiae* genes *PHO3/PHO5* were detected in the analysis, though there is a higher identity to *ScPHO3*. Their relation to cell wall integrity is not clear yet.

Table 7: Genes with an increased expression in the *Klrlm1* deletion as compared to wild type.

Identified ORF	*S. cerevisiae* homolog	Predicted function	Gene length	Wild type expression	*Δrlm1* expression	P-value
KLLA0D00264g	*YAL063c; FLO9*	flocculin	3015	0	10473	0
KLLA0D00275g	*YAL063c; FLO9*	flocculin	1176	0	248	1,91E-73
KLLA0D00269g	/	/	483	0	27	2,60E-05
KLLA0E20637g	*YER106W; MAM1*	chromosome attachment during meiosis	936	0	13	7,21E+00
KLLA0D00258g	*YBR092C; PHO3**	thiamin biosynthesis and uptake	1455	2	190	2,45E-52
KLLA0F22319g	*YDR213W; UPC2*	transcription of sterol biosynthetic genes	612	10	348	4,39E-90
KLLA0B14498g	*FLO1,FLO5*	flocculin	2301	24358	124180	0
KLLA0F06028g	*YOR003W; YSP3*	precursor to the subtilisin-like protease	1527	9	42	5,52E-02
KLLA0B00308g	*YAR050W; FLO1*	flocculin	1350	11	46	5,08E-03
KLLA0C19206g	/	oxygenase in *A. fumigatus*	1536	17	69	1,09E-03
KLLA0B11363g	*YIL107C; PFK26*	6-phosphofructo-2-kinase	819	19	75	3,36E-05
KLLA0F08745g	/	involved in cell wall biogenesis and oxidative stress response	1062	39	146	9,61E-13

Identified ORF	*S. cerevisiae* homolog	Predicted function	Gene length	Wild type expression	*Δrlm1* expression	P-value
KLLA0B00286g	*YBR092C; PHO3**	thiamin biosynthesis and uptake	1410	31	106	2,95E-07
KLLA0B10219g	/	/	540	46	152	2,07E-11
KLLA0B14916g	*YHR211W; FLO5*	flocculin	1653	14	43	3,10E+00
KLLA0B10373g	*YBR250W; SPO23*	meiosis	1704	114	331	2,08E-24
KLLA0C11517g	/	cell wall protein; PIR protein	1563	43	117	1,54E-04
KLLA0F04169g	*YBR296C; PHO89**	Na+/Pi cotransporter	1752	478	1247	4,89E-87
KLLA0B00517g	*YDR515W; SLF1*	regulation of mRNA translation	627	52	123	3,42E-04
KLLA0B14476g	*YGR234W; YHB1*	lavohemoglobin, putative role in oxidative stress response	1188	4019	9268	0
KLLA0A00176g	*YBR092C; PHO3**	thiamin biosynthesis and uptake	1410	35	79	5,50E-01
KLLA0E14477g	*YOR381W; FRE3*	ferric reductase	2151	3105	6929	0
KLLA0B04928g	*YLR445W; GMC2*	meisosis; response to alpha factor	537	31	69	2,76E+00
KLLA0F02610g	/	/	294	42	93	1,23E+00
KLLA0E09923g	*YBR200W; BEM1*	cell polarity; morpho genesis; scaffold protein for complexes that include Cdc24p, Ste5p, Ste20p	1572	89	192	9,30E-07
KLLA0D17952g	/	/	330	364	735	6,69E-31
KLLA0A12001g	/	/	381	68	133	2,85E-02
KLLA0B00495g	*YGL117W*	/	867	303	570	2,28E-19

Identified ORF	*S. cerevisiae* homolog	Predicted function	Gene length	Wild type expression	*Δrlm1* expression	P-value
KLLA0F08679g	*YOR313C; SPS4*	sporulation, stress response	1083	72	134	1,12E+00

Identified open reading frames with an increased mRNA level in the *Klrlm1* deletion mutant. The corresponding *S. cerevisiae* homologs and the putative protein function are shown. Genes marked with * have been described to be regulated by ScRlm1 in previous studies (Garcia, 2004).

A second group of genes with an increased mRNA level in the *RLM1* deletion mutant are the *PHO* genes, which encode homologs of *S. cerevisiae* acid phosphatases. Three different genes with homology to the highly similar *S. cerevisiae* genes *PHO3/PHO5* were detected in the analysis, though there is a higher identity to *ScPHO3*. Their relation to cell wall integrity is not clear yet.

The remaining upregulated genes (Table 7) cannot be attributed to a certain group, especially because knowledge about the function of most of the encoded proteins is scarce. Some genes are suspected to be transcriptionally upregulated in response to stress and/or to encode proteins that play a role in stress response, e.g. *YHB1*, *SPS4*, or *UTH1*. Genes for proteins that function in various other cellular processes were detected, ranging from mating (SPO23), meiosis (*GMC2; MAM1*) and translational control (*SLF1*) of sporulation (*SPS4*).

It should be noted, that many of the genes found to be upregulated in the *Klrlm1* deletion are not homologous to genes found in fungal databases, for example *KLLA0A12001g,* which apparently only exists in *K. lactis*.

Table 8 lists the genes that showed a decreased expression in the *Klrlm1* deletion mutant, and are thus presumed to be primary Rlm1 targets in *K. lactis*, whose transcription normally would be induced by Rlm1 in response to cell wall integrity signaling in wild-type cells.

Table 8: Genes with a decreased expression in the *Klrlm1* deletion as compared to wild type.

Identified ORF	*S. cerevisiae* homolog	Predicted function	Gene length	Wild type expression	Δrlm1 expression	P-value
KLLA0E24025g	*YPL089C; RLM1*	Transcription factor	2199	3598	4	0
KLLA0B14883g	/	*KlLAC4;* β-galactosidase	3078	20707	24	0
KLLA0C00374g	*YCL067C; HMLALPHA2*	Mating- type protein alpha2	672	491	4	1,2E-127
KLLA0C00352g	*YPL187W; MFALPHA1*	Mating- type protein alpha1	786	345	19	9,41E-69
KLLA0E01057g	*YDR077W; SED1*	Abundant cell surface glycoprotein	486	17411	1139	0
KLLA0B07447g	*YJL158C; CIS3*	Homology to Hsp150p and Pir1p and Pir3p; Pir protein	828	4528	393	0
KLLA0C00396g	/	Mating- type protein alpha3	2694	466	68	5,74E-63
KLLA0D07634g	*YFL014W; HSP12*	Involved in maintaining membrane organization; involved in response; regulated by the HOG and Ras-Pka pathways	321	3900	585	0
KLLA0F08107g	*YAL008W; FUN14*	/	507	528	98	1,13E-59
KLLA0B09900g	/	/	1362	328	61	2,35E-36
KLLA0B06347g	*YKL096W; CWP1*	Cell wall mannoprotein	747	1513	296	1,3E-171
KLLA0A08206g	*YNL144C*	PI and PE binding	693	353	90	5,77E-29

Identified ORF	*S. cerevisiae* homolog	Predicted function	Gene length	Wild type expression	Δrlm1 expression	P-value
KLLA0C16225g	*YPL223C, GRE1*	Hydrophilin; stress induced (osmotic, ionic, oxidative, heat, heavy metals); regulated by the HOG pathway	558	258	68	2,03E-19
KLLA0E15181g	/	Acyl-CoA dehydrogenase	1398	44	12	4,37E+00
KLLA0F00440g	*YOR374W; ALD4*	Mitochondrial aldehyde dehydrogenase	1569	8007	2315	0
KLLA0C05324g	/	Glucan 1,3-beta-glucosidase precursor	1290	297	86	3,44E-20
KLLA0A06556g	*YJL171C*	GPI-anchored CWP; response to cell wall damaging	1200	4980	1471	0
KLLA0F21736g	*YHR087W; RTC3*	RNA metabolism	348	2089	629	3,4E-154
KLLA0B13321g	*YKL001C; MET14*	Adenylylsulfate kinase; sulfate assimilation	597	46	14	9,07E+00
KLLA0F26818g	/	/	225	123	38	1,68E-04
KLLA0F18766g	*YDR034W-B*	Predicted tail-anchored plasma membrane protein; may be involved in response to stress	207	2567	850	7,4E-167
KLLA0D13926g	*YEL004W; YEA4*	UDP-GlcNAc transporter; cell wall chitin synthesis	1014	604	204	1,15E-34

Identified ORF	*S. cerevisiae* homolog	Predicted function	Gene length	Wild type expression	Δrlm1 expression	P-value
KLLA0C13321g	*YOR104W; PIN2*	Induces appearance of [PIN+] prion;	741	1322	447	8,42E-82
KLLA0C08173g	*YIL055C*	/	1494	1482	508	3,66E-90
KLLA0E07877g	*YIL136W; OM45*	/	1686	6358	2213	0
KLLA0D10439g	*YML128C; MSC1*	/	1458	2262	796	1,2E-133
KLLA0E19713g	/	/	432	250	89	1,71E-10
KLLA0F20966g	*YNL192W; CHS1*	Chitin synthase I	3027	4269	1563	4,9E-240
KLLA0F03036g	*YGR282C; BGL2*	Endo-beta-1 3-glucanase	936	6780	2500	0
KLLA0E20263g	*YBL061C; SKT5*	Activator of chitin synthase III; recruits Chs3p to the bud neck	2082	6685	2487	0
KLLA0C01738g	*YLR178C; TFS1*	Phosphatidyl ethanolamine-binding protein	594	167	63	1,41E-04
KLLA0A06996g	*YLR326W*	/	612	66	25	6,37E+00
KLLA0E24003g	/	/	843	2671	1058	4,8E-129
KLLA0A03740g	*YOL122C; SMF1*	Ion transporter; broad specificity for divalent and trivalent metals	1740	2591	1037	1,2E-121
KLLA0F17072g	*YMR138W; CIN4*	GTP-binding protein involved in beta-tubulin folding	573	286	115	2,06E-10
KLLA0C06743g	*YDR402C; DIT2*	Sporulation-specific enzyme; required for spore wall maturation	1542	1214	492	2,06E-54

Identified ORF	*S. cerevisiae* homolog	Predicted function	Gene length	Wild type expression	Δrlm1 expression	P-value
KLLA0F12298g	*YNL010W*	/	675	4088	1660	6,7E-190
KLLA0B11902g	*YHR030C; MPK1*	MAP kinase of CWI signaling	1488	5883	2393	1,2E-272
KLLA0F17116g	*YMR136W; GAT2*	GATA family of zinc finger motifs;	1176	255	106	3,98E-09
KLLA0A10967g	/	/	531	753	319	1,67E-28
KLLA0B13123g	/	/	282	864	371	2,61E-33
KLLA0A09933g	*YMR295C*	Localizes to cell periphery and bud; associates with ribosomes	594	4616	1987	8,3E-192
KLLA0C11187g	*YOL084W; PHM7*	/	2904	4831	2126	1,70E-192
KLLA0E03147g	*YOR137C; SIA1*	Involved in activation of the Pma1p plasma membrane H+-ATPase by glucose	1737	1749	771	9,74E-67
KLLA0F09790g	/	/	528	532	237	1,71E-17
KLLA0B07392g	*YKL164C; PIR1*	CWP	1089	55422	25083	0
KLLA0F26829g	*YKL065C; YET1*	/	624	1416	641	3,60E-50
KLLA0E10253g	*YDR070C; FMP16*	Possibly involved in responding to conditions of stress	273	925	419	1,39E-30
KLLA0E09593g	/	/	2511	200	91	5,46E-04
KLLA0F12078g	*YDR100W; TVP15*	Integral membrane vesicle protein	423	400	182	1,47E-10
KLLA0D06479g	/	/	522	92	42	8,55E-01
KLLA0F15862g	*YAL022C; FUN26*	/	1290	722	330	2,33E-23
KLLA0E08867g	*YNL015W; PBI2*	/	231	140	64	1,25E-01

Identified ORF	*S. cerevisiae* homolog	Predicted function	Gene length	Wild type expression	Δrlm1 expression	P-value
KLLA0D08481g	*YDL215C; GDH2*	Glutamate dehydrogenase	3099	15528	7122	0
KLLA0E02641g	/	Ceramide glucosyltransferase	1659	2187	1006	3,09E-76
KLLA0C00935g	*YDR525W-A*	Cation transport	228	1066	491	2,21E-35
KLLA0F02816g	*YJL108C; PRM10*	Pheromone-regulated protein; putative permease	2856	5104	2367	1,7E-177
KLLA0E19493g	*YDL169C; UGX2*	Transcript accumulates in response to any combination of stress conditions	558	222	103	1,88E-04

As expected, virtually no expression was found for the genes *RLM1, LAC4* and the mating type alpha typical genes in the *Klrlm1* deletion strain. They serve as a negative control since *RLM1* and *LAC4* were deleted in the mutant. Likewise, mRNA from three genes that encode for the mating type specific proteins alpha1, alpha2 and alpha3 was also not detected, since the *Klrlm1* mutant employed was of mating type a and thus lacked respective the coding sequences at the *MAT* locus.

Confirming the results obtained above (section 3.1.5.1 and Figure 13) the *PIR1* gene was amongst the ones being downregulated in the *Klrlm1* deletion as compared to wild-type cells. Two genes, whose promoter regions were also tested in the β-galactosidase assays in section 3.1.5.1, were *MPK1* and *BGL2*, known targets of Rlm1 in *S. cerevisiae*. They encode the MAP kinase of the CWI pathway and a glycosyl transferase in the yeast cell wall, respectively. Although both promoters were not differentially regulated in the *Klrlm1* deletion as judged from the ß-galactosidase assays, the respective mRNA levels of the encoding genes were found to be significantly decreased in the *Klrlm1* deletion mutant in

this transcriptome analysis. Thus, analogous to *S. cerevisiae*, they appear to be target genes of Rlm1 in *K. lactis*.

The list of Rlm1 target genes includes a stress responsive gene (*HSP12*), genes that have been shown to affect chitin synthesis (*CHS1, SKT5,* and *YEA4*), as well as cell wall proteins whose homologs are known to affect cell wall stability in *S. cerevisiae* (e.g. *PIR1*, *CIS3*, and *CWP1*).

Some of the *S. cerevisiae* homologs of the other protein coding genes identified in Table 8 have been shown to be involved in the general stress response (*UGX2, FMP16*, *YDR034W-B*) or to be related to high osmolarity signaling (*GRE1, HSP12*).

3.1.5.3 Genetic confirmation of the transcriptome data

To confirm the data obtained by mRNA sequencing, plasmids were constructed that contain different promoter-*lacZ* fusions. Four target promoters were chosen according to the likelihood of the controlled gene products to be involved in cell wall synthesis and to the strength of their regulation as judged from the differential transcriptome data. Thus, the strength of the *KlSED1*, *KlCIS3*, *KlHSP12* and *KlYNL144* promoters were assessed in the wild type strain and in the *rlm1* deletion mutant carrying the respective reporter constructs, as reflected by the specific β-galactosidase activities. Of four genes selected only *KlSED1* and *KlHSP12* yielded detectable ß-galactosidase activities in crude extracts prepared from the respective transformants. Of those, the *KlHSP12* promoter yielded similar specific activities in the *Klrlm1* deletion as in the wild type background, indicating that it is not responsive to KlRlm1 activation (Figure 16A).

In contrast, specific ß-galactosidase activities dropped approximately twofold in the *Klrlm1* deletion as compared to wild-type for transformants with the *KlSED1p-lacZ* construct, confirming that this gene is a likely target of KlRlm1 (Figure 16B). Moreover, cell wall stress led to a more than twofold increase in gene expression as judged from the specific enzyme activities in wild-type cells exposed to either caspofungin or to Congo red, as expected for a gene responding to KlRlm1 (Figure 16C). However, specific ß-galactosidase activities also doubled for the *Klrlm1* deletion strain as compared to non-stressed cells,

indicating another level of transcriptional regulation, which is independent of KlRlm1 (compare Figure 16B and C).

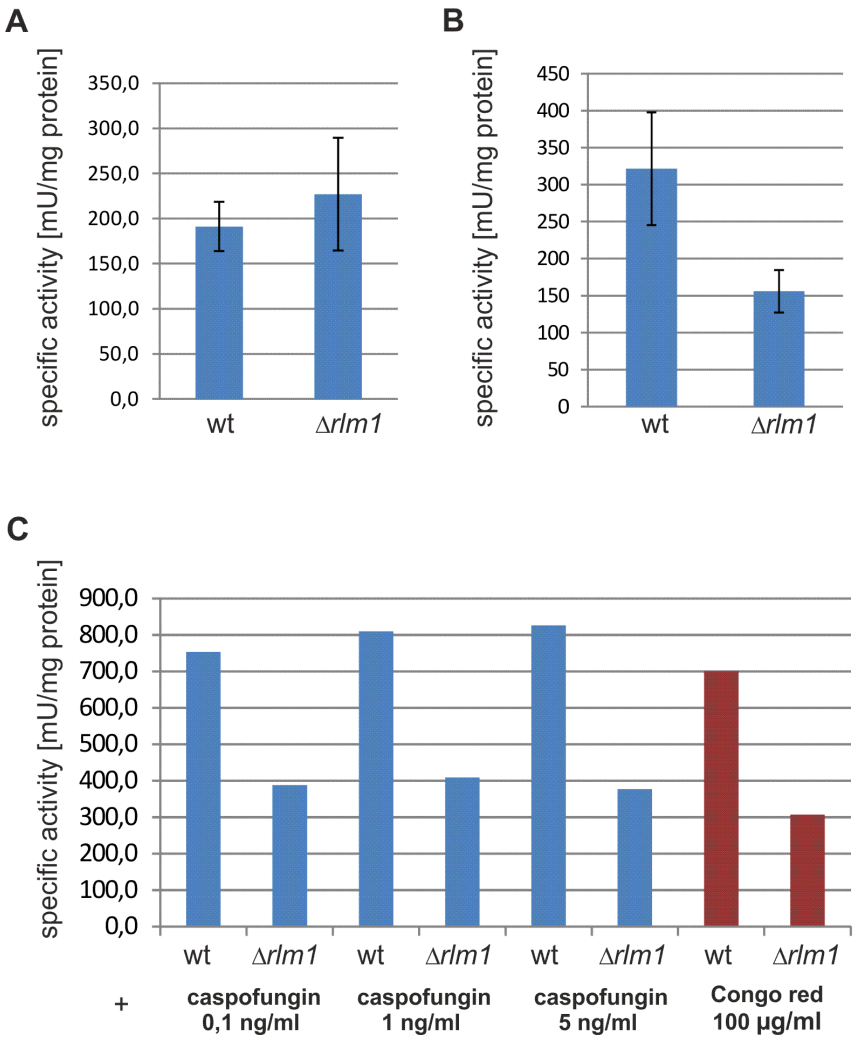


Figure 16: Detailed expression analysis using the *lacZ*-reporter fusion assay. Measurement of the specific β-galactosidase activity of the *K. lactis* wild type (wt, KDR1-6A) and the *rlm1* deletion (*Δrlm1*, KLSMO5-4B) employing **(A)** the plasmid pJJH1778 (*KlHSP12p-lacZ*) or **(B+C)** the plasmid pJJH1776 (*KlSED1p-lacZ*). The vector pJJH1564A was used as negative control (data not shown). The data were obtained in two independent experiments. The activities are given in mU/mg protein and were determined from pooled transformants grown in SC-URA medium + 1M sorbitol at 30°C to an OD_{600} of about 1. To induce cell wall stress **(C)** cells were treated with caspofungin and Congo red for three hours.

3.2 Distribution of CWI sensors in plasma membrane compartments of *K. lactis*

KlRlm1 is the most downstream component of the CWI signaling pathway in yeasts. Activation of this pathway in *K. lactis* is triggered by membrane-spanning sensors, KlWsc1, KlWsc2/3 and KlMid2, for which KlWsc1 has been shown to form discrete patches in the plasma membrane. In *S. cerevisiae* a well-established microcompartment of the plasma membrane is constituted by the so-called eisosomes, which has been thoroughly investigated in the decade. Since *K. lactis* disposes of many homologs of these proteins (Table 1), this part of the thesis first explores the existence of eisosomes in the milk yeast, and then relates them to the microcompartments occupied by the CWI sensors.

3.2.1 Identification and characterization of the eisosomal microcompartment

To investigate if eisosomes exist in *K. lactis,* the homolog of a gene encoding a major component of eisosomes in *S. cerevisiae,* the ORF of *KlPIL1,* was fused to the coding

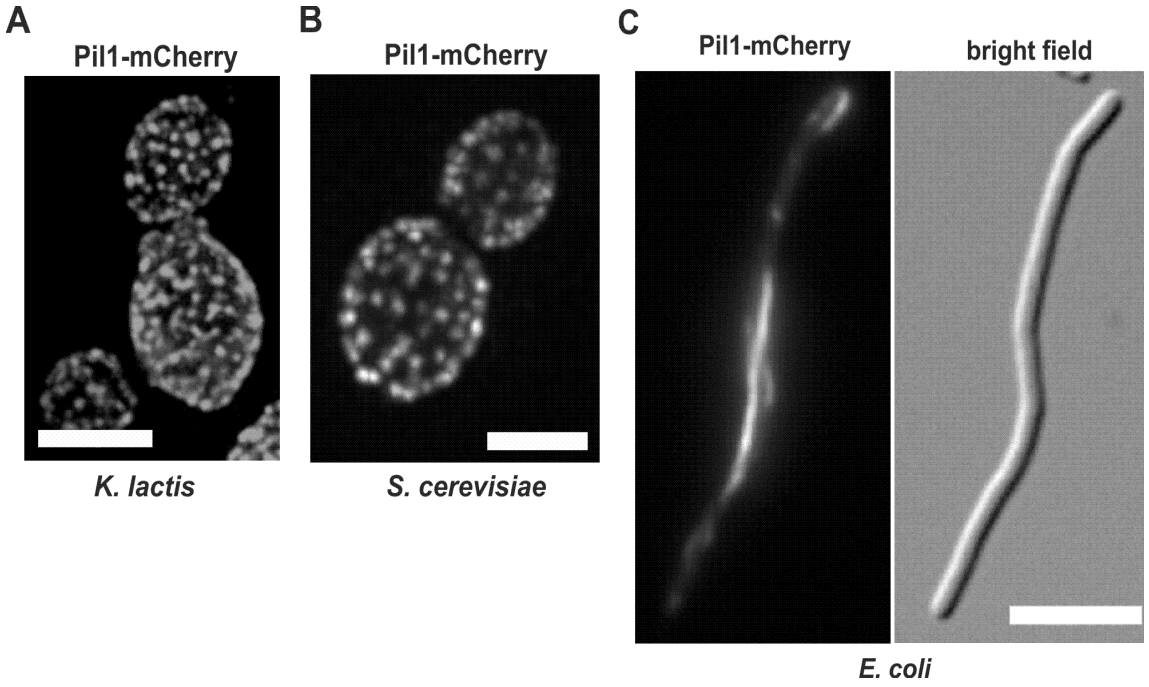


Figure 17: KlPil1-mCherry localizes in discrete patches within the fungal plasma membrane. KlPil1 was expressed from *CEN/ARS* vectors in **(A)** *K. lactis* wild type (strain KHO69-8C; plasmid pSMO58), **(B)** *S. cerevisiae* wild type (HD56-5A; plasmid pSMO51), and **(C)** *E. coli* (DH5α; plasmid pSMO51). Figure **A** shows a sum projection of about forty single planes of the whole cell, **B** shows a top view and in **C** an image of the middle section is presented. Scale bar 5µm.

sequence for the red fluorescent marker protein mCherry on a centromeric vector. Cells transformed with this construct were employed to determine the *in vivo* localization using fluorescence microscopy. Expression of KlPil1-mCherry in *K. lactis* revealed a patch-like localization of the protein at the cell cortex, with approximately 100 discrete spots within the entire cell (Figure 17A). The same localization pattern was observed for the KlPil1-mCherry fusion protein in *S. cerevisiae* (Figure 17B). Thus, KlPil1 in the milk yeast shows a similar distribution at the plasma membrane as has been reported for its homolog in *S. cerevisiae*. Furthermore, KlPil1-mCherry in *E. coli* formed tubular and dot-like structures in the bacterium (Figure 17C), indicating the ability of KlPil1 to self-assemble into higher order structures; a feature that was previously described for ScPil1 as well (Karotki *et al.*, 2011). These data can serve as a first experimental hint that *K. lactis* indeed disposes of eisosomes.

To further support the existence of eisosomes in *K. lactis*, two other genes encoding

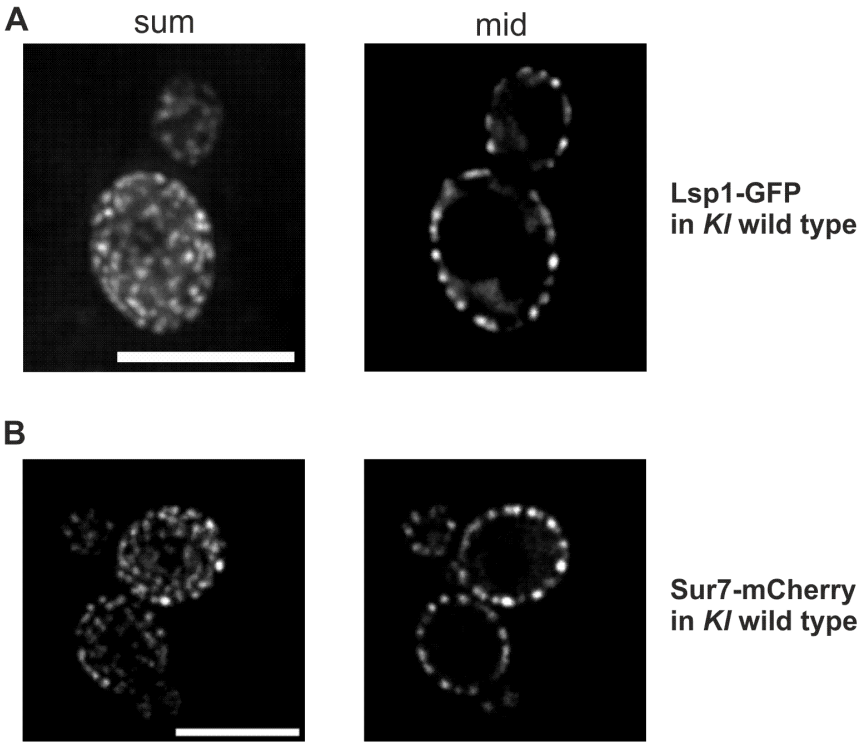


Figure 18: KlLsp1 and KlSur7 localize to dot-like structures at the cell surface. A: *KlLSP1-GFP* (pSMO103) and **B** *KlSUR7-mCherry* (pSMO102) were expressed from single copy plasmids in wild type cells of *K. lactis* (KHO69-8C). Localization of the fusion proteins was detected by fluorescence microscopy. In the left panel a summarizing view of about 40 images is gives. The images one the right show a single plane from the middle section. Scale bar 5 µm.

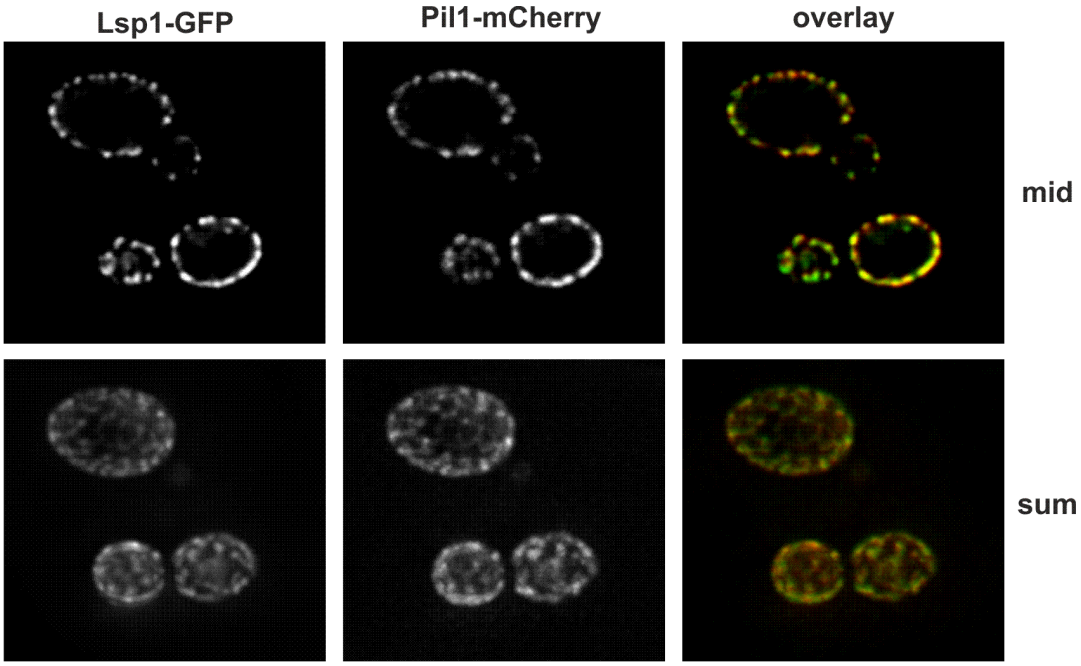


Figure 19: KlLsp1 colocalizes with KlPil1. Fluorescence microscopy of *K. lactis* wild type cells (KHO69-8C) expressing *KlLSP1-GFP* and *KlPIL1-mCherry* from *CEN/ARS* plasmids (pSMO108 and pSMO58, respectively). A view of the middle section is given in the top lane; the image in the lower section summarizes about 30 different planes. Scale bar 5 µm.

homologs of eisosomal proteins known in *S. cerevisiae*, *KlLSP1* and *KlSUR7,* were fused to fluorescent marker genes, again using an episomal plasmid carrying the constructs. Again, both fusion proteins, KlLsp1-GFP and Sur7-mCherry, localized to discrete patches at the cell cortex (Figure 18A and B).

Colocalization studies also confirmed that KlLsp1-GFP and KlPil1-mCherry, as well as KlSur7-mCherry and KlPil1-GFP largely occupy the same patches in the plasma membrane of *K. lactis* (Figure 19, Figure 20). Taken together, these findings support the notion, that KlLsp1 and KlSur7 are also components of eisosomes in *K. lactis*.

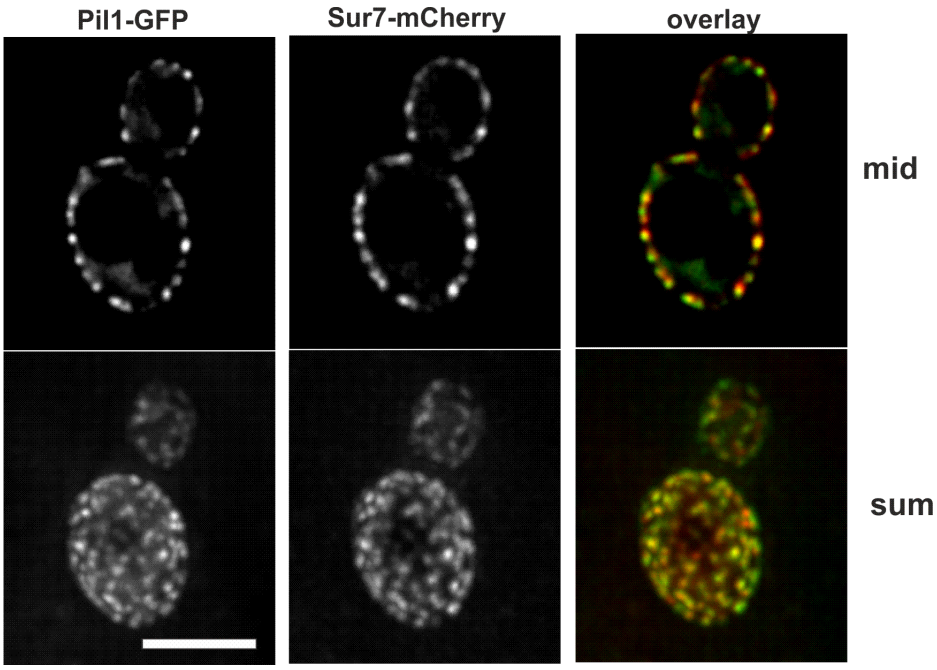


Figure 20: KlSur7 colocalizes with KlPil1. Fluorescence microscopy of *K. lactis* wild type cells (KHO69-8C) expressing *KlPIL1-GFP* and *KlSUR7-mCherry* from *CEN/ARS* plasmids (pSMO83 and pSMO100, respectively). A view of the middle section is given in the top lane; the image in the lower section summarizes about 30 different planes. Scale bar 5 µm.

Eisosomes were described to fall apart and occasionally cluster into eisosome remnants upon *PIL1* deletion in *S. cerevisiae* (Walther *et al.*, 2006). To further investigate the role of KlPil1 in eisosome formation in *K. lactis*, a *pil1* deletion mutant was constructed (Figure 21C). Expression of the *KlLSP1-GFP* and *KlSUR7-mCherry* constructs in the *Klpil1* deletion mutant demonstrated that both fusion proteins localized in three to five discrete patches at the cell cortex (Figure 21). This indicates that KlPil1 is responsible for the correct localization of the eisosomal proteins and thus probably for the proper formation and

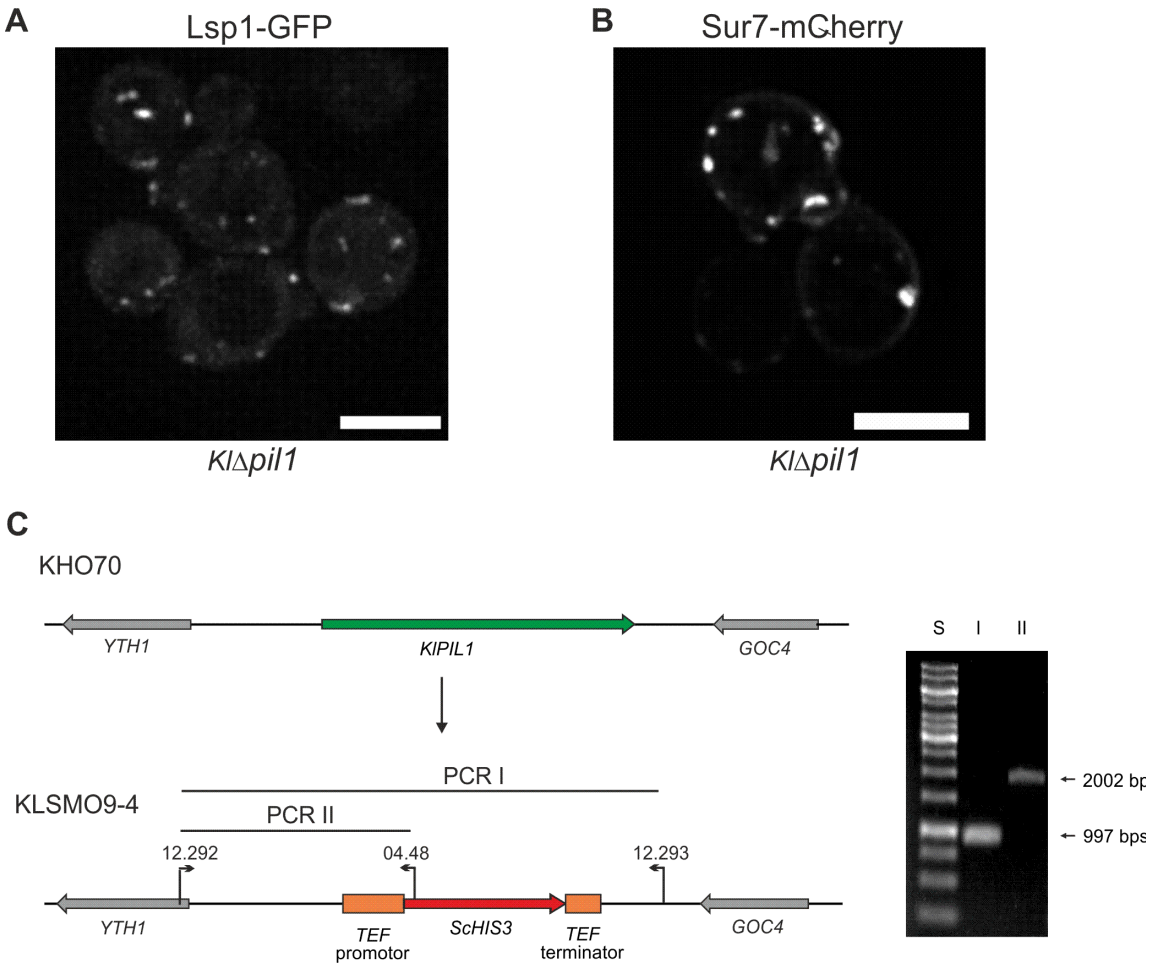


Figure 21: *KlPIL1* deletion prevents proper eisosome formation. C: *KlPIL1* was substituted in the *K. lactis* genome by the *ScHIS3* cassette (strain KHO70). Successful deletion of the gene was proven by PCR (DNA standard: 1kb ladder, Thermo Scientific). Fluorescence microscopy of *KlΔpil1* cells (strain KLSMO9-4) expressing (A) *KlLSP1-GFP* or (B) *KlSUR7-mCherry* from single copy plasmids (pSMO108 and pSMO83, respectively) revealed altered localization of both fusion proteins upon *KlPIL1* deletion. Both, Lsp1-GFP and Sur7-mCherry, appear as about five bright spots at the cell cortex. Scale bar 5 µm.

shape of eisosomes in *K. lactis*. Since Lsp1 is the other predominant protein in *S. cerevisiae* eisosomes, *KlLSP1* was also deleted from the *K. lactis* genome and the mutant was tested for the localization of the other eisosomal markers (C). Fluorescently tagged Pil1 and Sur7 showed that, unlike their behavior in the *Klpil1* null mutant, the localization patterns remained unaltered. Apparently, eisosomes formation does not depend on the presence of KlLsp1, indicating a more ancillary role of the protein in eisosome assembly. These data are in line with those reported for the respective deletion mutants in *S. cerevisiae*.

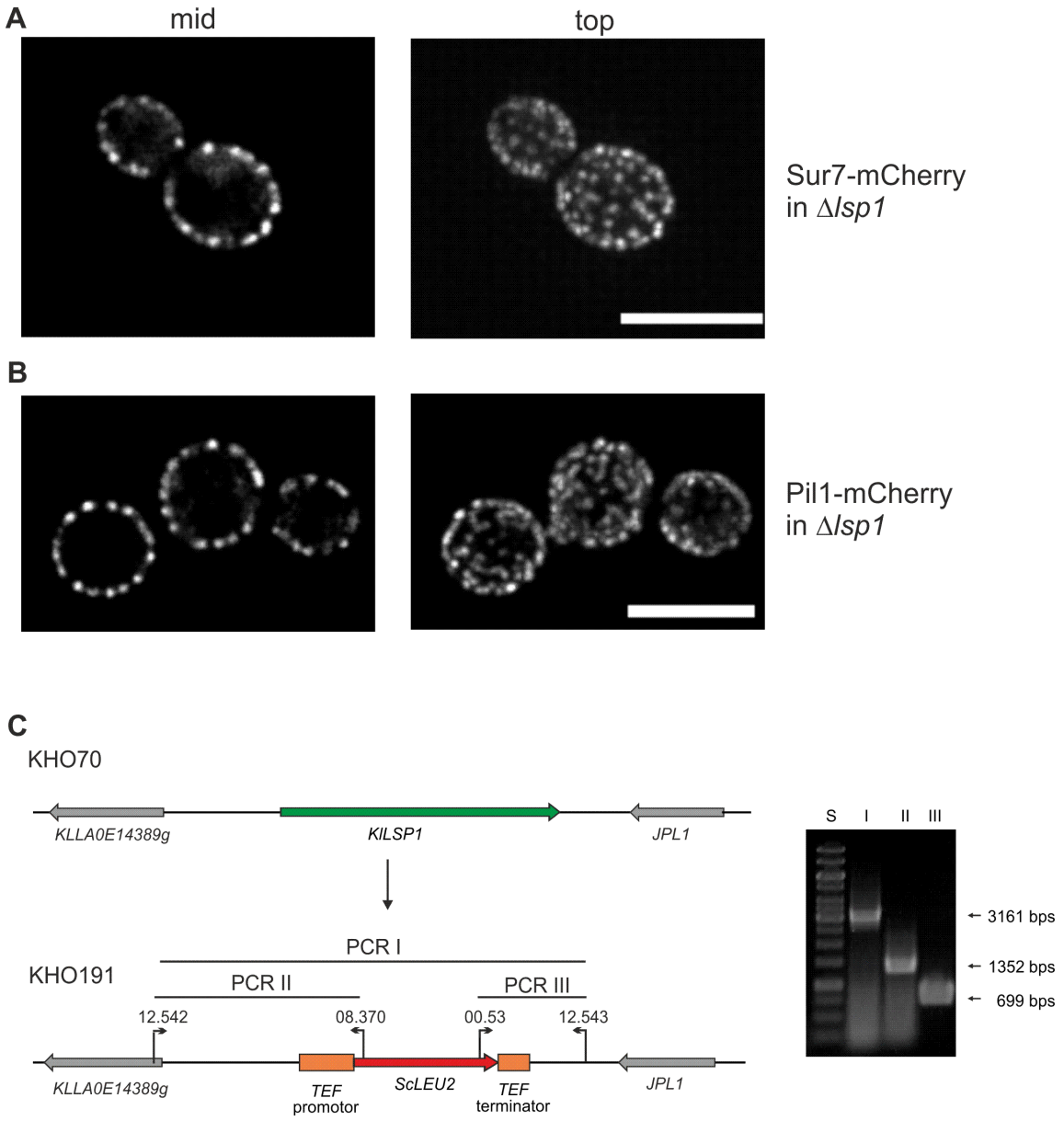


Figure 22: Deletion of *KlLSP1* does not influence eisosome assembly and shape. C *KlLSP1* was substituted in the *K. lactis* genome (strain KHO70) by the *ScLEU2* cassette. The cassette was amplified from the plasmid pSMO73 to obtain large homologous regions and thus to enhance the chances of successful deletion. Deletion of the gene was verified by PCR and agarose gel electrophoresis (standard: 1kb ladder, Thermo Scientific). Fluorescence microscopy of *KlΔlsp1* cells expressing (A) *SUR7-mCherry* or (B) *PIL1-mCherry* from single copy plasmids (pSMO58 and pSMO83, respectively) revealed the localization of the fusion proteins in an eisosome typical distribution pattern. Scale bar 5 µm.

Since Pil1 and Lsp1 have been shown to serve a similar purpose in the induction of membrane curvature in *S. cerevisiae*, the viability of the respective double deletions was also tested in *K. lactis.* The latter were obtained by crossing a strain carrying the *Klpil1*

allele to one with the *Kllsp1* deletion and subsequent tetrad analysis. There were some difficulties with spore germination and complete tetrads of four germinated spores were rare. Out of 54 tetrads segregated, only 5 yielded four and 16 three viable spores. Anyway, a total of 10 segregants were obtained carrying the markers used to substitute both genes independently of the number of spores geminated. Furthermore, the *Klpil1 Kllsp1* double deletion proved to be viable and did not display any obvious growth phenotype or morphological abnormalities (data not shown).

When the *SUR7-mCherry* construct was expressed as a marker protein for eisosomes in the *Klpil1 Kllsp1* double deletion strain, the protein clustered in eisosome remnants, much as in the single *Klpil1* deletion (Figure 21). Thus, the additional deletion of *KlLSP1* did not result in any phenotypic effect. This again underlines the minor role of Lsp1 in eisosome assembly.

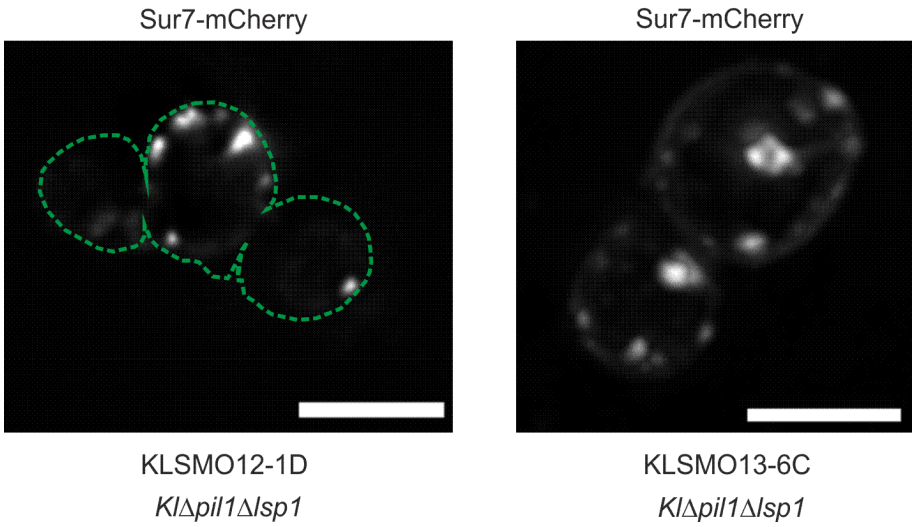


Figure 23: KlSur7 localization is altered in the *Klpil1 Kllsp1* double deletion strain. Fluorescence microscopy images of two independent *Klpil1 Kllsp1* deletion mutant strains (KLSMO12-1D and KLSMO13-6D, respectively) expressing *SUR7-mCherry* from single copy plasmids (pSMO83). Scale bar 5 µm.

In summary, the data obtained show that eisosomes are most likely existing in *K. lactis,* but are largely similar to the well-studied eisosomal microcompartments in *S. cerevisiae*.

3.2.2 The CWI sensors are excluded from the eisosomal microcompartment

While the existence of eisosomes could be shown in the milk yeast, the question of the cellular function of this microcompartment in fungi is still a matter of debate. A first claim for their role in endocytosis could not be verified (Brach *et al.*, 2011), but the heat sensitive phenotype of the *Scpil1* and the *Sclsp1* deletion mutants indicate a putative role in stress response. Genetic data in *S. cerevisiae* also link eisosome function to cell wall integrity signaling, but the exact nature of such interactions is still unclear (Mascaraque *et al.*, 2013). Intriguingly, the pattern observed above for fluorescently labeled eisosomal proteins resembles the distribution of the KlWsc1 sensor which triggers CWI signaling in *K. lactis* (Rodicio *et al.*, 2008). Thus, a possible relation was further investigated in the following.

In a first approach, the *Klpil1-,* the *Kllsp1-* and the *Klpil1 Kllsp1* null mutants were tested for growth in presence of several cell surface stressors. Neither the presence of caspofungin, nor the addition of sorbitol or sodium chloride, nor growth at elevated temperatures differentially affected the deletion mutants as compared to a wild-type strain (data not shown).

To investigate a possible influence of eisosomes on the distribution of the cell wall integrity signaling sensors, first sensor-GFP fusions were obtained and tested for their localization in a *Klpil1* deletion mutant. As described above, eisosomes are basically absent in such mutants and only eisosomal remnants can be detected (Figure 21). As shown in Figure 24, Wsc1-GFP, Mid2-GFP and Wsc2/3-GFP formed patches that were scattered all over the cell cortex, exactly like in the wild type. Clearly, the deletion of *PIL1* and the associated loss of eisosomes does not affect the distribution of the *K. lactis* CWI sensors, indicating that a lack of eisosomes has no general influence on the distribution of plasma membrane proteins, at least as far as the membrane-spanning sensors are concerned.

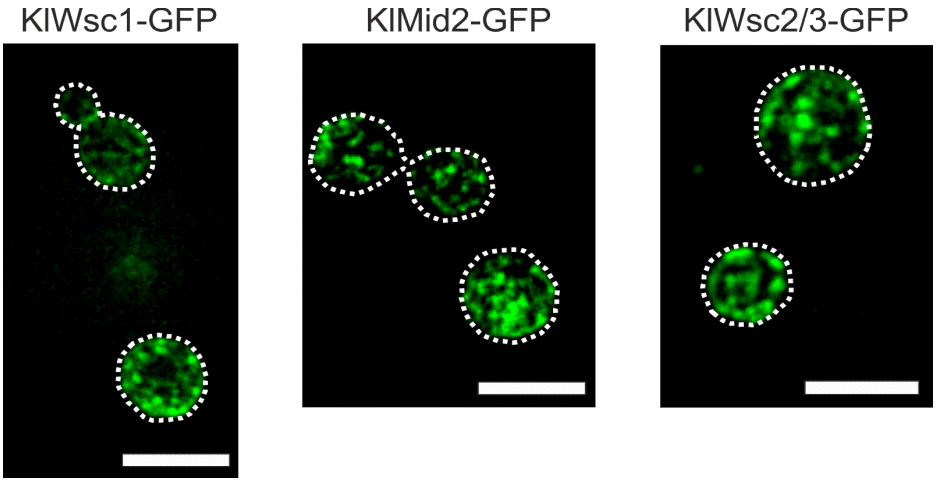


Figure 24: Localization of the cell wall integrity sensors in *K. lactis* does not change upon *KlPIL1* deletion. Fluorescence microscopy imaging of *KlΔpil1* mutant cells (KLSMO9-4) expressing the cell wall integrity pathway sensors Wsc1, Mid2 and Wsc2/3 respectively, fused to GFP from centromeric plasmids. Top views are shown. **(A)**: KlWsc1-GFP (pRRO73) **(B)**: Mid2-GFP (pRRO99) **(C)**: Wsc2/3-GFP (pULF20). Scale bar: 5µm.

In order to investigate a possible relation of CWI sensors with eisosomes in *K. lactis*, colocalization studies were performed. For this purpose, constructs with each one of the genes encoding the CWI sensors fused to the GFP coding sequence were expressed on centromeric vectors together with a strain producing a Pil1-mCherry fusion protein. As expected, all sensor-GFP fusion proteins, KlWsc1-GFP, KlWsc2/3-GFP and KlMid2-GFP , localized to the plasma membrane, where they were found in discrete spots that were evenly distributed all over the cell cortex (Figure 25, left panel). The same distribution was shown for the eisosomes as well (Figure 25, middle), but an overlay of the two images revealed that the patches of the two structures did not overlap (Figure 25, right). Thus, all three sensors seemed to be excluded from the microcompartment of eisosomes and rather form their own microcompartments. Due to time limitations, possible colocalizations of the different sensors have not been investigated, yet.

In summary, the data reported in the last three sections do not support a functional role of eisosomes in cell wall integrity signaling in *K. lactis*. Thus, the question of the physiological importance of eisosomes in fungi as a whole remains to be elucidated.

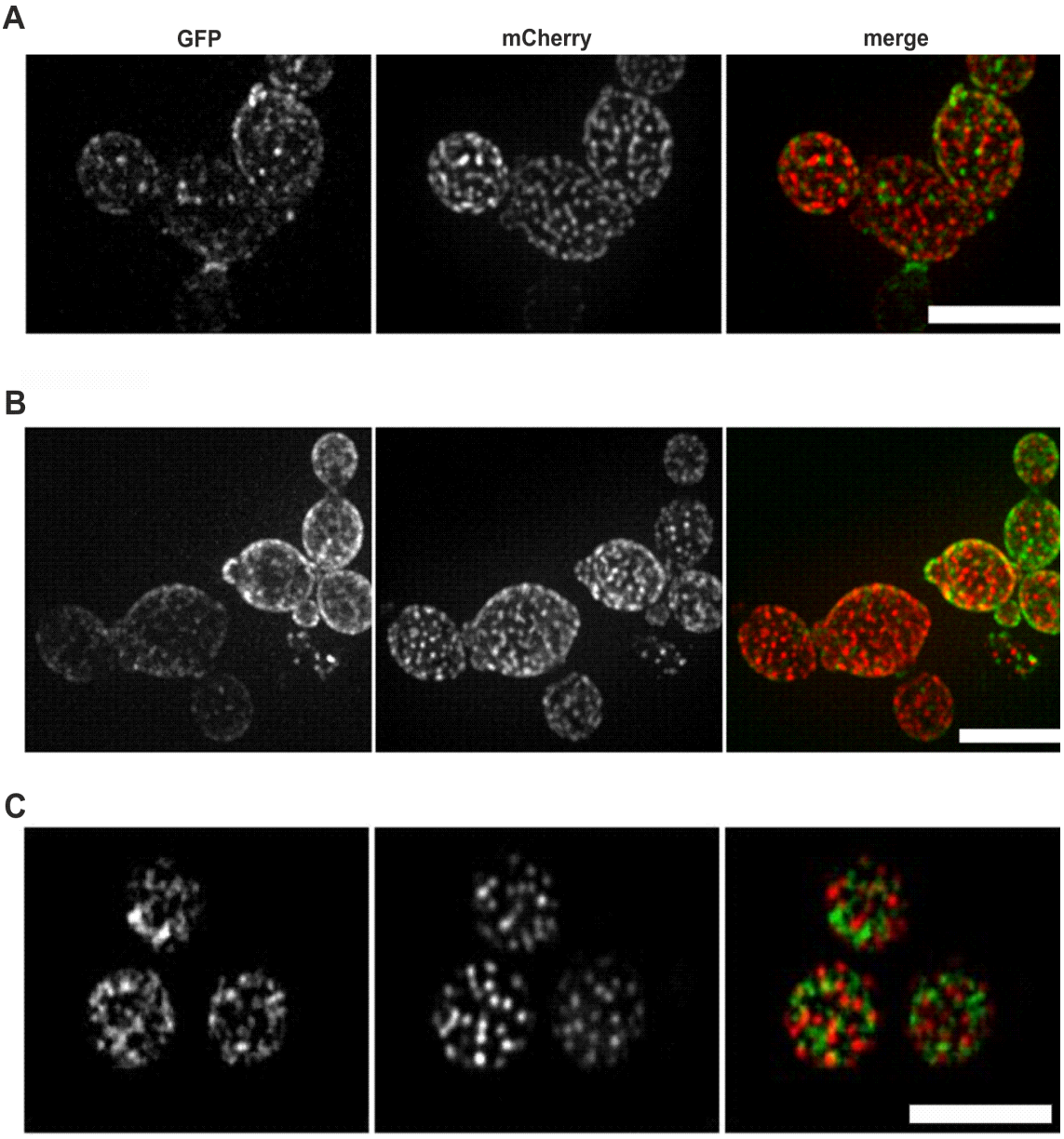


Figure 25: The sensors of the CWI pathway are excluded from the microcompartment of eisosomes. Fluorescence microscopy of *K. lactis* wild type cells (KHO69-8C) expressing Pil1-mCherry as a marker for eisosomes (pSMO58) and a sensor protein of the cell wall integrity signaling pathway fused to GFP. The left panel gives the respective GFP images, the middle section offers the Pil1-mCherry images and the colored panel provides an overlay of both images. **(A)**: Sum projection of 40 Z-stack images of cells expressing KlWsc1-GFP (pRRO73)**(B)**: Sum projection of 40 Z-stack images of cells expressing KlMid2-GFP (pRRO99). C: Top view of cells expressing KlWsc2/3 fused to GFP (pULF20). Scale bar: 5µm.

4 Discussion

The first part of this thesis was dedicated to the characterization of the transcription factor KlRlm1 and the identification of its putative target genes. In the second part of the thesis, the role of microcompartmentalization of the upstream sensors of the CWI pathway in the plasma membrane was investigated.

4.1 Characterization of the transcription factor Rlm1 in *K. lactis*

In a first approach, a BLAST search identified a homolog to the *ScRLM1* gene in the *Kluyveromyces lactis* genome, which encodes the central CWI transcription factor in *S. cerevisiae*, was identified. In the deduced amino acid sequence, the MADS-box mediating DNA binding, the Mpk1 binding site and the three phosphorylation sites were conserved between the two species. This indicated that the *K. lactis* homolog may also function in regulating the expression of specific target genes.

Growth analyses of the constructed deletion mutant then suggested a role of KlRlm1 in cell wall integrity signaling. Thus, the *rlm1* deletion mutant was sensitive to the cell wall perturbing agents Congo red, Calcofluor white and caspofungin. Interestingly, this phenotype differed from the one displayed by the deletion mutant of the *S. cerevisiae* homolog, which, in contrast to expectations, is not sensitive but in fact hyperresistant to Congo red and Calcofluor white (Lopez-Garcia *et al.*, 2010). Therefore, the reasons and underlying mechanisms of these differences in Rlm1 function were addressed in this thesis.

4.1.1 Differences in Rlm1 function between *K. lactis* and *S. cerevisiae*

Analyses of the *rlm1* deletion mutants transformed with the wild-type genes from both species revealed that expression of the heterologous gene does not complement the mutant phenotypes. Furthermore, the *S. cerevisiae RLM1* promoter does not confer sufficient expression in *K. lactis* to allow for growth, even if the native *KlRLM1* gene is introduced. In contrast, the *KlRLM1* promoter can drive sufficient expression of *ScRLM1* in

the *ScΔrlm1* mutant to complement the growth defect on caspofungin. Surprisingly, the mutant phenotypes in response to Congo red and Calcofluor white stress were not complemented, but rather amplified. This could be explained by an autoregulation, in that a low affinity of the *KlRLM1* promotor for ScRlm1 allows only a low-level expression of ScRlm1. In support of this notion, autoregulatory mechanisms have been shown to act on *ScRLM1* expression in response to Congo red and Zymolyase stress (Garcia, 2004). This is also consistent with the observation, that overexpression of *ScRLM1* reduces the growth rate (Yoshikawa *et al.*, 2011). Thus, the intracellular amount of ScRlm1 apparently needs to be tightly regulated. One would assume that at low Rlm1 concentrations only a few target genes with high Rlm1 binding affinity are activated, causing the hyperresistant phenotype. Alternatively, other pathways, which act in parallel to CWI signaling, may be activated when certain cell wall stressors are applied, but the Rlm1 level would remain low. This would lead to the expression of other target genes, which may result in the hyperresistance towards Congo red and Calcofluor white. Cross talk with other pathways has been demonstrated before (Fuchs and Mylonakis, 2009). Hypertonic solutions have been shown to induce transcription of *ScMPK1* in a Hog1 and Rlm1 dependent manner (Hahn and Thiele, 2002) and the need for coordination of both pathways has been described for Zymolyase treated cells (Garcia *et al.*, 2009). Thus, a low level of Rlm1 may impact Hog1 activity and thus expression of HOG1 responsive genes. For example, cells deleted for *HOG1* showed hyperresistance to Calcofluor white treatment (Lopez-Garcia *et al.*, 2010).

In summary, the *KlRLM1* promoter appears to mediate a lower but sufficient gene expression in *S. cerevisiae*, whereas the *ScRLM1* promoter is not strong enough to allow expression in *K. lactis*.

Nevertheless, it turned out that the Rlm1 proteins functioned only properly in their native yeast species, when using the appropriate promoters. Chimeric *RLM1* constructs were obtained to determine if the DNA binding domain or the transcriptional activation domain confers this species-specificity. The interpretation of the results obtained is hampered by the conflicting complementation abilities under different stress conditions (Table 9).

Table 9: Complementation of the *rlm1* deletion strains by expression of chimeric genes is depending on the applied stressor

		KlΔrlm1		
promoter	**gene**	**Congo red**	**Calcofluor white**	**Caspofungin**
KlRLM1p	***ScRLM1***	-	-	-
KlRLM1p	***KlRLM1***	+	+	+
KlRLM1p	***Sc/KlRLM1***	+	+	+
KlRLM1p	***Kl/ScRLM1***	+	hyperresistant	(+)
KlRLM1p	***Sc100/KlRLM1***	+	+	+
KlRLM1p	***Kl100/ScRLM1***	+	+	+

		ScΔrlm1		
promoter	**gene**	**Congo red**	**Calcofluor white**	**Caspofungin**
ScRLM1p	***ScRLM1***	+	+	+
ScRLM1p	***KlRLM1***	-	-	-
ScRLM1p	***Sc/KlRLM1***	(+)	(+)	+
ScRLM1p	***Kl/ScRLM1***	(+)	(+)	hyperresistent
ScRLM1p	***Sc100/KlRLM1***	-	-	+
ScRLM1p	***Kl100/ScRLM1***	(+)	(+)	hyperresistent

		ScΔrlm1		
promoter	**gene**	**Congo red**	**Calcofluor white**	**Caspofungin**
KlRLM1p	***ScRLM1***	hyperresistant	hyperresistant	(+)
KlRLM1p	***KlRLM1***	hyperresistant	hyperresistant	-
KlRLM1p	***Sc/KlRLM1***	hyperresistant	hyperresistant	(+)
KlRLM1p	***Kl/ScRLM1***	hyperresistant	hyperresistant	+
KlRLM1p	***Sc100/KlRLM1***	hyperresistant	hyperresistant	(+)
KlRLM1p	***Kl100/ScRLM1***	hyperresistant	hyperresistant	+

Full complementation of the *rlm1* deletion is indicated by +; no or poor complementation by -; partial complementation by the gene is displayed by (+).

In summary, all four chimeric constructs are generally functional in *S. cerevisiae.* A minor variation in complementation capacity could be observed for the chimeras that contain the *K. lactis* Rlm1 N-terminus. Especially the construct with the complete N-terminal half of *K. lactis* Rlm1 conferred a mild hyperresistance to caspofungin. This may be explained if additional target genes, presumably the homologs of KlRlm1 targets in *S. cerevisiae*, are recognized in response to caspofungin stress. These genes would not be upregulated by the native transcription factor. On the other hand, expression of all four chimeras from

the *K. lactis* promoter resulted in hyperresistance to Congo red and Calcofluor white, i.e. the deletion phenotype was enhanced by expression of the chimeric *RLM1* genes and rather than being suppressed. This result is reminiscent of the expression of *ScRLM1* from the *KlRLM1* promoter. This could again be caused by a low intracellular concentration of the respective chimera with a concomitant perturbed regulation of connected signaling pathways, as speculated above. Clearly, tagged versions of the proteins would be required to determine their relative abundance in immunoblots.

Like in *S. cerevisiae*, all chimeric constructs suppressed the phenotype of the *Klrlm1* mutant, indicating that they are functional in the milk yeast, too. Thus, they grew in the presence of cell wall stressors, some even slightly better than the wild type control. Results from the constructs expressed from the *ScRLM1* promoter in *K. lactis* were rather heterogenous, probably because of a low-level expression further varied by the feedback regulation discussed above. In contrast, overexpression of the full-length *ScRLM1* and *KlRLM1* genes from multicopy plasmids in the *Klrlm1* deletion mutant resulted in hyperresistance to the cell wall stressors, which was also true for the chimeric construct carrying the KlRlm1 N-terminus. Again, this indicates that the gene dosage of *RLM1* and the concomitant intracellular concentration of the transcription factor is a critical determinant in cell wall integrity signaling in *K. lactis*. Overproduction caused by the increase in gene copy number thus fully compensates for the variations caused by the different manipulations, including the effect on species-specificity.

In general, the drop dilution assays performed in this work raise the question of the reliability of the results. Small changes in growth and incubation conditions may lead to considerable variations. As an alternative, FACS analyses using live-cell staining was also performed in our lab to measure growth behavior of CWI mutants in *K. lactis* (Rodicio *et al.*, 2008), and could be recommended for further investigations, since despite its higher costs it ensures a much better statistical significance.

4.1.2 Target genes of the transcription factor KlRlm1

Growth analyses of *rlm1* deletion mutants, cross-complementation experiments and expression of the chimeric Rlm1 proteins in *K. lactis* and in *S. cerevisiae* indicated striking

differences in the number and/or nature of genes, which are regulated by Rlm1 in response to cell wall damage in the two yeast species. In bakers´ yeast, several studies have been dedicated to identify such target genes under various stress conditions (Jung and Levin, 1999; Boorsma *et al.*, 2004; Garcia, 2004). Similar studies have not been performed for KlRlm1 until now and thus promised to reveal new insights.

In a first approach to characterize KlRlm1 target genes, the promoters of some selected genes were fused to the *lacZ* reporter gene carried on episomal vectors, and β-galactosidase assays were performed. Only one construct, which carried the *KlPIR1p-lacZ* fusion, was identified to have a KlRlm1-dependent expression (section 3.1.5.1). Reporter gene expression from nine other promoters tested remained unaffected by the *Klrlm1* deletion, although their homologs are highly responsive to Rlm1 in *S. cerevisiae* (Jung and Levin, 1999). It should be noted that ß-galactosidase assays show a considerable degree of variation in the specific enzyme activities measured, so that differences of less than a vector of two may go undetected. Moreover, this approach relies on the selection of a limited number of target gene promoters. Therefore, in a more global approach, total mRNA sequencing was commissioned for four different samples (twice mRNA from the wild type, twice mRNA from a *rlm1* deletion mutant, in each case with and without the addition of caspofungin as a cell wall stressor). Thus, several putative target genes of KlRlm1 were identified which are discussed, below. It should be noted, however, that caspofungin treatment proved to be inefficient, since the expression of only four genes was upregulated in the treated versus the untreated sample. This indicates that the concentration of caspofungin applied was too low to trigger expression of all responsive genes, but rather elevated only the mRNA levels of the most prominent targets. Yet, the homologs of these four target genes in *S. cerevisiae* (*KlFLO1*, *KlGPI15*, *KLLA0C11517g* and *KLLA0C00374g*) are all listed in the CWI stress response, and thus indicate a similarity in this stress response between the two yeast species.

This is further supported by the fact that in the *rlm1* deletion mutant expression of only one gene was upregulated in response to caspofungin treatment. The latter was identified as *KLLA0F26818g* and the function of the encoded protein has not been characterized yet,

neither does it have a homolog in *S. cerevisiae*. Remarkably, expression of the same ORF was downregulated in the *rlm1* deletion strain as compared to the wild type.

Comparison of the mRNA sequencing data of the samples from the wild type and the *rlm1* deletion mutant identified a total of 87 genes whose transcription was significantly altered (note that the threshold for a significant difference was set at a twofold number of gene detection). Of those, 29 genes were upregulated in the deletion mutant and 58 were downregulated. This appears to be a rather small fraction of the entire genome, when compared with the number of genes which are differentially transcribed upon cell wall stress induced by Congo red in *S. cerevisiae*. There, 132 genes were found to be up- and 113 genes to be downregulated (Garcia, 2004). However, since the latter study included measurements from three different time points (2, 4, 6 hours of Congo red addition) and the threshold was set at 1.8-fold changes, the data presented here seem to be in the expected range.

Regarding the reliability of the transcriptome sequencing data obtained here, several genes can be used as controls. Thus, among the mRNAs whose abundance was drastically reduced in the *rlm1* deletion mutant as compared to the wild type, was *RLM1* itself. In the wild type it was detected approximately 3600 times, whereas it appeared only 4 times in the *rlm1* null mutant. Since the mRNA cannot be present in the deletion mutant, the latter number may therefore be regarded as the lower detection limit of this method. Likewise, the *LAC4* mRNA could not be detected in the *rlm1* deletion mutant, which can be attributed to the *lac4* deletion in this strain background as opposed to the wild-type gene carried in the control strain. The third internal control is provided by the different mating types of the wild type and the deletion strains, demonstrating the differential regulation of the respective genes.

4.1.2.1 Genes that are upregulated in the Klrlm1 deletion mutant

Target genes of KlRlm1 would be expected to be downregulated in the absence of the transcription factor. However, sequencing of the total mRNA identified 29 upregulated genes in the *Klrlm1* deletion mutant. The largest group of those genes belongs to the *FLO*

gene family. The amount of mRNA of five different genes of that cluster was significantly increased in the *rlm1* deletion mutant, i.e. with 10000-fold (0/10473; for the *KLLA0D00264g* gene) to 5-fold (24358/124180; for the *KLLA0B14498g* gene) changes as compared to the wild-type. Those genes encode homologs of the *S. cerevisiae* genes *FLO1*, *FLO5* and *FLO9* (two homologs), respectively. They all encode putative flocculins and share a high sequence identity. Nevertheless, deletions of the respective genes in *S. cerevisiae* display different phenotypes, such as decreased filamentous growth or decreased stress resistance. (Hodgson *et al.*, 1985; Teunissen and Steensma, 1995).

The *S. cerevisiae* homolog of *KlFLO1* encodes the lectin-like protein ScFlo1 that is expressed at the cell surface and leads to aggregation of cells by binding to the mannose chains in the cell wall of other cells (Kobayashi *et al*., 1998). Normally the expression of these genes is stimulated by nutrient limitation and results in the aggregation of cells into flocs, which rapidly sediment from the medium (Stratford, 1992) in a calcium dependent manner (Soares, 2011). *FLO* gene expression is generally silenced by the action of the histone deacetylase Hda1, the histone acetyltransferase Gcn5 (Dietvorst and Brandt, 2008) and the Set1 (COMPASS) methylation complex (Dietvorst and Brandt, 2010).

Increased flocculin expression in *S. cerevisiae* can be seen as part of a general stress response that eventually leads to invasive growth, which itself increases the cell/culture surface for nutrient uptake and precedes protection against adverse environments. Filamentous growth in general is regulated by different signaling pathways such as the Ras/cAMP protein kinase A (PKA) pathway (Mosch *et al.*, 1996; Mosch *et al.*, 1999), the TOR pathway (Rohde and Cardenas, 2004), or the MAPK pathway for mating and invasive growth (Roberts and Fink, 1994). The cell wall integrity pathway apparently also contributes to filamentous growth, mediated by components acting upstream of ScRlm1; e.g. the sensors ScWsc1p, ScWsc2p, ScMid2p, and the MAP kinase ScMpk1/Slt2 (Birkaya *et al.,* 2009). Interestingly, another MADS-box transcription factor, ScMcm1, was identified in the same screen as a prominent regulator of filamentous growth. A homolog also exists in *K. lactis*, indicating that both proteins may have overlapping activities. In line with this hypothesis, expression of flocculins in the fission yeast *S. pombe* is controlled in a complex

manner, involving various transcription factors, some of which are autoregulated at the transcriptional level (Kwon *et al.*, 2012).

Taken together, the upregulation of the *FLO* genes upon *KlRLM1* deletion may be an indirect way to compensate for the defect in cell wall synthesis. Alternatively, since KlRlm1 is not available as a target for KlMpk1 phosphorylation, one can imagine that the signal of the CWI pathway is re-routed to a different transcription factor which turns on *FLO* gene expression. That scenario would suggest a role of the CWI pathway in filamentous growth in the milk yeast under certain growth conditions, a process that has not yet been studied in *K. lactis*. In this context, previous transcriptome analyses in *S. cerevisiae* aimed at target genes of ScRlm1 revealed that 45% of the genes induced by Congo red treatment were also induced in cells overexpressing the transcription factor for pseudohyphal growth, ScTec1, (Madhani *et al.*, 1999), or when the cells were treated with pheromones (Roberts *et al.*, 2000). Thus, the MAPK pathways for mating and filamentous growth appear to be at least partially activated by cell wall damage which triggers the cell wall integrity pathway. A similar mechanism may therefore by operating in *K. lactis* as well. This is further supported by the observation that other genes related to mating and sporulation, e.g. *KlSPO23*, *KlGMC2* or *KlSPS4,* were also upregulated in the *Klrlm1* deletion (see section 3.1.5.2).

Expression of a second group of genes was also increased in the *Klrlm1* deletion mutant as compared to the wild type: Homologs of four genes of the *PHO* family in *S. cerevisiae*, whose products mediate phosphate homeostasis, were identified: *KlPHO3*, *KlPHO5* and *KlPHO89*. *ScPHO3* is highly similar to *ScPHO5* and encodes an acid phosphatase in *S. cerevisiae*. Pho3 hydrolyzes thiamin phosphates and thus affects the intracellular levels of this cofactor (Nosaka *et al*., 1989). The active form of thiamin, thiamin pyrophosphate, is required as a co-factor in the first step of alcoholic fermentation and for the regulation of several biosynthetic pathways. Recently, expression of thiamine biosynthetic enzymes has been shown to be upregulated in response to oxidative and osmotic stress, suggesting a role of thiamine metabolism in the general defense systems of yeasts (Kowalska *et al.*, 2012). The fourth *PHO* gene identified, *KlPHO89,* encodes a homolog of the high-affinity cation-dependent phosphate cotransporter in *S. cerevisiae*. ScPho89 is essential for the

regulation of phosphate homeostasis, especially under alkaline growth conditions (Pattison-Granberg and Persson, 2000; Sengottaiyan *et al.*, 2013)

How may *PHO* gene expression be related to CWI signaling? The fact that three different homologs of the *ScPHO3* gene were found in the screen, all with well FDR and p-values, indicates that the connection is not based on an artefact. Moreover, *ScPHO3, ScPHO5* and *ScPHO89*, were also identified in a previous transcriptome analysis in *S. cerevisiae* as being induced by Congo red treatment after 4h and 6h (Garcia, 2004). This seems to contradict the finding that in a *Klrlm1* deletion the homologs are upregulated. Yet, a shorter Congo red treatment of only 2h resulted in a of *ScPHO3* in the study of Garcia *et al.* (2008), which would be consistent with the transcriptome data of *K. lactis* presented in this thesis. It should be noted that the authors also show that the induction of *ScPHO89* expression by Congo red is not mediated by ScRlm1, but rather depends on the ScCrz1 transcription factor, which acts in calcineurin signaling (Garcia, 2004). The latter pathway converges with CWI signaling in regulating gene expression in *S. cerevisiae* (reviewed in Levin, 2011). The upregulation of the *PHO* genes in the *Klrlm1* deletion mutant thus indicates a similar connection of the calcineurin pathway and the CWI pathway in *K. lactis.* Again, the lack of KlRlm1 as the standard target for KlMpk1 phosphorylation may thus lead to an induction of the calcineurin pathway. Signaling pathways in the two yeasts may thus be similar, since the key components of the *S. cerevisiae* calmodulin/calcineurin signaling pathway are conserved in *K. lactis* (Zanni *et al.*, 2009). However, neither the target genes of this pathway nor the regulation of *KlPHO3* have been studied in *K. lactis* yet, so that experimental evidence is not available.

4.1.2.2 Genes that are downregulated in the Klrlm1 deletion mutant

Since MADS-box factors are known to activate transcription, the actual target genes of KlRlm1 are expected to show a decreased expression in the *Klrlm1* deletion as compared to wild-type cells. The genes which are conform with this criterion can again be divided into different functional groups related to cell wall remodeling, mainly those governing

cell wall biogenesis, general morphogenesis, signal transduction components and stress response.

The homologs of several genes identified in this thesis as KlRlm1 effectors have been described in previous studies to be involved in compensatory mechanism after cell wall damage in *S. cerevisiae*. Among these are the genes *KlSED1, KlCIS3, KlHSP12, KlCWP1 and KlPMR10,* which are all upregulated by ScMkk1 overexpression, in response to Calcofluor white or Congo red (Jung and Levin, 1999; Boorsma *et al.*, 2004; Garcia, 2004). In general, 18 out of the 53 genes identified as *K. lactis* homologs were also found when Congo red was applied to induce cell wall stress in *S. cerevisiae*. This strongly supports the notion that the transcriptome data obtained here are reliable and suggests that similar processes are regulated by ScRlm1 and KlRlm1 in the cell wall stress response of the two yeasts.

A gene identified in all previous studies that was found as a target of KlRlm1 by this approach is *KlPIR1.* Its expression was already shown in the promoter-*lacZ* fusions discussed above to be activated by KlRlm1. In contrast, *KlMPK1* and *KlBGL2*, which did not respond to the presence or absence of KlRlm1 in the reporter assay, were clearly downregulated in the *rlm1* deletion mutant according to the mRNA sequencing data.

As already indicated above, specific ß-galactosidase activities are usually prone to substantial variations (see for examples (Heinisch *et al.*, 1998)). These may be due to varying plasmid copy numbers in different cultures and activities are also highly sensitive to cultivation conditions. Moreover, the reporter plasmids were based on a multicopy vector in *K. lactis*, presenting a high number of promoter binding sites, which may titrate a limited amount of KlRlm1 present in a wild-type strain. A 2.5-fold change in gene expression as observed in the transcriptome data (e.g. for *KlMPK1*: 5883/2393 and for *KlBGL2*: 6780/2500 hits) may therefore easily go undetected. This is also true for four further genes, which were chosen from the transcriptome analysis for confirmation by promoter-*KlacZ* reporter fusions. Only *KlSED1p* displayed a KlRlm1 dependent expression. Interestingly, the same construct was not expressed in *S. cerevisiae*, indicating that ScRlm1 is not able to activate transcription from this promoter. The predicted Rlm1 binding site is not present in the promoter regions of the other three genes tested, namely *KlCIS3, KlHSP12* and KlGRE1, which in contrast to the transcriptome data did not show a

dependence on KlRlm1 in the lacZ-reporter assay. For verification of the transcriptome data one would therefore prefer to use an alternative method, such as quantitative real-time RT PCR assays for the selected genes.

Another subset of genes which are involved in cell wall construction, and are downregulated in a *Klrlm1* mutant as expected, relate to chitin synthesis as judged from their *S. cerevisiae* homologs. A comparative analysis of cell wall carbohydrates indicated that *K. lactis* and *S. cerevisiae* contain the same relative amounts of chitin, although the cell wall of the latter is approximately 30% thicker (Backhaus *et al.*, 2010). The genes downregulated in the *Klrlm1* deletion include *KlCHS1*, *KlSKT5* and *KlYEA4*. In *S. cerevisiae CHS1* encodes chitin synthase I, whose main function is the repair of the chitinous primary septum during cytokinesis, which is originally synthesized by its homolog Chs2 (Cabib *et al.,* 1992) Expression of *ScCHS1* is induced by mating factor, but also by stress like Congo red (Garcia, 2004).

The proteins encoded by the other two genes, although necessary for proper chitin synthesis, are not regulated in a Rlm1-dependent manner in *S. cerevisiae*. ScYea4 is a transporter for the chitin precursor uridine diphosphate N-acetylglucosamine (Roy *et al.*, 2000). Consequently, deletion mutants of *ScYEA4* have reduced amounts of cell surface chitin. ScSkt5 is an activator of the chitin synthase III (Bulawa *et al.*, 1986). ScChs3 is the major chitin synthase for the lateral cell wall, the chitin ring prior to budding, the spore wall chitin, and for linking chitin to β-1,3-glucan by β-1,4-linkages (Ziman *et al.*, 1996; Ono *et al.*, 2000). As expected, *Scskt5* deletions are hyperresistant to Calcofluor white, since they produce less chitin for interaction with the drug (Trilla *et al.*, 1997). It should be noted that Chs3 is relocated from intracellular storage chitosomes to the plasma membrane upon stress, e.g. a heat shock. (Valdivia and Schekman, 2003; Sanchatjate and Schekman, 2006; Starr *et al.*, 2012). Phosphorylation by protein kinase C (Pkc1), the upstream kinase in CWI signaling, is necessary to trigger this translocation. This may allow a compensatory chitin synthesis as a rapid first response to enforce the cell wall, with a subsequent glucan synthesis triggered by the downstream transcription factor ScRlm1 and its target gene products. Chitin synthesis does not appear to be any target for transcriptional regulation by the CWI pathway in *S. cerevisiae*.

In contrast, the transcriptome data discussed above indicate that Rlm1 in *K. lactis* activates the expression of a few genes which influence chitin levels. However, if the upstream regulation through chitosomes also exists in milk yeast has not yet been investigated. Taking into account that *Scrlm1* mutants are hyperresistant to Calcofluor white, whereas *Klrlm1* deletions are hypersensitive, one would deduce that the cell wall of the former mutants has an increased chitin content, whereas the latter has a lower one than the respective wild types. Indeed, for *Scrlm1* deletions a lower chitin content has been measured compared to a wild type strain in our laboratory (Marc Zuckermann, pers. communication), consistent with the phenotype. The apparent contradiction of the *Klrlm1* mutant phenotypes and the transcriptome data may be explained in two ways: i) The transcriptional regulation of the three genes observed may not be sufficient to result in a lower cell wall chitin content. This may be either due to the moderate expression changes or to the fact that the encoded proteins are not crucial for chitin synthesis. The latter is in line with the fact, that *KlCHS1* is downregulated in the *Klrlm1* mutant, but not *KlCHS2*, whose homolog encodes the more important isoform in *S. cerevisiae*. ii) Alternatively, the immediate response through chitin synthase liberation from chitosomes may be more important for the adjustment of cell wall chitin contents upon stress, rather than the transcriptional response mediated downstream through the CWI pathway. If so, it would be interesting to determine if *K. lactis* disposes of a similar chitosome-regulated system and how exactly it is controlled under cell wall stress.

The transcriptome analysis also turned up a number of genes related to other stress signaling pathways, which are downregulated in the Klrlm1 deletion as opposed to the wild type. Thus, two homologs of genes which are induced by a variety of different stress conditions (such as heat shock, osmotic stress, oxidative stress, etc.) in *S. cerevisiae*, *KlGRE1* and *KlHSP12* (Garay-Arroyo and Covarrubias, 1999; Rep *et al.,* 1999) were identified. The expression of the two genes is controlled by the HOG pathway, mediating the response to high osmolarity. This is also true for the *ScGAT2* gene (Proft *et al.*, 2005), whose *K. lactis* homolog was also identified as a putative KlRlm1 target here. *ScGAT2* encodes a GATA-transcription factor (Cox *et al.,* 1999), and its expression is regulated by a variety of other transcription factors acting in different stress response pathways, such as

Swi4/6, Msn4, Mbp1 and Ste12 (MacIsaac *et al.*, 2006; Workman *et al.*, 2006; Hu *et al.*, 2007). ScGat2 interacts with ScMpk1, the MAPK of the CWI signaling pathway, as a response to DNA damage. In *Candida albicans* the transcription factor Gat2 plays a critical role in biofilm formation, filamentous growth and virulence (Du *et al.*, 2012). Taken together with the data on the *KlFLO* gene expression, a picture emerges in which KlRlm1 could be involved in the regulation of cell wall remodeling related to the morphological changes necessary for filamentous growth. In this context, it would be interesting to identify the target genes of KlGat2 in future studies.

Two other genes, *KlFMP16* and *KlUGX2* were also identified here as KlRlm1 targets. Their *S. cerevisiae* homologs encode proteins of unknown functions, but the expression of the encoding genes is also increased under general stress conditions. Interestingly, ScFmp16 accumulates in mitochondria, suggesting a relation between CWI signaling and respiratory functions. Such a relation has also been proposed from data on genetic interactions between CWI signaling components and mitophagy in *S. cerevisiae* (Mao *et al.*, 2011), which are currently investigated in our group (Schmitz *et al*., manuscript submitted). Thus, KlRlm1 seems to control a variety of aspects related to other signaling pathways and physiological responses. However, one should keep in mind that global gene expression in a *Klrlm1* deletion may not only affect the direct target genes with binding sites for this transcription factor in their promoters, but could also be indirectly triggered by the related cell wall damage and the concomitant induction of other stress pathways.

A final group of genes which are downregulated in a *Klrlm1* deletion and have known homologs in *S. cerevisiae* encodes membrane associated proteins. They comprise *KlSIA1, KlTFS1* and *KlYNL422C*. *S. cerevisiae* Sia1 contains a peptide signal for membrane localization and activates the plasma membrane H^+-ATPase (de la Fuente *et al.*, 1997). An indirect genetic interaction with CWI signaling has been found for the encoding gene. It turned up in a high-copy suppressor screen of a temperature sensitive allele of the translation elongation factor *HYP2*, in which several other CWI pathway components, namely *PKC1*, *WSC1*, *WSC2*, and *WSC3* also appeared (Valentini *et al*., 2002). *ScTFS1* was also found to respond to cell wall stress, as it appeared to be upregulated upon Zymolyase treatment in a transcriptome analysis (Garcia *et al.* 2004). The Tfs1 protein that plays a

role in regulation of the protein kinase A (PKA) signaling pathway (Caesar and Blomberg, 2004) was shown to have a high affinity for anionic phospholipids, like phosphatidylserine, PI(3)P, PI(3,4)P_2, and PI(3,4,5)P3 (Mima *et al*., 2006). Moreover, transcription of *TFS1* is known to be upregulated in response to oxidative stress and heat shock by Msn1/Msn4 (Boy-Marcotte *et al.*, 1999). The third gene, *KlYNL144C,* encodes a homolog of a *S. cerevisiae* protein of unknown function, which has been described to bind phosphatidylinositols (PI) and phosphatidylethanolamine (PE) through its PH domain, and accumulates in mitochondria, again suggesting a relation to respiratory capacity (Gallego *et al.*, 2010).

A large group of genes, whose expression is reduced in the *Klrlm1* deletion mutant, either encode proteins with uncharacterized functions or do not have homologs in *S. cerevisiae* at all. These may in fact be the most interesting, since they promise to reveal new targets and possibly new interactions in CWI signaling in *K. lactis*. Future investigations should thus first concentrate on the verification of the transcriptome data, e.g. by real-time RT PCR as suggested above. This would be followed by genetic approaches, primarily by assessing the phenotypes of deletion mutants and subsequent epistasis analyses.

In summary, sequencing of the total mRNA turned out to be a powerful tool to reveal probable targets of KlRlm1 and to understand the mechanisms of regulating the transcriptional response to cell wall stress in *K. lactis*. As the sequencing technique becomes cheaper, faster and proved to be highly reliable, it´s a great possibility to increase the understanding of cell wall integrity signaling in yeast in future experiments. In addition, the data set already available should be subjected to further bioinformatic analyses, since transcriptome data on *K. lactis* are still scarce. Thus, the relative expression of the entire transcriptome in relation to some housekeeping genes would be of great interest. However, such analyses depend on a sufficient statistical significance of the data, so that at least two more biological replicates should be obtained.

4.2 Characterization of eisosomes in *K. lactis*

In the second part of this thesis the presence and characterization of the eisosome microcompartment in *K. lactis* and its possible relation to CWI signaling was addressed. Eisosomes are of great interest in *S. cerevisiae* and other fungi as prominent membrane

structures, but despite of numerous studies their cellular function is largely unknown. Nevertheless, it has been suggested that they fulfill an important structural role in organizing the yeast plasma membrane (Karotki *et al.*, 2011). This thesis provides the first evidence for the existence of eisosomes in the milk yeast *K. lactis*.

4.2.1 Imaging of eisosomes in life cells of *K. lactis*

Using fluorescence microscopy approaches, the presence of eisosomes was verified in *K. lactis*. The proteins KlPil1 and KlLsp1, which are homologous to the central and highly abundant core proteins of eisosomes in *S. cerevisiae* (Walther *et al.*, 2006), were shown to localize to several discrete patches at the cell cortex, a distribution that is typical for eisosomes. Further, colocalization of the two proteins was observed. Both also showed colocalization with a third eisosomal marker protein, KlSur7. Sur7 is a transmembrane protein that localizes to eisosomes. It represents a significant component of eisosomes in fungi and is therefore often used as an eisosomal marker protein in fungal species (Walther *et al.*, 2006; Douglas *et al.*, 2011; Wang *et al.*, 2011). Although its detailed function is not known, ScSur7 and three other family members (ScFmp45, ScYnl194c, and ScPun1) are mediating stress response in *S. cerevisiae* (Young *et al.*, 2002; Douglas *et al.*, 2011). A *Klsur7* deletion mutant constructed here did not differ from wild-type under a variety of conditions tested (e.g. in its sensitivity towards elevated temperature, Caspofungin, Calcofluor white or Congo red; data not shown). Colocalization of KlPil1, KlSur7 and KlLsp1 in the plasma membrane of *K. lactis* strongly suggests that eisosomes have a similar composition as their counterparts in *S. cerevisiae* and may serve a similar function. This notion is further supported by the fact, that except for six proteins, all eisosomal components identified in *S. cerevisiae* have encoding homologs in the *K. lactis* genome (Table 1).

A first line of functional studies performed in this thesis also suggested a similar role for eisosomes in both yeast species. ScPil1 is the central eisosomal protein in *S. cerevisiae* and essential for eisosome assembly and shape (Moreira *et al*., 2009). Deletion of Sc*PIL1* leads to the disassembly of eisosomes and formation of eisosome remnants, a phenotype that was not observed for a deletion of the gene encoding its isoform ScLsp1, which has a

similar abundance but seems to be less important (Walther *et al.*, 2006).The same is true for the two *K. lactis* proteins. Deletion of *KlPIL1* resulted in collapsed eisosomal structures, whereas deletion of *KlLSP1* did not alter eisosome abundance and shape. In addition, double deletion mutants lacking both genes, *KlLSP1* and *KlPIL1,* are viable and do not present additive phenotypes as compared to the *Klpil1* single deletion strain (see discussion in the next section), again reminiscent of the observations in *S. cerevisiae*. Like their *S. cerevisiae* counterpart, KlPil1 self-assembles into linear, tubular structures, further underlining its conserved function. It has been demonstrated, that this tubule-formation is caused by membrane curvature induced by the BAR domain located near the amino-terminus of the ScPil1 protein (Olivera-Couto *et al.*, 2011), which is highly conserved in KlPil1. Thus, KlPil1 seems to carry out the same functions in the milk yeast while the role of KlLsp1 is also only of minor importance.

4.2.2 The role of eisosomes in maintenance of cellular integrity

The fungal plasma membrane is composed of different microcompartments and eisosomes are thought to be an essential factor in the process of organization and establishment of these domains. This has been attributed to eisosome mediated invagination and curvature of the lipid bilayer, triggered by the two core proteins Lsp1 and Pil1, as discussed above (Karotki *et al.*, 2011). Eisosomes have also been connected to cell wall integrity signaling and to stress response in fungi. In *S. cerevisiae* Pil1 has been described as a target of the protein kinases Pkh1 and Pkh2 (Zhang *et al.*, 2004), which function as mediators in cell wall integrity signaling. Here, a putative influence of eisosomes on cell wall integrity signaling and especially on the localization of the cell wall integrity pathway sensors in *K. lactis*, KlWsc1, KlWsc2/3 and KlMid2, was addressed. The findings and their physiological implications are discussed in the following.

In agreement with published data (Rodicio *et al.,* 2008) GFP fusions of the membrane spanning sensors KlWsc1, KlWsc2/3 and KlMid2 were found to localize to discrete spots within the plasma membrane; a distribution that is reminiscent of eisosomes in size and shape of the spots. Yet, the data presented here show that all three sensors did not localize to eisosomes, but rather seemed to be excluded from this membrane

microcompartment. This findings contradicts the assumption that eisosomes represent a protective area for the sensor molecules within the plasma membrane; a theory, which has been presented before for proteins of the endocytic machinery (Grossmann *et al.*, 2008; Brach *et al.*, 2011). Sensor distribution remained largely unaltered in the *Klpil1* deletion mutant, so the absence of eisosomes did not possess a noticeably effect on their localization. Moreover, *Klpil1, Kllsp1, Klsur7* deletion mutants and the *Klpil1 lsp1* double deletion mutant were not hypersensitive to cell wall stress. A heat resistant phenotype, which had been described in *S. cerevisiae* for the *Scpil1* and *Sclsp1* deletion strains (Luo *et al.*, 2008) could not be detected in *K. lactis* either. Thus, a direct influence of eisosomes on sensor distribution and cell wall integrity signaling is unlikely in the milk yeast. Alternatively, only one or two sensors could be affected by loss of the eisosomes, with a minor reduction in signaling capacity. In this case, an impact would be hard to detect by growth assays, especially since the three sensors are redundant in *K. lactis* and the single mutants present comparatively mild phenotypes (Rodicio *et al.*, 2008). In fact, the triple deletion of all CWI sensors in *K. lactis* proved to be viable, indicating either the presence of yet another unidentified sensor gene or of a compensatory mechanism for the entire CWI signaling pathway (Jürgen Heinisch, personal communication). Evidence for the latter hypothesis is provided by the activation of alternative stress pathways represented in the transcriptome data discussed above.

4.2.3 The function of eisosomes in plasma membrane microcompartmentalization

Although the original interest of this part of the thesis related to the role of eisosomes in the distribution of the CWI sensors, conclusions can also be drawn on the general organization and microcompartmentalization of the plasma membrane in the milk yeast. In analogy *S. cerevisiae,* one may assume that eisosomes organize the plasma membrane in *K. lactis*, even though the consequences remain highly speculative. In *S. cerevisiae* it has been proposed that eisosomes contribute to microcompartmentalization by segregating membrane lipids, since an increased concentration of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) was detected in these structures (Karotki *et al.*, 2011). However, stable lipid domains were also formed in the yeast vacuolar membrane in response to starvation, changes in the pH of the growth medium and other stresses (Toulmay and

Prinz, 2013) One of these domains is probably raft-like and similar to domains in the plasma membrane (Eggeling *et al*., 2009; Owen *et al*., 2012). Eisosomes are not found in the vacuolar membrane, so sterol–sphingolipid interactions are thought to provide the basic driving force for their plasma membrane distribution. In addition, protein–lipid and protein–protein interactions could lead to the formation of non-eisosomal structures of higher complexity with more, smaller and highly flexible microcompartments (Spira *et al*., 2012; Toulmay and Prinz, 2013). The membrane curvature maintained by eisosomes may provide a skeleton for this domain formation, since high-resolution fluorescence imaging with giant unilamellar vesicles (GUVs) indicated a correlation between domain composition and membrane curvature (Baumgart *et al*., 2013).

The lack of strong phenotypes in the mutants lacking eisosome components in *K. lactis* limits the speculation on their possible functions to the comparison with other fungal systems. Thus, in the fission yeast *Schizosaccharomyces pombe* an increased hydrolysis of phosphatidylinositol (4,5)-bisphosphate has been detected in eisosomes(Kabeche *et al.*, 2014). According to the authors, this eisosome-dependent regulation of PI(4,5)P2 levels would influence the membrane environment leading to an upstream signal in CWI and TOR signaling. Yet, like in *K. lactis,* they could not detect typical CWI phenotypes in their eisosome mutants.

In contrast, a *sur7* mutant in *C. albicans* displays several such phenotypes. As expected, CaSur7 localizes to eisosomes in the plasma membrane of *C. albicans*. Cells deleted for *CaSUR7* are hypersensitive to Calcofluor white and have decreased levels of cell wall β-glucan. Furthermore, cells display an abnormal morphogenesis with unusual intracellular cell wall structures (Alvarez *et al.*, 2008; Douglas *et al.*, 2011; Wang *et al.*, 2011). The authors thus conclude that CaSur7 is involved in plasma membrane organization, maybe by acting as a scaffold for other proteins, e. g. the Pkh1/2 kinases. By deletion of *CaSUR7* cell wall damage is mimicked, leading to activation of the compensatory mechanism and subsequent cell wall growth (Alvarez *et al.*, 2008). In *Ashbya gossypii* the *pil1*Δ mutant also displays strong defects in morphogenesis. Absence of eisosomes resulted in abnormal polarized growth (Seger *et al.*, 2011). It would

therefore be interesting to examine the cell wall structure of the *K. lactis* eisosome mutants described herein by transmission electron microscopy.

In contrast to these examples, eisosomes in the filamentous fungus *Aspergillus nidulans* are only present in conidiospores and seem to differ largely from other fungi. The Lsp1 homolog shows a rather diffused distribution in the cytoplasm and the Sur7 homolog is found in vacuoles and endosomes, and its lack does not cause any obvious phenotype (Vangelatos *et al.*, 2010).

In *S. cerevisiae,* eisosomes are associated with the Pkh1/2 protein kinases, with Pkc1 as one of their targets, and have an influence on the phospholipid composition in the plasma membrane (especially of $PI(4,5)P_2$) .The Pkh kinases are thought to control eisosome assembly and organization (Walther *et al.*, 2007) by responding to changes in the plasma membrane and transmitting this signal to eisosomes *via* ScPil1 and ScLsp1 phosphorylation (Luo *et al.*, 2008).

In mammalian cells, the Pkh1/2 homologs regulate cell integrity, actin localization and response to heat stress (Dickson *et al.*, 2006; Dickson, 2008). The organization of PI(4,5)P2 by eisosomes may also influence stress response and cell wall integrity in yeast (Karotki *et al.*, 2011). An enriched membrane pool of PI(4,5)P2 is also crucial for anchoring and function of other signaling complexes, such as the one coordinated by ScSte5 in pheromone signaling (Garrenton *et al.*, 2010). This is also suggested by the phenotype of *Scmss4* null mutants: Mss4p is essential for generating plasma membrane PI(4,5)P2 and is critical for plasma membrane targeting of components of the yeast pheromone response and the yeast cell wall integrity pathways. Consequently, *Scmss4* mutants are also defective in CWI signaling (Guillas *et al.,* 2013). A different study from *S. cerevisiae* offers a hypothesis, where lateral rearrangements of membrane compartments are necessary for survival of rapid cell dehydration. Alterations of the Sur7-GFP distribution and formation of many plasma membrane invaginations suggested that eisosomes play a crucial role in this process (Dupont *et al.*, 2010).

In summary, the preliminary data gathered here suggest a similar composition and function of eisosomes in *K. lactis* and *S. cerevisiae*. Regarding the fact that their exact function remains elusive, the mutants generated here may serve as a basis for further

genetic screens, which may help to better define this role. *K. lactis* may be better suited for such an approach than the extensively studied baker's yeast, since its genome has not undergone a duplication during evolution and is thus much less redundant, thereby facilitating the detection of mutant phenotypes (Rodicio and Heinisch, 2013).

5 Conclusions

From the results described in this thesis, one can draw a number of conclusions:

1. The MADS box transcription factor KlRlm1 is a homolog of the well-described CWI transcriptional activator ScRlm1 from *S. cerevisiae* and has a similar domain structure and as expected, it localizes to the nucleus.

2. KlRlm1 functions in maintaining cell wall integrity in *K. lactis.* Thus a *Klrlm1* deletion mutant is sensitive to typical cell wall stressors like caspofungin, Calcofluor white and Congo red.

3. The capacity of the *RLM1* genes to complement the deletions in the respective heterologous hosts was assessed. In general, overexpression confers growth to the deletion mutant in the heterologous host although variations in the sensitivities of the strains to a specific stress agent, and the different responses to different stress agents were found. This indicates that the proteins are indeed functional homologs but also that there is a certain degree of species specificity.

4. Chimeric constructs between the presumed DNA-binding domain and the transcriptional activation domain of Rlm1 from *K. lactis* and *S. cerevisiae* indicate that they are all functional to some degree.

5. A set of eight *K. lactis* genes whose *S. cerevisiae* homologs are known to be transcriptionally upregulated by activation of the CWI pathway were tested as putative targets of Rlm1 in *K. lactis* using *lacZ*-promoter fusions. Only one of them, *KlPIR1,* was downregulated in the *Klrlm1* mutant. This indicates that Rlm1 in the two species share some common gene targets, but that there are also different targets in *K. lactis.*

6. Transcriptome sequencing identified 58 such putative target genes of KlRlm1 (including *KlPIR1*), since their expression was downregulated in the *Klrlm1* deletion mutant as compared to wild type. A number of the identified genes can be directly related to cell wall biosynthesis, while others seem to be involved in more general stress response mechanisms and were not previously identified as targets of the CWI pathway in *S. cerevisiae*.

7. A further 29 genes displayed an increased expression in the deletion mutant. They contain a number of genes encoding flocculins, which may serve as a safeguard mechanism for cell wall integrity.

8. *K. lactis* has the capacity to form eisosomes, as shown by the distribution of KlPil1 and KlLsp1, which are homologs of the major eisosome constituents in *S. cerevisiae*.

9. The distribution of *LSP1-GFP* and *SUR7-mCherry* is altered in a *Klpil1* deletion mutant. This indicates that KlPil1 is responsible for the proper formation and shape of eisosomes in *K. lactis*.

10. Single and double deletion mutants of the *KlPIL1* and *KlLSP1* genes are viable, indicating that eisosomes do not serve an essential function in *K. lactis*.

11. The three CWI sensors of *K. lactis*, KlWsc1, KlWsc2/3, and KlMid2, form distinct membrane microdomains, which are excluded from the regions occupied by the eisosomes. A lack of KlPil1 does not affect the distribution of the sensors in the plasma membrane. This indicates that microdomains in the plasma membrane of *K. lactis* are formed independent from each other and may solely be determined by the constituent protein properties.

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7 List of abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microliter
aa	Amino acid
Ac	Acetate
Amp	Ampicillin
bp	Base pair
CSM	Complete supplement mixture
CWI	Cell wall integrity
CWP	Cell wall protein
D	Glucose
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DIC	Differential interference contrast
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FDR	False Discovery Rate
g	Gram
G418	Geneticin
GAP	GTPase activating proteins
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GlcNAc-PI	N-acetylglucosaminyl phosphatidylinositol
GPI	Glycosylphosphatidylinositol
GTP	Guanosine triphosphate
GUV	Giant unilamellar vesicle
h	Hour
H_3PO_4	Phosphoric acid
HA	Human influenza hemagglutinin
HOG	High osmlolarity glycerol
K_2HPO_4	Dipotassium phosphate
kb	Kilo bases
KH_2PO_4	Monopotassium phosphate
l	Liter
LB	Lysogeny broth

M	Molar
MAPK	Mitogen activated protein kinase
MCC	Membrane compartment occupied by Can1
MCP	Membrane compartment occupied by Pma1
MCT	Membrane compartment occupied by TorC2
mg	Milligram
Min	Minute
ml	Milliliter
mM	Millimolar
NaOH	Sodium hydroxide
no.	number
OD	Optical density
ONPG	Ortho-Nitrophenyl-β-galactoside
ORF	open reading frame
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
pH	Decimal cologarithm of hydrogen
PI	Phosphatidylinositol
PI(3)P	Phosphatidylinositol 3-phosphate
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PI(3,4,5)P3	Phosphatidylinositol 3,4,5-trisphosphate
PIR	Proteins with internal repeats
RNA	Ribonucleic acid
rpm	Rounds per minute
SBF	SCB binding factor
SC	Synthetic complete
SCB	SBF complex binding
SDS	Sodium dodecyl sulfate
sec	Second
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
UV	Ultraviolet radiation
W	Watt
w/o	without
Wt	Wild type
x	Fold
YEP	Yeast extract peptone
G418	Geneticin

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9 Curriculum vitae

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„Physiology and dynamics of cellular microcompartments"
Poster: "Dynamics of sensors triggering cell wall integrity signaling in *S. cerevisiae* & *K. lactis*" Christian Kock, **Sascha Meyer** und Jürgen Heinisch

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1. Joint meeting, Osnabrück, Germany, 11/2010
2. Joint meeting, Oviedo, Spain, 11/2011
Talk: "The role of the transcription factor Rlm1"
3. Joint meeting, Oviedo, Spain, 11/2012
Talk: "Microcompartments in the yeast plasma membrane"
4. and final meeting, Madrid, Spain, 04/2014
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Meyer, S., Schmitz, H.-P., Rodicio, R., and Heinisch, J.J.: **Characterization of the MADS-box transcription factor Rlm1 and its targets in *Kluyveromyces lactis.***

Meyer, S., Rodicio, R., and Heinisch, J.J.: **Cell wall integrity sensors in Kluyveromyces lactis form distinct microdomains in the plasma membrane, which are distinct from eisosomes.**

10 Statutory declaration

Erklärung über die Eigenständigkeit der erbrachten wissenschaftlichen Leistung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Bei der Auswahl und Auswertung des aufgeführten Materials haben mir keine weiteren Personen geholfen.

Personen waren an der inhaltlichen materiellen Erstellung der vorliegenden Arbeit nicht beteiligt.

Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Osnabrück, 26.06.2014

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