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Characterization of synthetic regulatory elements for protein expression in *Pichia pastoris*

Master Thesis

written by

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ABSTRACT

P. pastoris is a methylotrophic yeast widely used in industrial biotechnology due to its amenability to genetic manipulation, its capability of high level protein production, the propensity to post-translationally modify proteins and the availability of well characterized vectors and strains for protein expression. Additionally *P. pastoris* also provides strong, methanol inducible promoters that are tightly regulated. The most widely employed promoter for protein expression in *P. pastoris* is the P_{AOX1}. However, the practicability of this system is limited by safety requirements to store and handle large amounts of methanol, a toxic and flammable compound. Therefore, any process allowing methanol free, controlled expression of P_{AOX1} is highly desirable. In this work, several putative regulatory factors comprising of zinc finger transcription factors, chromatin remodeling/histone modifying enzymes and catabolite repression master regulators (kinases, phosphatases) were evaluated for the usability in the design of synthetic genetic circuits for protein expression. The overexpression of one of three zinc finger transcription factors (Mxr1, Prm1 or Mpp1) under the derepressed catalase promoter (P_{CAT1}) allowed methanol-free induction of P_{AOX1} reaching 75 % of full methanol induction. Subsequently the three activator overexpression strains were analyzed on the transcriptional level using microarrays. In summary, this study resulted in a tightly regulated, auto-inductive expression system capable of high level P_{AOX1}-driven protein expression without the need to use methanol. In contrast to earlier studies in which Mxr1 overexpression lead to a drastic decrease in cell viability, P_{CAT1} regulated expression retained viability and allowed methanol free protein expression. From a synthetic biology view point these factors may also become valuable tools for genetic circuit design in *P. pastoris*.

KURZFASSUNG

Die methylotrophe Hefe *Pichia pastoris* wird in der industriellen Biotechnologie aufgrund ihrer Zugänglichkeit für genetische Manipulationen, ihrer Fähigkeit Proteine in großen Mengen zu produzieren, der Fähigkeit post-translationale Modifikationen durchzuführen und der Verfügbarkeit von gut charakterisierten Vektoren und Stämmen sehr häufig für die Proteinexpression verwendet. Zusätzlich besitzt *P. pastoris* starke und stringent regulierte Promotoren, die mit Methanol induziert werden können. Dabei stellt der P_{AOX1} den am häufigsten verwendeten Promoter für die Protein Expression dar. Nichtsdestotrotz ist die Anwendbarkeit dieses Systems limitiert durch die Lagerung und Handhabung großer Mengen an Methanol, einer toxischen und hochentzündlichen Chemikalie. Deshalb ist ein Prozess, der die P_{AOX1} getriebene Expression ohne Methanol erlaubt höchst wünschenswert. Im Rahmen dieser Arbeit wurden putative regulatorische Faktoren in Form von Zinkfinger-Transkriptionsfaktoren, Chromatin-Remodellierungskomplexen und Histonmodifizierenden Enzymen für die Zusammensetzung zu synthetischen genetischen Schaltkreisen auf ihre Eignung zur Proteinproduktion überprüft. Die Katalase Promoter (P_{CAT1}) getriebene Überexpression einer der drei Zinkfinger-Transkriptionsfaktoren (Mxr1, Prm1 oder Mpp1) erlaubte die Methanol freie Induktion des P_{AOX1} auf ein Level von 75% der Aktivität unter Methanol Induktionsbedingungen. In weiterer Folge wurden die drei Aktivator-Überexpressionsstämme mittels Microarrays auf transkriptioneller Ebene analysiert. Das Ergebnis dieser Studie ist ein auto-induktives Expressionssystem, das eine effiziente und Methanol freie Proteinproduktion mittels *AOX1* Promoter erlaubt. Im Gegensatz zu früheren Studien, die zeigen dass Mxr1 Überexpression die Lebensfähigkeit von Zellen stark einschränkt, erlaubt eine durch den Katalase Promoter (P_{CAT1}) regulierte Expression sowohl die Lebensfähigkeit der Zellen als auch die Methanol freie Protein Expression. Vom Standpunkt der synthetischen Biologie betrachtet, können diese Faktoren als wertvolle Werkzeuge für das Design von genetischen Schaltkreisen in *P. pastoris* verwendet werden.

CONTENTS

1. INTRODUCTION

1.1 Synthetic Biology as a tool for rewiring transcriptional networks

Over the last decade the field of synthetic biology and overlapping disciplines such as systems biology and metabolic engineering have experienced a burst in attention of the scientific community accompanied by a surge in research funding. Commonly studied topics of synthetic biology range from novel genetic engineering tools and the generation of artificial cellular building blocks to highly sophisticated integrated systems. The cross-fertilization of disciplines, that in the past used to be seen as strictly separated, resulted in an explosion of ideas and enthusiasm creating a momentum which pushed other areas along (Brent 2004, Purnick 2009).

One of the most challenging problems that synthetic biologists face at the moment lies in a shortage of well characterized parts, which would seriously shorten design cycles resulting in less efforts required to obtain fully functional systems. For a proper implementation into coherent systems, synthetic parts must fulfill the criteria of independence, reliability, tunability, orthogonality and composability (Lucks 2008, Canton 2008). To which extent all these single characteristics need to be present in a system to be fully functional has to be evaluated individually. However, before starting to discuss the desired features of standardized biological parts we need to accumulate large enough repositories containing components with above mentioned qualities. At this stage of development the individual and highly dispersed nature of synthetic biology represents both its greatest asset and its biggest liability. Drew Endy pointed out, that after having established a vibrant and open research community[1], the crucial next step for further advancing synthetic biology lies in strategic leadership. Without the existence of centralized repositories collecting, storing and curating important contributions made in labs around the world synthetic biology will be thwarted in its potential to solve problems (Endy 2005).

However, the collection of "bio bricks" with desired traits mentioned above is not strictly limited to either isolation from natural sources or creation from scratch. Frances H. Arnold and coworkers were successful in implementing directed evolution experiments for the generation of synthetic parts thereby complementing the purely rational approach (Yokobayashi 2002). For many applications it might be more efficient to divert the function of

[1] see conferences such as the BioBricks Foundation SB conferences and the yearly held iGEM competition

already available parts for a certain task rather than searching them from natural sources. Often iterative retrofitting of newly assembled parts is necessary to functionalize nonfunctional circuits which could possibly be circumvented by combining rational design and directed evolution experiments (Dougherty 2009, Ellis 2009).

The usefulness of modular architecture and therefore the possibility to start from a single point and move into many different directions to end up with a specific activity has already been shown in the construction of TALENs (transcription activator like effector nucleases) which allow targeted genome editing (Mussolino 2012, Slusarczyk 2012). Recently the development of new targeting systems in the form RNA guided nucleases, which circumvent the need to tune specificity via protein-DNA interactions, has been shown. CRISPR, short for clustered, regularly interspaced, short palindromic repeats are components derived from bacteria and archaea. In these organisms CRISPR form part of an adaptive immune system against invading DNA. Once infected with foreign DNA small stretches get incorporated into CRISPR and will be transcribed into RNA. These transcripts are subsequently used to guide Cas9 to the invading DNA to inactivate it by cleavage. By incorporating the guide RNA against a desired DNA molecule into the CRISPR region selective cleavage can be achieved. (Carroll 2013). A slightly different approach to control gene expression was taken by Qi and coworkers. They employed catalytically dead Cas9 from CRISPR-Cas9 systems to target it to a specific gene in order to interfere with transcriptional elongation, RNA polymerase binding and transcription factor binding (Qi 2013). Modifications of this approach have also been implemented by Gilbert *et al* 2013 or Mali *et al.* 2013 by fusing transcriptional repressor or activator domains with catalytically dead Cas9 thereby rewiring the transcriptional regulation (Gilbert 2013, Mali 2013, Perez-Pinera 2013).

Considering synthetic biology breakthroughs in the narrower field of transcriptional regulation we are now at a stage at which we are able to systematically construct eukaryotic transcription functions. Developments such as TALENs and CRISPR-Cas9 allow us to combine different functionalities and thereby rewire whole transcriptional circuits. By selectively changing on the one hand DNA specificity and promoter affinity and on the other hand protein interactions at the regulatory domains of transcription factors, synthetic zinc finger proteins for specific purposes can be built (Khalil 2012).

After 10 years of research providing proof of principle data, synthetic biology finally is at a stage at which it can tackle pressing societal issues such as alternative means for the production of green fuels and cheap drugs. In 2009 and 2010 first Purnick and Weiss and later Khalil and Collins published two very comprehensive reviews about applications for synthetic biology that have already come of age, ranging from issues like biofuels and biopharmaceuticals (metabolic flux control) to biosensing devices. This shows that synthetic biology has finally left the "training ground" and is set to succeed in solving existing problems (Khalil 2010, Purnick 2009).

1.2 Design of synthetic genetic circuits for auto-inductive protein expression

One aim of this thesis is the construction of an auto-inductive system for high level protein expression in *P. pastoris*. Crucial for the successful realization of a synthetic genetic circuit capable of auto-inducing protein expression is the selection of suitable parts. The standardization facilitates such approaches. Figure 1.1 depicts a schematic representation of such a genetic circuit that could potentially be used for auto-inductive protein expression. The single elements comprise of a promoter $P_{(specific)}$, which has the ability of inducible high level gene expression (GOI, gene of interest), an activator, which regulates the function of said promoter and a second promoter $P_{(core)}$ controlling the expression behavior of the activator. By choosing $P_{(core)}$ accordingly we would theoretically be able to adapt the circuit to a desired signal which triggers activator and therefore GOI expression. This strategy is similar to a transcriptional amplification strategy (TAS) already described in higher eukaryotes (Liu 2008).

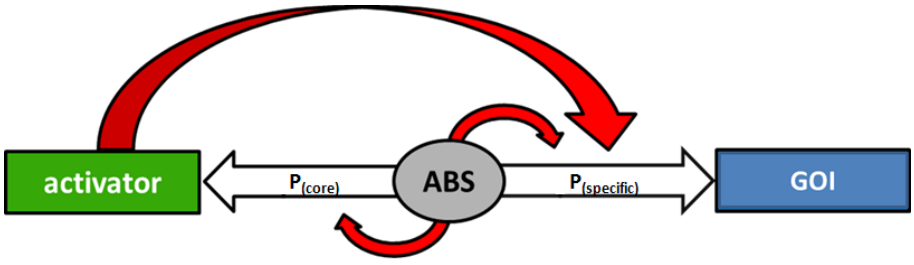

Figure 1.1.: Genetic Circuit for an auto-inductive protein expression amplifier. Expression of a desired protein is driven by a strong, tightly regulated and inducible promoter ($P_{(specific)}$). An activator is controlled by a promoter which exhibits desired features and acts on $P_{(core)}$. This activation can either be engineered by choosing an already existing activator for $P_{(core)}$ or by designing activator binding sites (ABS). Upon signal triggering (glucose depletion, addition of inducer, heat changes) activator will be transcribed and is free to act on $P_{(specific)}$ via binding to ABS. The binding of activator will then allow expression of the gene of interest and ideally acts in a feed-back loop manner to further push activator expression.

The most widely employed promoter for protein expression in *P. pastoris* is the *alcohol oxidase 1* promoter (P_{AOX1}) (Vogl and Glieder, 2013). However, the usage of this promoter is limited by safety issues regarding the storage and handling of large amounts of methanol, a toxic and flammable compound and therefore industry is looking for alternatives. So far protein expression using *P. pastoris* in methanol free conditions was possible with constitutive promoters such as P_{GAP}, with the *FLD1* promoter and methylamine as an inductor or with derepressed synthetic P_{AOX1} variants (Hartner et al. 2008). Furthermore Prielhofer *et al* 2013 identified six novel glucose-limit inducible promoters which were identified by shifting the cells from a glycerol batch phase to a glucose fed-batch phase. One of these promoters P_{G1} was determined to be a high-affinity glucose transporter. These promoters could be employed as alternatives for methanol free protein expression (Prielhofer 2013). However, while first generation *AOX1* promoter variants show derepression behavior upon glucose depletion they lacked the strong upregulation observable upon methanol induction (Hartner 2008). From an industrial point of view a system combining both, on the one hand strong induction without usage of methanol and on the other hand derepression upon glucose depletion would be highly desirable. One way to reach this effect was to amplify activating regions in the *AOX1* promoter (Mellitzer 2012, Hartner 2008). Alternatively, to retain the system's capability of induction a trigger signal and the corresponding inducible promoter should be employed to drive expression of the activator.

In order to conform to the system's ability to work in methanol free culture conditions the signal most likely functional in *P. pastoris* is glucose depletion. Therefore in our system pictured in figure 1.2 we chose the glucose repressed and methanol inducible catalase promoter P_{CAT1} to drive the expression activator promoters. Upon glucose depletion the expression of activator would commence and activate P_{AOX1} by binding to activator binding sequences (ABS). This activation would eventually lead to the expression of the sTomato reporter gene. Subsequently, glucose limited feed strategies can be used to maintain the system in a state of glucose depletion, while still complying with carbon and energy requirements.

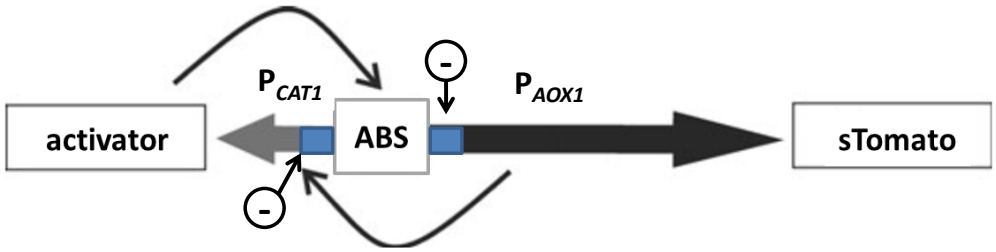

Figure 1.2.: Depiction of promoters and reporter used in the construction of the genetic circuit for auto-inductive protein expression. Due to its tight repression on glucose and strong upregulation upon glucose depletion P_{CAT1} was chosen as activator-regulating promoter. The strongly inducible *AOX1* promoter, which is repressed in the presence of carbon sources such as glucose and glycerol, was selected to drive expression of the fluorescent protein single Tomato (sTomato) as a reporter. sTomato provides the ability to qualitatively evaluate expression using bare eyes due to its fluorescence at visible light wavelengths. ABS and "-" signs refer to activator and repressor binding sites, respectively. Figure adapted from Ruth and Glieder ChemBioChem 2010.

1.3 Transcriptional Regulation of Carbon Metabolism

1.3.1 Systems taking part in the regulation of glucose catabolite repression in yeast

P. pastoris is a yeast species extensively used in industrial biotechnology and as such has been subject to optimization on a number of levels ranging from cell-engineering, media formulations and bioprocess-engineering (Macauley-Patrick 2005, Lin-Cereghino 2002, Cregg 2000, Lin-Cereghino 2000, Krainer 2012). Methanol inducible promoters such as P_{AOX1} are widely used to achieve high level production of recombinant proteins including industrially used biocatalysts and biopharmaceuticals (Vogl *et al.*, 2013). The understanding of the exact molecular regulation of the *P. pastoris AOX1* promoter is still incomplete (Vogl and Glieder, 2013). Yet, the selection of parts for the desired auto inductive circuit requires at least basic understanding of the underlying natural regulation. Therefore incorporating information from related carbon source regulated promoters from other yeast species appears crucial to select promising parts for autoinduction of P_{AOX1}. Engineering approaches in *P. pastoris* in general have largely relied on homologies drawn from *S. cerevisiae*, which has been studied extensively for several decades as a eukaryotic model organism (e.g. Stadlmayr 2010, Sauer 2004). Due to the lack of any feasible alternative I abided by this approach and performed a thorough literature research on carbon-source dependent transcriptional regulation in *S. cerevisiae*. After pinpointing *S. cerevisiae* key regulators involved in this process I

tried to find possible homologues in *P. pastoris* and other methylotrophic yeasts. These factors could potentially behave in a very similar way in *P. pastoris*.

The yeast *S. cerevisiae* is able to metabolize a broad array of carbohydrates like glucose, fructose, galactose, sucrose, glycerol, ethanol and oleate. However, the usage of easily fermentable carbon sources like glucose or fructose is favored and reflected by the cellular regulation behavior upon availability of this carbon source (Barnett 1976). In the presence of glucose, the genes for enzymes involved in the metabolism of alternative carbon sources are transcriptionally repressed in a process termed "catabolite repression". Catabolite repressed genes are regulated by different modes of regulation: (De-)repression and activation (Gancedo 1998). In the presence of glucose, genes for the metabolization of non-glucose carbon sources are repressed. After glucose is depleted, repressors are deactivated resulting in a derepressed state. Yet, for full expression of the respective promoters, activation by the respective alternative carbon source is needed (Zaman 2009). In general, five systems are known so far in taking part in the regulation of glucose catabolite repression in *S. cerevisiae* – Ras/PKA, Gpr1/Gpa2, Sch9, Snf1 and Rgt2/Snf3 (Zaman 2009).

The first step in realizing this capability is sensing the presence of glucose in the vicinity of the cell, which is performed by glucose sensors embedded in the cell membrane of yeast (Rolland 2002). Two prominent examples of proteins fulfilling this task are Snf3p and Rgt2p which transmit the glucose signal to Rgt1p. Under glucose depleted conditions this transcription factor acts as a repressor blocking the transcription of *HXT1*, *HXT2*, *HXT3* and *HXT4* and is subject to hyperphosphorylation under high glucose concentrations, thereby acting as a transcriptional activator (Rolland 2002, Gancedo 1998).

One major component of the glucose signaling system is the Ras/PKA system, reacting to changes in glucose concentrations and diverting transcription accordingly (Zaman 2009). Ras proteins are part of the G-protein family and act as switches according to their bound cofactor, whereby GTP bound protein is in its active form and GDP-bound inactive (Colombo 1998). Additionally posttranslational modifications responsible for membrane localization are important. Active Ras-GTP proteins stimulate cAMP production through adenylate cyclase. The increase in cAMP activates PKA which itself acts as a kinase regulating a host of cellular functions such as carbon metabolism and transcription (Santangelo 2006). Under high PKA activity transcription of genes containing carbon source responsive ele-

ments (CSRE), like many gluconeogenic and TCA genes, is repressed (Schüller 2003). The third important contributor to glucose regulation is the Gpr1/Gpa2 system, which is also part of the G-protein coupled receptor family (GPCR) and acts in parallel to Ras/PKA via cAMP production (Zaman 2009, Colombo 1998). The role of Snf1p as a regulatory protein will be discussed at a different place. Sch9 is an AGC family kinase and by overexpression was shown to suppress lethality due to absent PKA signaling high-lighting its importance in glucose signaling (Toda 1988).

1.3.2 Comparison of glucose regulation in *S. cerevisiae* and methylotrophic yeasts

To make the complex transcriptional regulation of carbon metabolism in *S. cerevisiae* easily accessible and facilitate evaluation of possible activator targets the state-of-knowledge in summarized in figure 1.3 (A). This figure is contrasted with a putative network based on my homology search in methylotrophic yeasts in figure 1.3 (B). It is important to mention that I tried to back the function of the single regulation partners and their possible interactions by experimental data available to me. Only in a few cases I was not able to find a *S. cerevisiae* homologue of *P. pastoris* specific activators as in the case of Mpp1, an activator of genes necessary for methanol metabolization in *H. polymorpha* (Leao-Helder 2003, van Zutphen 2010). In some cases homologous sequences from *S. cerevisiae* found in *P. pastoris* clearly have different functions in this methylotrophic yeast. Most prominent example is the *P. pastoris* homologue of *ScGAL4*, which is annotated as activator for genes in the metabolization of galactose – a capability that this *P. pastoris* does not possess (Bobrowicz 2012).

The central regulator of carbon metabolism and more specifically in glucose repression and derepression is the protein kinase Snf1, the yeast homolog to mammalian AMP-activated PK (Zaman 2009). The kinase Snf1p and its regulatory protein Snf4p are responsible for the derepression of glucose repressible structural genes which are needed for processing of alternative non-glucose carbon sources. Additional factors interacting with Snf1p have been identified (coded by *SIP1*, *SIP2*, *GAL83*) with all of them sharing the central kinase-interacting sequence (Jiang 1996, Schüller 2003). Upon glucose exhaustion the Snf1p upstream kinases (Sak1p, Tos3p, Elm1p) activate Snf1p and thereby allow *S. cerevisiae* to react to the presence of non-glucose carbon sources such as glycerol or ethanol (Hedbacker 2004, Hong 2003). Phosphorylation of transcriptional regulators downstream of Snf1p has

different outcomes such as activation in the case of Adr1p and Cat8p but inactivation of Mig1p in *S. cerevisiae* (Papamichos-Chronakis 2004). In cells grown under glucose conditions Snf1p is found in the cytosol whereas upon shift to non-fermentable carbon sources Snf1p gets imported into the nucleus (Hedbacker 2004). The antagonistically acting phosphatase system Glc7p/Reg1p is regulating amongst other substrates also Snf1p, negatively affecting its kinase activity. Reg1p denotes the regulatory subunit which is known to participate in glucose repression (Tu 1994). As is the case for Snf1p and Glc7p the specificity of substrate interaction is mediated not by the kinase/phosphatase itself but rather by the regulatory subunit acting as targeting intermediaries (Santangelo 2006).

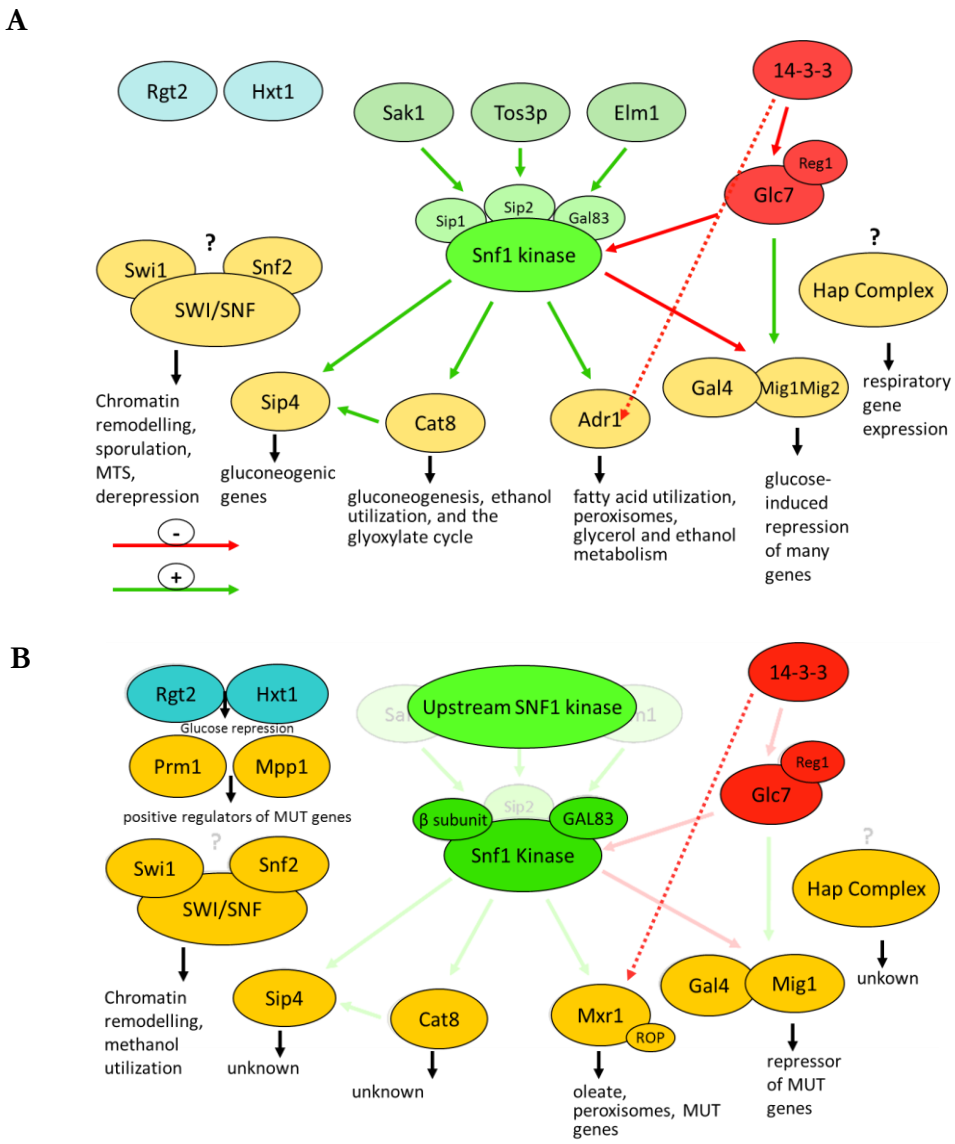

Figure 1.3.: Comparison of carbon source regulation in *S. cerevisiae* and other methylotrophic yeast species. A: Carbon source regulation in *S. cerevisiae*. **B:** Putative factors involved in carbon source regulation in the methylotrophic yeasts *P. pastoris, H. polymorpha* and *C. boidinii*. Depicted three-letter gene names conform to SGD (Saccharomyces Genome Database) naming and cited literature or as annotated in the genome of *P. pastoris* and deposited single files in the case of *H. polymorpha* and *C. boidinii*. The key regulator of carbon metabolism is shown in the center of the image and denoted Snf1 kinase with its counter-acting phosphatase Glc7/Reg1 shown in red. The red and green arrows indicate activation and deactivation, respectively. Note that kinase-mediated phosphorylation is not necessarily an activating input signal as for instance in the case of Mig1/Mig2 where phosphorylation leads to inactivation. Possible interactions that still remain to be elucidated are marked with a question mark as in the case of the SWI/SNF and Hap complex. Only experimentally verified interactions are shown in bright red/green while putative interactions are drawn in pale red/green. Pale bubbles depicting interaction partners from *S. cerevisiae* could not be found via bioinformatics-assisted homology search (BLAST). The black arrows in the bottom part of the figure relate each TF with its involvement in cellular functions as described in literature. Note that experimental data showing area of involvement is spread across all three yeast species. See table 1.1 for further details.

After having established the corner stones of transcriptional regulation of catabolite repression, I will elaborate more specifically on single factors involved in glucose repression and metabolization of non-glucose carbon sources. The overview so far has been mainly deducted from research in the yeast *S. cerevisiae* but due to the conserved nature of these key regulators from yeast to higher eukaryotes it seemed sensible to rely on this information for discussing possible implications for *P. pastoris* or other methylotrophic yeasts species.
If we take a closer look at the carbon source regulation in methylotrophic yeasts in figure 1.3 (B) the most striking difference between *S. cerevisiae* and *P. pastoris* obviously lies in the latter yeast's ability to metabolize methanol. This metabolization requires a set of transcription factors regulating the expression of genes involved in methanol assimilation and dissimilation, including the generation of compartments and associated cellular adaptions.

Since major parts of the process of methanol utilization is taking place inside of peroxisomes *P. pastoris* is a preferred model organism for the elucidation of peroxisome biogenesis and function (Gould 1992). Major contributors to this process are the so called *PEX* genes, which comprise of roughly 20 peroxins involved e.g. in peroxisomal protein import (van der Klei 2006). The metabolization of peroxisomal substrates like methanol and oleate is mainly regulated on the transcriptional level in a combination of repression/derepression under glucose depleted conditions and methanol-specific induction (Lin-Cereghino 2006). The gene regulation of methanol metabolization in *P. pastoris* and fatty acid utilization in *S. cerevisiase* share many similarities, ranging from subcompartmentalization in peroxisomes to the mode of repression and induction in both yeasts (Sloan 1999, Lin-Cereghino 2006). To better evaluate possible targets in *P. pastoris* possibly acting positively on P_{AOX1} expression I have compiled them with their respective function in table 1.1 (if known in any methylotrophic yeast).

Table 1.1.: Synopsis of proteins involved in the regulation of catabolite repression and activation listed with their respective function in methylotrophic yeasts. Listed in this table is condensed information of published data in methylotrophic yeasts *P. pastoris*, *H. polymorpha* and *C. boidinii*. In case I was unable to find experimental data from methylotrophic yeast species, references to the homologous protein function in *S. cerevisiae* are shown.

Protein Name	Function	References
Rgt2	Rgt2p (Gss1) is a glucose sensor in *P. pastoris,* Δ*gss1* showed impaired glucose catabolite repression	**Polupanov 2012**
Mxr1	Master regulator of methanol and peroxisomal genes in *P. pastoris* (Mxr1p) and *C. boidinii* (Trm2p), the *S. cerevisiae* homolog Adr1p is also involved in activation of catabolite repressed genes	**Parua 2012, Sasano 2010**
Prm1	Prm1p in *P. pastoris*, its homologue Trm1p in *C. boidinii* and Mut3p in *H. polymorpha* are positive regulators of MUT genes	**Sasano 2008, Vallini 2000 Takagi 2012**
Swi1 **Snf2**	*Δswi1Δsnf2* double knockout showed defective methanol utilization in *H. polymorpha*	**Ozimek 2004**
Snf1	*S. cerevisiae* Snf1p kinases is a master regulator of carbon catabolite-derepression	**Wilson 1996**
Cat8	Zinc cluster transcriptional activator involved in activation of catabolite repressed genes in *S. cerevisiae*.	**Young 2003**
Reg1	annotated as regulatory subunit of Glc7p master phosphatase in *P. pastoris*, Glc7p acts antagonistically to master kinase Snf1p by dephosphorylation	**Zaman 2009**
Gal4	*S. cerevisiae* regulator of galactose metabolism, in *Pichia pastoris* annotated as *LAC9*, unknown function as *P. pastoris* is unable to grow on galactose	**Traven 2006**
Mpp1	Mpp1p (methylotrophic peroxisomal protein 1) in *H. polymorpha* important for growth on methanol, annotated as YLL054C in *P. pastoris*	**van Zutphen 2010 Nishi 2012**
Rpd3 **Hda1**	Histone deacetylases from *S. cerevisiae* which regulate transcription, silencing, autophagy and other processes by influencing chromatin remodeling, *Δrpd3* and *Δhda1* genes allowed constitutive promoter binding of Adr1p and Cat8p	**Rundlett 1996 Tachibana 2007**
Zta1	ssDNA binding protein present in *P. pastoris*, binds to -288 to -115 region of P_{AOX1}, possible regulatory function	**Kranthi 2006**
Rop1	in *P. pastoris* methanol- and biotin-starvation-inducible zinc finger protein, repressor of phosphoenolpyruvate carboxykinase (PEPCK), represses P_{AOX1} on methanol containing full media, binds with higher affinities to the same sequences as Mxr1p	**Kumar 2011 Kumar 2012**
Mig1 **Mig2**	Mig1 is in *H. polymorpha* is responsible for catabolite repression and pexophagy, *ΔHpMig1* and *ΔHpMig1ΔHpMig2* showed no impairment on glucose but only on MeOH + 2-deoxyglucose suggesting glucose repression impairment, in *C. boidinii* involved in negative regulation of methanol inducible genes	**Stasyk 2007, Zhai 2012**
Bmh	in *P. pastoris* 14-3-3 protein regulates transcriptional activity of methanol inducible transcription factor Mxr1p by binding to a conserved serine residue, that is phosphorylated on ethanol and thereby inhibiting Mx1p	**Parua 2012**
Hap	Subunit of the heme-activated, glucose-repressed Hap2p/3p/4p/5p CCAAT-binding complex in *P. pastoris,* regulation of respiration in *S. cerevisiae*	**Schüller 2003**
Hxt1	Hxt1p glucose transporter in *P. pastoris*, *Δhxt1* showed impaired glucose catabolite repression	**Zhang 2010**

1.3.3 Factors involved in the regulation of MUT (methanol utilization) genes

1.3.3.1 The role of Mxr1p in the regulation of MUT genes

The regulatory system in *S. cerevisiae* governing oleate response is comprised of two independently functioning levels. Adr1p together with its regulatory kinase Snf1p and Oaf1p-Pip2p mediate fatty acid and peroxisome biogenesis (Ratnakumar 2010). Interplay between both systems is found in a set of promoters with overlapping oleate response elements and UAS. These overlapping sequences are bound in a coordinate fashion and subsequently induce transcription upon shift to oleate (Simon 1991). In accordance with its role in peroxisomes the *S. cerevisiae* homologue of Mxr1p, *Sc*Adr1p is a positive regulator of peroxisomal genes such as *CTA1*, *FOX2*, *POT1* and *ADH2* (Tachibana 2005). Also the expression of three peroxisomal proteins ketothiolase, trifunctional enzyme and peroxisome assembly protein 1, was negatively affected in *adr1* null mutant strains (Simon 1991). In *S. cerevisiae* Adr1p therefore acts both on a global and local level to induce transcription of genes involved in dissimilation of alternative carbon sources (Tachibana 2005).

The functional similarities between *Pp*Mxr1p and *Sc*Adr1p are striking, since both are necessary for gene activation of several non-glucose carbon dissimilation-associated genes. Western and Northern-Blot experiments of wildtype and *mxr1*Δ strains showed that Mxr1p is indispensable for expression of methanol-utilization related and peroxisomal biogenesis genes in response to methanol (Lin-Cereghino 2006, Parua 2012). Both *adr1*Δ and *mxr1*Δ are growth deficient under oleate conditions but *mxr1*Δ still shows expression of some proteins necessary to metabolize oleate. Additionally *adr1*Δ is not capable to grow on glycerol or ethanol (Sloan 1999). Interestingly, while *mxr1*⊿ cannot grow on the peroxisomal substrate oleic acid Lin-Cereghino *et al.* were nevertheless unable to find causes of this deficiency at the molecular level. In *mxr1*Δ strains the mRNA levels of aco (acyl-CoA oxidase), catalase, Pex8, Pex5 and thiolase showed only slightly lower levels compared to the wildtype when grown on methanol. Subcellular fractionation and subsequent catalase and acyl-CoA oxidase enzyme assays show the majority of enzyme activity within the peroxisomes ruling out any deficiencies in transport (Lin-Cereghino 2006). Parua *et al.* 2012 came to a similar conclusion, since expression of β-oxidation genes in *mxr1*Δ stains on oleate is only modestly or hardly effected at all. Therefore Mxr1p appears to be specifically regulating methanol utilization, while Adr1p also regulates other gluconeogenic substrates like ethanol and glycerol (Sloan 1999, Young 2003). Interestingly, *P. pastoris* strains grown on ethanol, glycerol and glucose are all capable of repressing Mxr1-dependent genes *AOX1* and

DHAS, while in *S. cerevisiae* glycerol did not repress expression of *PEX* and fatty acid genes (Parua 2012).

Both genes *PpMXR1* and *ScADR1* are constitutively expressed at low levels in all carbon sources which suggests possible post translational regulation (Lin-Cereghino 2006). The major difference so far is the nuclear localization of *Pp*Mxr1p and *Sc*Adr1p since the latter seems to be constantly localized to the nucleus while *Pp*Mxr1p is subjected to carbon-dependent shuttling between the cytosol and nucleus (Sloan 1999, Lin-Cereghino, 2006). Mxr1p acts in opposition to the repressor of gluconeogenic genes Mig1p, since it is found exclusively in the cytosol on glucose (Lin-Cereghino 2006). While Mxr1p is nuclear under methanol and oleate conditions and cytoplasmic under glucose, in ethanol and glycerol conditions this TF is also localized to the nucleus although *PEX* and *MUT* genes stay repressed. This suggests an additional level of regulation possibly through coordinative binding of several transcription factors or post translational modifications of Mxr1p (Parua 2012).

If we compare both key transcriptional regulators *Pp*Mxr1p and *Sc*Adr1p we see that both of them encode C_2H_2 type zinc finger transcription factors with Mxr1p having 70% DBD (DNA binding domain) sequence identity to *Sc*Adr1p. Despite showing a highly similar DBD, the DNA sequences recognized by Mxr1p and Adr1p are different as demonstrated by Kranthi *et al.* (Kranthi 2009). Mxr1p is also unable to cross-react with *ADR1*-UAS (Parua 2012). This suggests also different target gene specificity between Mxr1p and Adr1p. Outside of the DNA binding domain (DBD) the similarity between Mxr1p and Adr1p is rather low except for a short stretch harboring a core 14-3-3 binding region. 14-3-3 proteins are regulators in numerous cellular processes and bind typically phosphoserine and phosphothreonine regions in interaction partners (van Heusden 2006). In *S. cerevisiae* the function of several of these proteins is described thoroughly amongst them Bmh1p/Bmh2p being involved in glucose repression (Hahn 2011). In *S. cerevisiae* binding of Bmh1p to Reg1p, the regulatory subunit of Glc7p phosphatase inactivates protein kinase Snf1p necessary for Adr1p activation. Additionally 14-3-3 protein also binds Adr1p and represses activation of Adr1p-regulated genes (Parua 2010). In *P. pastoris* Parua *et al.* 2012 were able to show 14-3-3 interaction with the predicted region in Mxr1p, through pull-down assays with Glutathione-S-transferase. By using anti-phosphoSer-215 antibodies they were able to show that Mxr1p is phosphorylated at this residue. Upon mutation of

Ser215 to Alanine neither binding nor pull-down could be observed. Homology could further be proved by complementing a *S. cerevisiae bmh1⊿* strain with the *P. pastoris* 14-3-3 gene (Parua 2012).

Screening of *Pp*Mxr1p mutant at serine residue 215 (S215A), a crucial residue for 14-3-3 interaction, showed only a modest derepression effects on glucose, glycerol and ethanol. The addition of methanol to these three carbon sources showed a very strong gene expression on ethanol medium, while in the presence of only glucose or glycerol *AOX1* and *DHAS* remained repressed (the wildtype Mxr1 protein shows tight repression under all conditions). This data suggests that the 14-3-3 protein is involved in repressing Mxr1p activity in the simultaneous presence of ethanol and methanol conditions by directly binding phosphorylated Mxr1p. Parua *et al.* found that the major activation domain of Mxr1p is inhibited by 14-3-3 binding (Bmh) and by deleting the corresponding region on Mxr1p inhibition could be overcome. In the same study by Parua *et al* 2012 carbon-source dependent phosphorylation studies showed diminished phosphorylation on glucose and methanol while Mxr1p A215 was phosphorylated on ethanol as sole carbon source. Still, both on glucose and methanol weak 14-3-3 binding to Mxr1p was observed suggesting a second line of regulation independent from 14-3-3 binding and that 14-3-3 in *P. pastoris* is specifically needed for ethanol repression (Parua 2012).

Focusing on the possibility of post-DNA binding regulation Parua *et al.* were able to show that wildtype Mxr1p and S215A-Mxr1p show comparable binding affinity on methanol and ethanol, while in glucose this binding was considerably weaker. Weaker binding in glucose is most likely due to the cytoplasmic localization of Mxr1p on glucose. However, comparable level of DNA binding under both methanol and ethanol point into the direction of a post-DNA interaction possibly in the form of RNA pol II recruitment or pre-initiation complex formation. This factor could potentially act in the form of heterodimer binding in combination with Pip2/Oaf1, which was already observed for *S. cerevisiae* Adr1p (Parua 2010).

The growth and gene expression phenotypes of constitutive allels of *ADR1*, termed *ADR1^c* (lacks S-230 phosphorylation), in a *snf1*Δ background show activation of peroxisomal genes and are also able to compensate for lack of *OAF1* and *PIP2*. Contrasting these results with wildtype Adr1p, we can observe lower promoter occupancy and lower recruitment of pol-

ymerase II (Ratnakumar and Young 2010). Ratnakumar and Young could further show that promoter binding did not correlate with transcription activation, which points into the direction of binding-independent activation. To explain their results obtained with *ADR1*^{c} they suggest a mechanism involving recruitment of transcriptional coactivators (Ratnakumar 2010).

Noteworthy, Mxr1p homologues have also been found in other methylotrophic yeast species such as *C. boidinii* where *trm2*Δ*trm1*Δ showed growth defect on methanol but not on glucose. Interesting to mention is the fact that *trm2*Δ (*PpMXR1* homologue) also showed growth defects on glycerol as opposed to *trm*1Δ (*PpPRM1* homologue) suggesting that these two factors are regulating two different sets of genes. In terms of nuclear localization and its ability to induce expression of MUT genes Trm1p behaves very similar to Trm2p (Sasano 2010).

The comparison of Adr1-dependent regulation in *S. cerevisiae* and Mxr1-mediated regulation in *P. pastoris* differs fundamentally in the former factor's ability to allow expression of glucose repressed genes in glycerol and ethanol medium, while Mxr1-dependent genes show a tight repression even in the presence of ethanol or glycerol (Sloan 1999, Parua 2012). This tight repression and the distinctive subcellular localization under different carbon sources in the case of Mxr1p suggest that while both factors function in a similar way to regulate repression of genes on glucose, Mxr1p-function contains one or more layers of regulation. This would allow *P. pastoris* to specifically adapt to methanol conditions (Lin-Cereghino 2006).

1.3.3.2 The role of Prm1p in the regulation of MUT genes

In *P. pastoris* the existence of a second positive activator of methanol utilization genes was described as Prm1p (positive regulator of methanol 1) by Takagi *et al.* in a US patent "Method for methanol independent induction from methanol inducible promoters in *Pichia pastoris*". Tagaki *et al.* were able to show methanol independent expression using P_{GAP} and p*TEF1* for *PRM1* expression thereby enabling methanol free phytase expression from P_{AOX1} and P_{DAS}. On glucose the P_{GAP} -driven *PRM1* expression gave a 7.6-44.2 fold change and a 1.86-3.0 fold change for $P_{DAS\ 1}$ and P_{AOX1} driven phytase expression, respectively. In comparison methanol induction of the *AOX1* promoter was described to result in more

than 1000-fold induction compared to growth in presence of glucose (Tschopp 1987). The P_{GAP} -driven expression of *MXR1* on the other hand gave a 7.6 fold phytase activity. The issue of normalization to calculate fold-change was not addressed, which could seriously affect the outcome dependent on $P_{DAS\,1}$ and P_{AOX1} expression on glucose. Since P_{AOX1} activity under glucose corresponds to 2-4% of activity under methanol conditions (Hartner and Glieder 2006), even small changes in phytase activity would lead to large numbers in fold-change activity. Tagaki and coworkers nevertheless claim the usage of this positive factor to activate methanol inducible genes and therefore perform methanol independent protein production (Takagi 2012). In a mutation screening for genetic factors positively affecting *MOX* gene expression in the methylotrophic yeast *H. polymorpha* Vallini *et al.* found 12 complementation groups with significantly reduced *MOX* activity (Vallini 2000). The protein sequence of *MUT3* (possible Prm1p homologue) fell into one of those groups and was deposited on NCBI by that research group at a later point (GenBank: AAK84946.1). However, the reference linked to the deposited file does not exist; the article was apparently never published. Additionally I was unable to find any experimental data on Mut3p.

1.3.3.3 The role of Mpp1p in the regulation of MUT genes

In *H. polymorpha* microarray studies comparing gene expression on glucose and methanol, the highest upregulation of any transcriptional activator was found for *MPP1* (van Zutphen 2010). Leao-Helder *et al* 2003 found that *mpp1*Δ strains are unable to grow on methanol but showed no growth deficiency on glucose, ethanol, glycerol or dihydroxyacetone. In these deletion strains the level of *PEX3*, *PEX5*, *PEX10* and *AO* gene expression was reduced or in the case of *DHAS* (dihydroxyacetone synthase) absent altogether. However, the import of other peroxisomal proteins like Cat1p and Pex14p was not affected. Methanol induced *mpp1*Δ strains showed a single large peroxisome which was not susceptible to glucose-induced selective peroxisome degradation (pexophagy). The subcellular localization of Mpp1p was examined in a *mpp1*Δ strains expressing a *MPP1-eGFP* fusion gene. During cell growth on glucose no fluorescence signal could be detected, but upon shift to methanol fluorescence was localized to the nucleus (Leao-Helder 2003, van der Klei 2006). Interestingly, the genomic location of *MPP1* in both *P. pastoris* and *H. polymorpha* is upstream of the 3' end of the *DAS2* gene as in figure 1.5 (Küberl 2012 and Leao-Helder 2003). The distance of close to 2000 bp suggests an own promoter of the *MPP1* gene for separate regulation of transcription.

Figure 1.5.: Genomic locus showing *MPP1*, *DAS2* and *DAS1* in *P. pastoris.* The locus information has been retrieved from the genome sequence of *P. pastoris* CBS 7435 from NCBI, more specifically region PP7435_Chr3-0349 (Küberl 2010). In *H. polymorpha* MPP1 and DAS are organized in the identical fashion (Figure 1 of Leao-Helder 2003).

In 2012 Nishi *et al* filed a patent application with the title "YEAST FOR TRANSFORMATION AND PROCESS FOR PRODUCING PROTEIN" (WO2012102171) in which they describe the use of P_{GAP} -controlled overexpression of *MPP1* to use for P_{AOX1} driven expression of secreted anti-TNF-α Fab. In this patent application there is however no reference to methanol-free expression systems. The application of *MPP1* is restricted to methanol induction systems under a broad array of methanol inducible promoters. By overexpression of *MPP1* they could improve production capacity of P_{AOX1} driven anti-TNF-α Fab secretion.

1.3.3.4 Additional factors with possible influence on the regulation of MUT genes

If we focus again on table 1.1 we see other factors being involved in the process of glucose repression. Two of these regulators Snf2p and Swi1p are part of the SWI/SNF complex, which in *S. cerevisiase* is a chromatin remodeling complex responsible for the regulation of DNA replication, stress response and transcription of *ADH2*. (Peterson 1992) In *H. polymerpha* Ozimek *et al.* 2004 report growth defects on methanol (fully impaired), glycerol and ethanol (decreased growth rate) of both knock-out strains *swi1*Δ and *snf2*Δ while growth on sucrose remained similar to the wildtype. Subsequently performed Western-blot analysis and beta-lactamase assay showed a dramatic decrease in the activity of the *MOX* promoter. Additionally peroxisome biogenesis and maintenance were effected by either *swi1*Δ or *snf2*Δ, showing a reduction in diameter compared to WT but no difference in peroxisome number. Due to the similar growth phenotype compared to *S. cerevisiae* and the sequence similarity to *Sc*Swi1p and *Sc*Snf2p the authors suggest that these two proteins are also components of the SWI/SNF complex in *H. polymorpha* (Ozimek 2004).

The binuclear zinc cluster protein Cat8p is specifically activating carbon source responsive elements (CSRE) containing promoters in *S. cerevisiae* (Schüller 2003). In the presence of high amounts of glucose, Cat8p will be prevented from activating promoters containing CSRE motifs by Mig1p. Upon glucose depletion derepression effects mediated through

inactivation of Mig1p and activation through Snf1p, Cat8p will be capable of commencing transcription (Young 2003). Whether Cat8p is a directly interacting substrate of Snf1p remains to be determined (Schüller 2003). The inhibitory effect of Mig1p was further examined by Stasyk *et al.* and Zhai *et al.* in *H. polymorpha* and *C. boidinii* respectively. The generation of *mig1*Δ strains in *C. boidnii* did not result in any growth difference on glucose but showed increased P_{AOX1} activation in the early stages of methanol induction. Concerning the subcellular localization, Mig1p shows antagonistic translocation behavior compared to Mxr1p since on glucose Mig1p resides in the nucleus while upon shift to methanol Mig1p translocates to the cytosol, suggesting a possible role in negative regulation of methanol genes (Zhai 2012).

The role of Mig1p in *H. polymorpha* is similar to *Cb*Mig1p, however Stasyk *et al* investigated its role in macro- and microautophagy more closely and were able to show an impairment in macroautophagy in the *mig1*Δ*mig2*Δ strains. Additionally an increasing role in microautophagy, showing Mig1p's involvement in glucose repression and possible pleiotropic effects in cell component turnover could be observed (Stasyk 2003).

Focusing again more closely on *P. pastoris* reports from Kranthi *et al.* propose the existence of a ssDNA binding protein termed zeta crystalline (*ZTA1*), which binds to upstream sequences of the *AOX1* promoter. Zta1p's function in the cell is two-fold, on the one hand it reduces naturally occurring quinones and on the other hand it acts as a ssDNA binding protein under low NADPH conditions. It might be tempting to speculate about a possible NADPH sensing function, whereby low levels of NADPH translocate Zta1p to the nucleus to regulate expression of MUT genes (Kranthi 2006).

In 2011 Kumar and Rangarajan identified a TF involved in the regulation of biotin metabolism related genes. Their primary goal was to identify proteins similar to Mxr1p. The outcome of this search resulted in a protein termed Rop1, which shared 58% identity with the zinc finger domain of Mxr1p. Interestingly *ROP1* mRNA was only present under methanol conditions, but not on glucose, glycerol or oleic acid which prompted them to speculate about a possible involvement in MUT gene regulation. However, *rop1* deletion showed no effect on MUT gene expression but an upregulation of genes involved in biotin metabolism (Kumar 2011).

As is the case for the SWI/SNF complex and its chromatin remodeling capability, the involvement of factors acting on histones can also be observed in the case of histone deacetylase Hda1p and Rpd3p. The covalent modification of histones above all acetylation and deacetylation has been shown to influence transcription regulation by changing nucleosome packaging (Suka 1998). Both histone deacetylases show high sequence similarity in *S. cerevisiae* and regulate distinct but partially overlapping genes. Both *hda1*Δ and *rpd3*Δ show increased acetylation of the repressed *S. cerevisiae ADH2* promoter chromatin by destabilizing the structure of the TATA box-containing nucleosome (Verdone 2002, Rundlett 1996). This data suggest that histone acetylation status influences transcription regulation.

1.4 The zinc finger protein motif as a central feature of transcription factors

So far no regulating RNAs are known for *P. pastoris* which might contribute to the regulation of methanol inducible promoters or be involved in methanol or glucose sensing. The vast majority of transcriptional regulators fall into the group of zinc finger proteins. Three categories of zinc finger proteins are classified according to their zinc binding motif and are Cys_2His_2, Cys_4 or Zn_2Cys_6 (Iuchi 2001). The latter protein group harbors only one zinc finger that binds two zinc atoms, termed zinc cluster proteins. Zinc finger transcription factors are the largest families of transcriptional regulators in eukaryotes and form finger-like shapes with one α-helix and a pair of antiparallel β-strands. The binding of zinc stabilizes the protein and ensures proper structure and function (Laity 2001). Zinc cluster proteins are most often comprised of several domains: cysteine rich DNA-binding domain (DBD), regulatory middle homology region (MHR) and activation domains (acidic region). The DBD is more precisely built up of the zinc finger, the linker and the dimerization domain and is usually located at the N terminus. Very frequently zinc cluster proteins recognize CGG nucleotide triplets via major groove interactions and specificity is determined by orientation and spacing between triplets (MacPherson 2006).

Zinc finger proteins form homo- or heterodimers to differentially regulate genes by interacting in a combinatorial way (eg. a homodimer of Oaf1p sufficient for basal transcription, but Pip2p/Oaf1p heterodimer induces gene expression to metabolize oleate) (Ratnakumar 2010). Further examples for zinc finger protein features include self-regulation and feedback loop behavior to down- or- upregulate the expression of the respective transcription factor, like in the case of Cat8p and Sip4p in *S. cerevisiase* (Haurie 2001).

For a transcription factor to exert its effect on gene expression it has to be present in the nucleus. Two groups of transcription factors according to their localization mechanism can be determined. One group is permanently present in the nucleus and only activated by binding of an activating molecule (e.g. *ILV* genes for leucine production). The other group is imported into the nucleus by binding to nuclear localization sequences mostly located at the N terminus close to the DBD (Kaffmann 1999).

A prominent feature of many zinc cluster transcription factors is their activation by phosphorylation. The best available examples are transcription factors regulateod by the Snf1 kinase, which phosphorylates many TFs and thereby contributes to their activation or inactivation (Kaffmann 1999). Zinc cluster proteins require in certain cases the support of chromatin-remodeling complexes, histone modifying enzymes or cofactors to overcome the repressive nature of DNA packaging (MacPherson 2006). Well established examples in *S. cerevisiae* are SAGA complex histone acetyltransferases. Others are the chromatin-remodeling complexes SWI/SNF (Biddick 2008). Processes involving negative regulation in the case of histone deacetylases also exist with prominent examples Rdp3p and Hda1p in *S. cerevisiae* (Rundlett 1996, Tachibana 2007). In some cases regulatory chromatin remodeling mechanisms require ATP hydrolysis for their action (MacPherson 2006).

1.5 Homology search via BLAST® in four different yeast species

In order to acquire a more complete picture about putative factors involved in the regulation of carbon metabolism in methylotrophic yeast species, I tried to find possible homologues of *S. cereviasiae* key regulators in *P. pastoris* and other methylotrophic yeasts and vice versa. Table 1.2 shows the outcome of the homology search via p-BLAST® search in four different yeast species: *S. cerevisiae, H. polymorpha, C. boidinii* and *P. pastoris*. The search space of *H. polymorpha* and *C. boidinii* was severely restricted since a complete genome is not available in NCBI. Therefore only sequences deposited in the NCBI database could be considered, mostly arisen from complementation and knock-outs studies. Table 1.2 shows the outcome of this search with query coverage, sequence identity and E-value for all sampled sequences.

Table 1.2.: BLAST results within 4 yeast species. Protein sequences from *S. cerevisiae* (taxid: 4932) were blasted (NCBI blastp) against the genome of *P. pastoris* CBS 7435(taxid: 4922), *C. boidinii* (taxid: 5477) and *H. polymorpha* (taxid: 870730). Due to the lack of complete genome sequences in the case of *H. polymorpha* and *C. boidinii* only single deposited files could be used to perform BLAST search. For reasons of avoidance of false positive hits, sequences from the latter two species without any corresponding experimental reference were omitted from the table. Three-letter protein abbreviations in *H. polymorpha*, *C. boidinii* and *S. cerevisiae* were taken from referenced publications and SGD (Saccharomyces Genome Database), respectively. All BLAST queries resulted in E-values <1e-25. For a more thorough analysis including E-value, query coverage, max/total scores and protein size see Appendix. n.s.a. no sequence available. n.h. no homologue. * PpZta1 is a medium-chain dehydrogenase/reductase, in *S. cerevisiae* 20 of these proteins with highly similar sequences are known. Therefore the query hit in *H.polymoprha* might not be a functional homologue.

	Protein name (if annotated) - GenBank accession number			
Internal name	***P. pastoris***	***H. polymorpha***	***C. boidinii***	***S. cerevisiae***
Mxr1	Mxr1 - CCA40655.1	n.s.a.	Trm2 - BAJ07608.1	Adr1 - AAA73863.1
cov./seq.ident.			88%/37%	34%/55%
Prm1	Prm1 - CCA40959.1	Mut3 - AAK84946.1	Trm1 - BAF99700.1	Asg1 - NP_012136.1
cov./seq.ident.		96%/53%	75%/74%	56%/53%
Swi1	Swi1 - CCA37890.1	Swi1 - AAQ75382.1	n.s.a.	Swi1 - NP_015309.1
cov./seq.ident.		99%/29%		75%/29%
Snf1	CCA38457.1	AAN84785.1	n.s.a.	Snf1 - NP_010765.3
cov./seq.ident.		51%/56%		98%/65%
Cat8	Cat8 - CCA38204.1	n.s.a.	n.s.a.	Cat8 - NP_014007.1
cov./seq.ident.				74%/32%
Reg1	Hex2 - CCA36537.1	n.s.a.	n.s.a.	Reg1 - NP_010311.1
cov./seq.ident.				44%/61%
Gal4	Lac9 - CCA37633.1	n.s.a.	n.s.a.	Gal4 - NP_015076.1
cov./seq.ident.				55%/29%
Mpp1	CCA39317.1	Mpp1-AAO72735.1	n.s.a.	n.h
cov./seq.ident.		68%/33%		
Snf2	CCA40198.1	n.s.a.	n.s.a.	Snf2 - NP_014933.3
cov./seq.ident.				76%/54%
Rpd3	Rpd3 - CCA37028.1	n.s.a.	n.s.a.	Rpd3 - NP_014069.1
cov./seq.ident.				90%/87%
Hda1	Hda1 - CCA38680.1	n.s.a.	n.s.a.	Hda1 - NP_014377.1
cov./seq.ident.				92%/62%
Zta1	CCA38647.1	ADH - ADM49192.1	n.s.a.	Zta1 - NP_009602.1
cov./seq.ident.		99%/28%*		99%/59%
Rop1	Zms1 - CCA39607.1	n.s.a.	n.s.a.	Tda9 - NP_013630.1
cov./seq.ident.				78%/32%
Mig1	CCA40819.1	Mig2 - ABU63593.1	Mig1 - BAM38481.1	Mig1 - CAA39084.1
cov./seq.ident.	16%/74%	82%/41%	23%/87%	
Mig2	Mig1 - CCA37444.1	Mig2 - ABU63593.1	Mig1 - BAM38481.1	Mig2 - NP_011306.1
cov./seq.ident.	16%/63%	29%/46%	30%/66%	

2. OBJECTIVES

The methylotrophic yeast *Pichia pastoris* is widely used in industry as an efficient recombinant protein production system. The simplicity of molecular genetic manipulation and the ability to produce post-translationally modified proteins in high amounts makes it an ideal host for industrial protein expression. The P_{AOX1} is one of the strongest promoters found in nature and due to its tight regulation and the possibility to induce it with methanol widely used in the production of recombinant proteins. However, the applicability of P_{AOX1} on an industrial scale is limited by the necessity to store and handle large amounts of methanol. Therefore additional new processes and tools that allow P_{AOX1} driven high level expression without methanol induction would be highly desirable. Additionally any alternative system devised should retain the capability of inducible protein expression.

The aim of this thesis was the construction of an auto-inductive or auto-amplifying system for high level protein expression in *P. pastoris* without using methanol. By testing novel regulators from *P. pastoris* and assembling them into a synthetic genetic circuit I aimed to accomplish a system that allows the methanol-free and P_{AOX1} driven expression of recombinant proteins. Using a transcriptomics approach on the basis of microarrays the underlying transcriptional responses of such regulator overexpression strains should be elucidated and used for application refinement. To further study the transcriptional regulation by key regulators in the carbon metabolism, I aimed to construct knock-out strains and check their gene expression behavior under different conditions. Furthermore the thesis should result in a set of activators, which could in the future be employed for the design of different synthetic genetic circuits.

3. MATERIAL AND METHODS

3.1 Equipment and Devices

All equipment and devices used in this thesis are listed in table 3.1. Smaller vessels like Eppendorf tubes, "Greiner" tubes and pipette tips are not included.

Table 3.1.: All equipment and devices used in this thesis.

Instrument	Company
Plate Reader	
SynergyMx Plate Reader	Biotek Inc., Winooski, United States
Centrifuge	
Eppendorf Centrifuge 5415R	Eppendorf AG, Hamburg, Germany
Eppendorf Centrifuge 5810R	Eppendorf AG, Hamburg, Germany
Eppendorf Centrifuge 5415D	Eppendorf AG, Hamburg, Germany
PCR Cycler	
Applied Biosystems 2720 Thermal Cylcer	Applied Biosystems, Inc., Foster City, CA, Unites States
Shaker	
HT Infors Multitron Shaker	Infors AG, Bottmingen, Switzerland
HT Infors RS306 shaker	Infors AG, Bottmingen, Switzerland
HT Infors Orbitron shaker	Infors AG, Bottmingen, Switzerland
Heidolph Titramax 1000 Plate Shaker	Heidolph Instruments, Schwabach, Germany
Fermenter	
DASGIP Parallel Bioreactor System	DASGIP Information and Process Technology GmbH, Jülich, Germany
Other Devices	
MT PG12001-S DeltaRange Balance	Mettler Toledo Inc., Greifensee, Switzerland
Sartorius B2 120S Analytical Lab Scale	Sartorius AG, Göttingen, Germany
Heidolph MR 2002 Mangentic Stirrer	Heidolph Instruments, Schwabach, Germany
Ikamag RCT Magnetic Stirrer	IKA-Werke GmbH & Co. KG, Staufen, Germany
Heidolph MR 3000 Magnetic Stirrer	Heidolph Instruments, Schwabach, Germany
inoLab pH 720 pH-Meter	WTW GmbH, Weilheim, Germany
Bio-Rad BioRad Gene Pulser 1652076	Bio-Rad Laboratories Inc., Hercules, CA, United States
Capacitance Extender 1652087	Bio-Rad Laboratories Inc., Hercules, CA, United States
Pulse Controller P/N 1652098	Bio-Rad Laboratories Inc., Hercules, CA, United States
Vortex Genie 2	Scientific Industries Inc., Bohemia, NY, United States
Eppendorf Thermomixer Comfort	Eppendorf AG, Hamburg, Germany
Certoclav LVEL 12L	CertoClav GmbH, Traun, Austria
BioRad PowerPac Basic Power Supply	Bio-Rad Laboratories Inc., Hercules, CA, United States

UVP gel doc-it Imaging System	UVP. LLC, Upland, CA, United States
Eppendorf BioPhotometer plus	Eppendorf AG, Hamburg, Germany
Thermo Scientific Nanodrop 2000c	Thermo Fisher Scientific Inc., Waltham, MA, United States

Pipettes	**Company**
Denville Xl 3000i single channel pipette 0.1-2 µL	Denville Scientific Inc., Metuchen, NJ, United States
Denville Xl 3000i single channel pipette 2-20µL	Denville Scientific Inc., Metuchen, NJ, United States
Denville Xl 3000i single channel pipette 20-200µL	Denville Scientific Inc., Metuchen, NJ, United States
Denville Xl 3000i single channel pipette 100-1000µL	Denville Scientific Inc., Metuchen, NJ, United States
Biohit Proline 50-1200 µL	Biohit Oyj, Helsinki, Finnland
Biohit Proline 5-100µL	Biohit Oyj, Helsinki, Finnland

Microtiter Plate	**Company**
96 well PS Microplater sterile	Greiner Bio-One GmbH, Frickenhausen, Germany
96 well PS Microplater unsterile	Greiner Bio-One GmbH, Frickenhausen, Germany
Nunc™ MicroWell™ 96-Well Optical-Bottom Plates with Polymer Base	Thermo Fisher Scientific Inc., Rochester, NY, United States
Bel-Art 96-Well Deep Well Plates	Bel-Art Products, Wayne, NJ, United States
Cell Projects Electroporation Cuvettes	Cell Projects Limited, Kent, United Kingdom

3.2 Strains, Plasmids and PCR reactions

3.2.1 *P. pastoris* Strains

Table 3.2 contains all *P. pastoris* strains generated in this thesis. The host strain for all transformations was *P. pastoris* CBS 7435 (wildtype, Mut^+)(IMBT strain collection 3132) and resistance markers used for selection are tabulated as well.

Table 3.2.: All *P. pastoris* strains generated during this thesis. All numbers appearing in this list (plasmid and strain) refer to the number of our internal strain collection. If strains are not present in a single glycerol stock vile but in a DWP glycerol stock the word DWP was used instead. * This clone was used for the reactor cultivation, *this clone was used for screenings.

Host Strain	*Pichia pastoris* Strain	Plasmid#	Strain#	Resistance
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Mxr1-pAOX1-sTomato	677	620	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT1-Mxr1-pAOX1-sTomato	678	621	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Prm1-pAOX1-sTomato	679	623	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT-Prm1-pAOX1-sTomato	680	624	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Mpp1-pAOX1-sTomato	681	626	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT-Mpp1-pAOX1-sTomato	682	627	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Mxr1c-pAOX1-sTomato	687	631	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT1-Mxr1c-pAOX1-sTomato	688	632	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT-Snf1-pAOX1-sTomato	761	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT-Snf2-pAOX1-sTomato	762	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT-Cat8-pAOX1-sTomato	763	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT-Reg1-pAOX1-sTomato	764	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT-Gal4-pAOX1-sTomato	765	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Snf2-pAOX1-sTomato	766	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT-Rpd3-pAOX1-sTomato	767	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT-Hda1-pAOX1-sTomato	768	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Swi1-pAOX1-sTomato	769	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Snf1-pAOX1-sTomato	770	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Reg1-pAOX1-sTomato	771	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Gal4-pAOX1-sTomato	772	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT-Swi1-pAOX1-sTomato	773	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Rpd3-pAOX1-sTomato	774	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Cat8-pAOX1-sTomato	720	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pGAP-Hda1-pAOX1-sTomato	721	DWP	Zeo
CBS 7435	pPpT4mutZeoMlyI-intArg4-pGAP-eGFP-pAOX1-sTomato	305	629	Zeo
CBS 7435	pPpT4mutZeoMlyI-intArg4-pCAT1-eGFP-pAOX1-sTomato*	306	630	Zeo

Host Strain	*Pichia pastoris* Strain	Plasmid#	Strain#	Resistance
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT1-Mxr1-pAOX1-LuHNL	716	DWP	Gen
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT1-Prm1-pAOX1-LuHNL	717	DWP	Gen
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT1-Mpp1-pAOX1-LuHNL	718	DWP	Gen
CBS 7435	pPpT4mutZeoMlyI-intUra3-pCAT1-eGFP-pAOX1-LuHNL	719	DWP	Gen
CBS 7435	pPpT4mutZeoMlyI-intUra3-pMxr1-sTomato	683	633	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pPrm1-sTomato	684	634	Zeo
CBS 7435	pPpT4mutZeoMlyI-intUra3-pMpp1-sTomato	685	635	Zeo
CBS 7435	pPpT4mutZeoMlyI-intArg4-pCAT1-eGFP-pAOX1-sTomato*[1]	306	814	Zeo

3.2.2 *E. coli* Strains and Plasmids

In table 3.3 all plasmids used in this master thesis are listed. All of this plasmids are present both in *E. coli* TOP10F' as a 20% glycerol stock and as isolated plasmid. The numbers in this list refer to our internal strain collection.

Table 3.3.: This table contains all plasmids generated during the work of this thesis.

Host strain	Plasmid	Strain number#	Resistance Marker
TOP 10 F'	pPpCK1-Kan-Mxr1	292	Gen
TOP 10 F'	pPpCK1-Kan-Prm1	293	Gen
TOP 10 F'	pPpCK1-Kan-Swi1	294	Gen
TOP 10 F'	pPpCK1-Kan-Snf1	295	Gen
TOP 10 F'	pPpCK1-Kan-Cat8	296	Gen
TOP 10 F'	pPpCK1-Kan-Reg1	297	Gen
TOP 10 F'	pPpCK1-Kan-Gal4	298	Gen
TOP 10 F'	pPpCK1-Kan-Mpp1	299	Gen
TOP 10 F'	pPpCK1-Kan-Snf2	300	Gen
TOP 10 F'	pPpCK1-Kan-Rpd3	301	Gen
TOP 10 F'	pPpCK1-Kan-Hda1	302	Gen
TOP 10 F'	pPpCK1-Kan-PrmI short	303	Gen
TOP 10 F'	pPpCK1-Kan-Reg1 short	304	Gen
TOP 10 F'	pPpCK1-Mxr1	703	Zeo
TOP 10 F'	pPpCK1-Prm1	704	Zeo
TOP 10 F'	pPpCK1-Swi1	705	Zeo
TOP 10 F'	pPpCK1-Snf1	706	Zeo
TOP 10 F'	pPpCK1-Cat8	707	Zeo
TOP 10 F'	pPpCK1-Reg1	708	Zeo
TOP 10 F'	pPpCK1-Gal4	709	Zeo
TOP 10 F'	pPpCK1-Mpp1	710	Zeo
TOP 10 F'	pPpCK1-Snf2	711	Zeo
TOP 10 F'	pPpCK1-Rpd3	712	Zeo
TOP 10 F'	pPpCK1-Hda1	713	Zeo
TOP 10 F'	pPpCK1-PmrI short	714	Zeo
TOP 10 F'	pPpCK1-Reg1 short	715	Zeo
TOP 10 F'	pPpCK1-Kan.	309	Gen
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP-Mxr1-pAOX1-sTomato	677	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT1-Mxr1-pAOX1-sTomato	678	Zeo

Host strain	Plasmid	Strain number#	Resistance Marker
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP-Prm1-pAOX1-sTomato	679	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT-Prm1-pAOX1-sTomato	680	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP-Mpp1-pAOX1-sTomato	681	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT-Mpp1-pAOX1-sTomato	682	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP-Mxr1c-pAOX1-sTomato	687	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT1-Mxr1c-pAOX1-sTomato	688	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT-Snf1-pAOX1-sTomato	761	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT-Snf2-pAOX1-sTomato	762	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT-Cat8-pAOX1-sTomato	763	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT-Reg1-pAOX1-sTomato	764	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT-Gal4-pAOX1-sTomato	765	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP-Snf2-pAOX1-sTomato	766	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT-Rpd3-pAOX1-sTomato	767	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT-Hda1-pAOX1-sTomato	768	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP-Swi1-pAOX1-sTomato	769	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP-Snf1-pAOX1-sTomato	770	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP-Reg1-pAOX1-sTomato	771	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP-Gal4-pAOX1-sTomato	772	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT-Swi1-pAOX1-sTomato	773	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP-Rpd3-pAOX1-sTomato	774	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP1-Cat8-pAOX1-sTomato	720	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pGAP1-Hda1-pAOX1-sTomato	721	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intArg4-pGAP-eGFP-pAOX1-sTomato	305	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intArg4-pCAT1-eGFP-pAOX1-sTomato	306	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT1-Mxr1-pAOX1-LuHNL	716	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT1-Prm1-pAOX1-LuHNL	717	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT1-Mpp1-pAOX1-LuHNL	718	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pCAT1-eGFP-pAOX1-LuHNL	719	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pMxr1-sTomato	683	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pPrm1-sTomato	684	Zeo
TOP 10 F'	pPpT4mutZeoMlyI-intUra3-pMpp1-sTomato	685	Zeo
TOP 10 F'	pPpKan	-	Gen

3.2.2.1 Construction of transcription regulator overexpression plasmids

The basic vector for construction of all transcription factor overexpression plasmids is depicted in figure 4.1. Plasmid maps of constructs used for transcriptional regulator over-expression are provided in genbank format on the accompanying microSD card, also all other maps are provided there. The plasmids denoted pPpT4mutZeoMlyI-intUra3- P_{GAP} -TF-pAOX1-sTomato and pPpT4mutZeoMlyI-intUra3-pCAT1-TF-pAOX1-sTomato, where TF denotes the respective insert as listed in table 3.4. The construction was achieved by PCR-amplifying the P_{GAP} (P13297/P13298) and P_{CAT1} (P13301/P13302), the P_{AOX1} (P13299/P13300), the eGFP CDS (P13295/P13296), the *AOX1*TT (P13293/P13294) and the *URA3 (*P13291/P13292) integration sequence. The T4-plasmid based pPpT4mutZeoMlyI-intArg4-pAOX1-sTomato was cut with *Pci*I and *Eco*RI and together with the PCR fragments subjected to Gibson Assembly. 3µL of this reaction were used to transform *E. coli* TOP10 F' and plated on selective media. The single colonies appearing after overnight incubation at 37°C were streaked for plasmid isolation and isolated plasmids Sanger-sequenced by LGC Genomics. This procedure resulted in plasmids pPpT4mutZeoMlyI-intUra3-pGAP-eGFP-pAOX1-sTomato (#305) and pPpT4mutZeoMlyI-intUra3-pCAT1-eGFP-pAOX1-sTomato (#306). For the construction of all other plasmids e.g. plasmids #677-688, #721+ #720 and #761-774 listed in table 3.4 these two plasmids were cut with enzymes *Asc*I and *Spe*I and the respective PCR-amplified insert harboring overlapping regions to P_{GAP} / P_{CAT1} on the 5' end and *URA3* on the 3' end (table 3.4) were again cloned into the vector via Gibson Assembly. The resulting assembly reaction was processed as already described above and finally all inserts sequenced.

Table 3.4.: Primers used for PCR amplification of transcription regulator inserts.

Construct	Primer#	Primer Name
pCAT1-Mxr1	P13303	TT-AscI-Mxr1 fwd
	P13305	CAT1-Spe1-Mxr1 rev
pCAT-Prm1	P13306	TT-AscI-Prm1 fwd
	P13308	CAT1-SpeI-Prm1 rev
pCAT-Mpp1	P13309	TT-AscI-Mpp1
	P13311	CAT1-SpeI-Mpp1
pCAT-Swi1	P13599	AOX1TT-SWI1 fwd
	P13600	CAT1-SWI1 rev
pCAT-Snf1	P13601	AOX1TT-SNF1 fwd
	P13602	CAT1-SNF1 rev
pCAT-Cat8	P13603	AOX1TT-Cat8 fwd
	P13604	CAT1-Cat8 rev
pCAT-Reg1	P13605	AOX1TT-Reg1 fwd
	P13606	CAT1-Reg1 rev
pCAT-Gal4	P13607	AOX1TT-Gal4
	P13608	CAT1-Gal4 rev
pCAT-Snf2	P13609	AOX1TT-Snf2 fwd
	P13610	CAT1-Snf2 rev
pCAT-Rpd3	P13611	AOX1TT-Rpd3 fwd
	P13612	CAT1-Rpd3 rev
pCAT-Hda1	P13613	AOX1TT-Hda1 fwd
	P13614	CAT1-Hda1 rev
pGAP-Mxr1	P13303	TT-AscI-Mxr1
	P13304	Mxr1-Spe1-GAP
pGAP-Prm1	P13306	TT-AscI-Prm1 fwd
	P13307	GAP-SpeI-Prm1 rev
pGAP-Mpp1	P13309	TT-AscI-Mpp1 fwd
	P13310	GAP-SpeI-Mpp1 rev
pGAP-Swi1	P13599	AOX1TT-SWI1 fwd
	P13615	GAP-Swi1 rev
pGAP-Snf1	P13601	AOX1TT-SNF1 fwd
	P13616	GAP-Snf1 rev
pGAP-Cat8	P13603	AOX1TT-Cat8 fwd
	P13617	GAP-Cat8 rev
pGAP-Reg1	P13605	AOX1TT-Reg1 fwd
	P13618	GAP-Reg1 rev
pGAP-Gal4	P13607	AOX1TT-Gal4
	P13619	GAP-Gal4 rev
pGAP-Snf2	P13609	AOX1TT-Snf2 fwd
	P13620	GAP-Snf2 rev
pGAP-Rpd3	P13611	AOX1TT-Rpd3 fwd
	P13621	GAP-Rpd3 rev
pGAP-Hda1	P13613	AOX1TT-Hda1 fwd
	P13721	GAP-Hda1 rev

For the construction of the constitutive variant *MXR1*^{c} into P_{GAP} and P_{CAT1} background I incorporated two point mutations by designing the mutations into primers and joined together the three fragments with via Gibson Assembly. Further processing was performed as described above which included sequencing to confirm the double point mutation (see Table 3.5).

Table 3.5.: Primer for the construction of *MXR1*^{c} variant

Construct	Primer#	Primer Name	Fragment
pGAP-Mxr1^{c}	P13304	Mxr1-Spe1-GAP	**1**
	P13449	Mxr1cmut rev	
	P13368	MxrIcmut 1 fwd	**2**
	P13369	MxrIcmut rev	
	P13370	MxrIcmut 2 fwd	**3**
	P13303	TT-AscI-Mxr1 fwd	
pCAT1-Mxr1^{c}	P13305	CAT1-Spe1-Mxr1 rev	**1**
	P13449	Mxr1cmut rev	
	P13368	MxrIcmut 1 fwd	**2**
	P13369	MxrIcmut rev	
	P13370	MxrIcmut 2 fwd	**3**
	P13303	TT-AscI-Mxr1 fwd	

3.2.2.2 Construction of transcription regulator overexpression plasmids for *Lu*HNL expression

The construction of plasmids expressing *MXR1*, *PRM1*, *MPP1* and *eGFP* under the control of P_{CAT1} and *LuHNL* controlled by P_{AOX1} was achieved by cutting vectors #678 (P_{CAT1}-*MXR1*), #680 (P_{CAT1}-*PRM1*), #682 (P_{CAT1}-*MPP1*) and #306 (P_{CAT1}-*eGFP*) with *Eco*RI and *Not*I and Gibson assemble the *LuHNL* PCR fragment (P13622/P13623). The proceeding steps were performed as described as above and finally inserts were sequenced. This procedure resulted in plasmids #716 -#719 (P_{CAT1}-*MXR1*- P_{AOX1}-*LuHNL*, P_{CAT1}-*PRM1*- P_{AOX1}-*LuHNL*, P_{CAT1}-*MPP1*- P_{AOX1}-*LuHNL* and P_{CAT1}-*eGFP*- P_{AOX1}-*LuHNL*).

3.2.2.3 Construction of plasmids to investigate native promoters of methanol activators

To test for the promoter activity of the three methanol activator genes *MXR1*, *PRM1* and *MPP1* I cloned 1000bp upstream of these three genes upstream of the *sTomato* gene by primers depicted in Table 3.6. All primers harbored an overlapping sequence to *URA3* on the 3' end and *sTomato* on the 3' end of the PCR products. Plasmids #305 (pPpT4mutZeoMlyI-intArg4-pGAP-eGFP-pAOX1-sTomato) was cut with *Eco*RI and *Sbf*I and PCR products inserted via Gibson Assembly. The subsequent work was done as already described and sequenced. The result of this cloning work were plasmids #683, #684 and #685 (pPpT4mutZeoMlyI-intUra3-pMxr1-sTomato, pPpT4mutZeoMlyI-intUra3-pPrm1-sTomato, and pPpT4mutZeoMlyI-intUra3-pMpp1-sTomato)

Table 3.6.: Primer used to amplify the native promoters of methanol activators *MXR1*, *PRM1* and *MPP1*.

p***MXR1***	P13624	URA3-pAdr1 fwd
	P13625	sTomato-Adr1 rev
p***PRM1***	P13626	URA3-pPRM1 fwd
	P13627	sTomato-pPRM1 rev
p***MPP1***	P13628	URA3-pMpp1 fwd
	P13629	sTomato-pMpp1 rev

3.2.2.4 Construction of *P. pastoris* knock-out plasmids containing flipper cassettes

As a basis for the construction of knock-out plasmids for *P. pastoris* plasmid #309 (pPpCK1-Kan) was taken and digested with *Sfi*I. The PCR products listed in table 3.7 were joined together via oePCR and cloned into the digested vector with T4 DNA ligase. The subsequent procedures were performed as already described. The final confirmation of correct cloning was obtained via sequencing. Due to difficulties with Geneticin selection I recloned all existing plasmids with #292-#304 by digestion with *Sfi*I. The same PCR products as mentioned above (Table 3.7) were cloned into the digested vector by ligation. The final conformation of correct cloning was reached via sequencing. This resulted in plasmids #703-#715.

Table 3.7: Primers used for the construction of 5'UTR and 3'UTR fragments to be joined by oePCR and cloned into *Sfi*I digested pPpCK1-Kan.

Construct	Primer#	Primer Name	Fragments
Mxr1	*P13214*	3UTRMxr1fwd	3'UTR
	P13215	3UTRMxr1rev	
	P13216	5UTRMxr1fwd	5'UTR
	P13217	5UTRMxr1rev	
Prm1	*P13220*	3UTRPmr1fwd	3'UTR
	P13221	3UTRPmr1rev	
	P13222	5UTRPmr1fwd	5'UTR
	P13223	5UTRPmr1rev	
Swi1	*P13226*	3UTRSwi1fwd	3'UTR
	P13227	3UTRSwi1rev	
	P13228	5UTRSwi1fwd	5'UTR
	P13229	5UTRSwi1rev	
Snf1	*P13232*	3UTRSnf1fwd	3'UTR
	P13233	3UTRSnf1rev	
	P13234	5UTRSnf1fwd	5'UTR
	P13235	5UTRSnf1rev	
Cat8	*P13238*	3UTRCat8fwd	3'UTR
	P13239	3UTRCat8rev	
	P13240	5UTRCat8fwd	5'UTR
	P13241	5UTRCat8rev	
Reg1	*P13244*	3UTRReg1fwd	3'UTR
	P13245	3UTRReg1rev	
	P13246	5UTRReg1fwd	5'UTR
	P13247	5UTRReg1rev	
Gal4	*P13250*	3UTRGal41fwd	3'UTR
	P13251	3UTRGal41rev	
	P13252	5UTRGal41fwd	5'UTR
	P13253	5UTRGal41rev	
Mpp1	*P13256*	3UTRMpp1fwd	3'UTR
	P13257	3UTRMpp1rev	
	P13258	5UTRMpp1fwd	5'UTR
	P13259	5UTRMpp1rev	
Snf2	*P13262*	3UTRSnf2fwd	3'UTR
	P13263	3UTRSnf2rev	
	P13264	5UTRSnf2fwd	5'UTR
	P13265	5UTRSnf2rev	
Rpd3	*P13268*	3UTRRpd3fwd	3'UTR
	P13269	3UTRRpd3rev	
	P13270	5UTRRpd3fwd	5'UTR

	P13271	5UTRRpd3rev	
Hda1	*P13274*	3UTRHda1fwd	3'UTR
	P13275	3UTRHda1rev	
	P13276	5UTRHda1fwd	5'UTR
	P13277	5UTRHda1rev	
Prm1short	*P13220*	3UTRPmr1fwd	3'UTR
	P13221	3UTRPmr1rev	
	P13222	5UTRPmr1fwd	5'UTR
	P13280	5UTRPmr1rev	
Reg1	*P13281*	3UTRReg1fwd	3'UTR
	P13245	3UTRReg1rev	
	P13246	5UTRReg1fwd	5'UTR
	P13247	5UTRReg1rev	

3.2.2.5 Construction of vectors expressing two different transcriptional regulators simultaneously

To be able to simultaneously overexpress two transcription factors I took vector #306 (pPpT4mutZeoMlyI-intArg4-pCAT1-eGFP-pAOX1-sTomato) and vector pPpKan and digested both with *Bam*HI and *Kpn*I and joined the vector backbone from #306 and Kan resistance marker from pPpKan together via ligation. The subsequent sequencing gave correct cloning results. The resulting vector was digested with *Eco*RI and *Bgl*II, PCR amplified fragments containing P_{PEX5} (P13683/P13684) and *LuHNL* (P13685/P13686) werejoined together via Gibson Assembly. After confirming the correctness of cloning by sequencing I digested this vector with *Eco*RI and *Not*I and cloned in *MXR1* (P13719/ P13303) and *MPP1* (P13720/ P13309) via Gibson Assembly. After confirmation by sequencing I digested these two vectors with *Asc*I and *Nhe*I. The *MXR1* and *MPP1* genes were amplified with P_{CAT1} and parts of P_{PEX5} using primers P13742/P13303 for *MXR1* and P13742/P13309 for *MPP1* and joined together via Gibson Assembly. The correctness of cloning has to be checked by sequencing in the near future.

3.3 Primer

Table 3.8 lists all primers used in this master thesis. The primers were ordered from IDT Integrated DNA Technologies, Inc., Coralville, Iowa. The delivered viles containing lyophilized primers were dissolved to 100 pmol/μL and stored at -20°C.

Table 3.8.: Primers used in this thesis. All primer listed here are stored in our primer collection at -20°C with their respective number listed in the column "Internal number".

Primer name	Sequence 5' --> 3'	Internal Number
3UTRMxr1fwd	tcggccgatcaggccttaatgaattatgattttgtttgactatagattagtcaatacata	P13214
3UTRMxr1rev	tccggtggtagagtagtctgaatttaaatgtatatccacgcataagacttgtttaagac	P13215
5UTRMxr1fwd	taaacaagtcttatgcgtggatatacatttaaattcagactactctaccaccggataagc	P13216
5UTRMxr1rev	tcggccctagtggcctgtgcgtgggataaagtcatcaaac	P13217
5UPMxr1fwd	tcaccttccttgcagctagtgcta	P13218
3DOWNMxr1rev	acgtctcatatcacaagagtttccttcc	P13219
3UTRPmr1fwd	ttcggccgatcaggccctctgtaaattaattgataatttcaaacgtgaatggacataa	P13220
3UTRPmr1rev	aagataggacgaagtattatcaatttaaattataggggcaaacctggaccttatataaa	P13221
5UTRPmr1fwd	tccaggtttgcccctataatttaaattgataatacttcgtcctatctttatcgccattaa	P13222
5UTRPmr1rev	tcggccctagtggcccgacattcatcacaagctctggtga	P13223
5UPPmr1fwd	gacctgtaaaggcttcagcaagac	P13224
3DOWNPmr1rev	aacttcgatggtcaatatcatcgcaattct	P13225
3UTRSwi1fwd	tcggccgatcaggccttttgatcccactaggatatacaaaaaaatatgggc	P13226
3UTRSwi1rev	attcttctgatgaacgataaatttaaatgaacaagagacttttaaccctagtaacgg	P13227
5UTRSwi1fwd	ttaaaagtctcttgttcatttaaatttatcgttcatcagaagaatagaggtttatgaagc	P13228
5UTRSwi1rev	tcggccctagtggccatgactggcgtggtagtgaac	P13229
5UPSwi1fwd	cagatcaaattgttattgagaaatttgacgctagaa	P13230
3DOWNSwi1rev	tatttacgcatcgcgcgcg	P13231
3UTRSnf1fwd	tcggccgatcaggccgctttttttatatctattttaatagtataccagtggtatcaatatt	P13232
3UTRSnf1rev	ggacaaagagcacggcaaaagatttaaattaggttggtacaactgaagcatatctctaag	P13233
5UTRSnf1fwd	gatatgcttcagttgtaccaacctaatttaaatcttttgccgtgctctttgtcctctt	P13234
5UTRSnf1rev	tcggccctagtggcctagcttgatgaagaatgagtggagtgc	P13235
5UPSnf1fwd	tctctttctgacctaatacgatcatgttcc	P13236
3DOWNSnf1rev	gttaatctggaagatttccaggatctcagag	P13237
3UTRCat8fwd	tcggccgatcaggccttcaaaggactacagtttaaagacttcacatgt	P13238
3UTRCat8rev	agttgttagtagacaattggctagaaacaatttaaatacccaaggagtcaaggggattc	P13239
5UTRCat8fwd	tccccttgactccttgggtatttaaattgtttctagccaattgtctactaacaactctt	P13240
5UTRCat8rev	tcggccctagtggcctttaaggagatgaatcgagacactaatcaattaaaataaaa	P13241
5UPCat8fwd	atagagacaaaattcatgacaacatgctcg	P13242
3DOWNCat8rev	ttcctgagaggtatgaccccac	P13243

Primer name	Sequence 5' --> 3'	Internal Number
3UTRReg1fwd	tcggccgatcaggccagaccaatggtcaactccagaagc	P13244
3UTRReg1rev	cctccaaggtatccgggatatcaatttaaatggaagcattgtttcgttagaaggtcttga	P13245
5UTRReg1fwd	agaccttctaacgaaacaatgcttccatttaaattgatatcccggataccttggaggaa	P13246
5UTRReg1rev	tcggccctagtggcctgtctctagttggggaaagagaaggtaa	P13247
5UPReg1fwd	cgagaggcgatgggtgaatt	P13248
3DOWNReg1rev	ttaattagctttagagataggcatttcatgacaag	P13249
3UTRGal41fwd	tcggccgatcaggccgagcgattcaaagagaaacacttgaaaaagatc	P13250
3UTRGal41rev	tcatgtctggtcacaagattgaggtatttaaatctgcattgtcatcgaaaagaaaccc	P13251
5UTRGal41fwd	gggtttcttttcagatgacaatgcagatttaaatacctcaatcttgtgaccagacatgat	P13252
5UTRGal41rev	tcggccctagtggcctttgcagcgggcctgttt	P13253
5UPGal41fwd	gtagaatcatgacgcctcacaacc	P13254
3DOWNGal4rev	caacacattgatccaaagattttggaacc	P13255
3UTRMpp1fwd	tcggccgatcaggcctggctgttatatagaatctttgagaggagg	P13256
3UTRMpp1rev	tagggagttggtaggaaaggtttttatttaaatttgatcgaagcgagctacaagtccata	P13257
5UTRMpp1fwd	ttgtagctcgcttcgatcaaatttaaataaaaacctttcctaccaactccctaacatata	P13258
5UTRMpp1rev	tcggccctagtggccaaatcacaatcaccattacatatccaaccaac	P13259
5UPMpp11fwd	aaggatgcatgtactacgggttattgg	P13260
3DOWNMpp1rev	atctgaaaactggggtaaagaactccttaa	P13261
3UTRSnf2fwd	tcggccgatcaggccaaattatgccacataatgagatatatagcctcgc	P13262
3UTRSnf2rev	atgggatgtgacattgctgttatttaaattttgattagccttctagggaacattctcata	P13263
5UTRSnf2fwd	cctagaaggctaatcaaaatttaaataacagcaatgtcacatcccataatatatatcaac	P13264
5UTRSnf2rev	tcggccctagtggccttgttgctagataagacttggctatgaca	P13265
5UPSnf2fwd	aacagaatctctgatgtgatcggtagg	P13266
3DOWNSnf2rev	atatttatgtcatggtctaagccatatcatttggtt	P13267
3UTRRpd3fwd	ttcggccgatcaggcctacgaggggaacaagcaggaata	P13268
3UTRRpd3rev	cagagaatgagtcattgaagaaccagatttaaatatactacctgacatccgcagtctgag	P13269
5UTRRpd3fwd	actgcggatgtcaggtagtatatttaaatctggttcttcaatgactcattctctgtttac	P13270
5UTRRpd3rev	tcggccctagtggccatgaaagatgattataaaaaccgtagtaatgttatggttt	P13271
5UPRpd3fwd	atgctatggtaggcctgataaaccatattc	P13272
3DOWNRpd3rev	aacctacttcgacataggagccaag	P13273
3UTRHda1fwd	tcggccgatcaggccttaaattaggaatgaaatattattagtctttagctatatctgctt	P13274
3UTRHda1rev	cttcatacgaggtaaaaggaatgtaatttaaatgtcaccagaggagaattgcgattg	P13275
5UTRHda1fwd	cgcaattctcctctggtgacatttaaattacattccttttacctcgtatgaagcaatccc	P13276
5UTRHda1rev	tcggccctagtggcccacagattgcagcgctgttgt	P13277
5UPHda1fwd	ggaaattggatcggtcacctgaaat	P13278
3DOWNHda1rev	gactccgactgtggaagtaaatcatatgc	P13279
5UTRPmr1rev	tcggccctagtggcctttagttataaagaagggagattaatacagggcaaa	P13280
3UTRReg1fwd	tcggccgatcaggccggtatatatgtaattattttaggtttcacacgacatcttc	P13281
PucSeqF	ctttttacggttcctggccttttgc	P13282

Primer name	Sequence 5' --> 3'	Internal Number
PAox1SeqR	ggtttcattcaacctttcgtctttggatg	P13283
PciI-GAP fwd	cttttgctggccttttgctcaaacatgtttttttgtagaaatgtcttggtgtcctcgtc	P13284
pUC-URA3 fwd	tggccttttgctcacatgtatttaaatgcaaaatccgaaaaattgtcgattgg	P13291
TT-SbfI-URA3 rev	agattaagtgagaccttcgtttgtgccctgcaggaagttcaaacggaacaacctatgagg	P13292
URA3-SbfI-TT fwd	tcataggttgttccgtttgaacttcctgcagggcacaaacgaaggtctcacttaatcttc	P13293
TT-AscI-EGFP rev	atggcatggatgaattgtacaagtaaggcgcgcctcaagaggatgtcagaatgccatttg	P13294
TT-AscI-EGFP fwd	tggcattctgacatcctcttgaggcgcgccttacttgtacaattcatccatgccatgtgt	P13295
EGFP-SpeI-pGAP rev	tgaacaactatcaaaacacaactagtcgaaacgatggctagcaaaggagaagaacttttc	P13296
EGFP-SpeI-pGAP fwd	ttgctagccatcgtttcgactagttgtgttttgatagttgttcaattgattgaaataggg	P13297
pGAP-BglII-pAOX rev	cattcaacctttcgtctttggatgttagatctttttttgtagaaatgtcttggtgtcctcg	P13298
pGAP-BglII-pAOX1 fwd	ggacaccaagacatttctacaaaaaagatctaacatccaaagacgaaaggttgaa	P13299
pAOX1-EcoRI-Kozak-Tomato rev	cttgataacttcctcacccttagaaaccatcgtttcggaattcaataattagttgttttt	P13300
EGFP-SpeI-CAT1 fwd	tctcctttgctagccatcgtttcgactagttttaattgtaagtcttgactagagcaagtg	P13301
pAOX1-BglII-CAT1 rev	cgtctttggatgttagatctagtgtgtaatcatatatataataaatgaggaataataatt	P13302
TT-AscI-Mxr1	caaatggcattctgacatcctcttgaggcgcgccctagacaccaccatctagtcggtttt	P13303
Mxr1-Spe1-GAP	attgaacaactatcaaaacacaactagtcgaaacgatgagcaatctacccccaacttttg	P13304
CAT1-Spe1-Mxr1 rev	ctagtcaagacttacaattaaaactagtcgaaacgatgagcaatctacccccaacttttg	P13305
TT-AscI-Prm1 fwd	tggcattctgacatcctcttgaggcgcgccttaactgtcaaaatttattgtatctggcgc	P13306
GAP-SpeI-Prm1 rev	atcaattgaacaactatcaaaacacaactagtcgaaacgatgcctcctaaacatcggctg	P13307
CAT1-SpeI-Prm1 rev	ctagtcaagacttacaattaaaactagtcgaaacgatgcctcctaaacatcggctg	P13308
TT-AscI-Mpp1 fwd	attctgacatcctcttgaggcgcgccctattcttcaacattccagtagtcaattaactcc	P13309
GAP-SpeI-Mpp1 rev	atcaattgaacaactatcaaaacacaactagtcgaaacgatgagtaccgcagccccaa	P13310
CAT1-SpeI-Mpp1	ctagtcaagacttacaattaaaactagtcgaaacgatgagtaccgcagcccc	P13311
Mxr1cmut rev	tctgacagacaaataagcgtagctttcctgcgggtgtggt	P13449
MxrIcmut 1 fwd	cccgcaggaaagctacgcttatttgtctgtcagacatg	P13368
MxrIcmut rev	taatgggcataattatttccactaacggcggagaaggcagctcttcttag	P13369
MxrIcmut 2 fwd	ctgccttctccgccgttagtggaaataattatgcccattatgtgaataat	P13370
MxrIcmut 2 fwd	ctgccttctccgccgttagtggaaataattatgcccattatgtgaataat	P13371
CAT1-Spe1-Mxr1 rev	ctagtcaagacttacaattaaaactagtcgaaacgatgagcaatctacccccaacttttg	P13305
Mxr1-Spe1-GAP	attgaacaactatcaaaacacaactagtcgaaacgatgagcaatctacccccaacttttg	P13305
AOX1TT-SWI1 fwd	gcaaatggcattctgacatcctcttgaggcgcgccctaatcgacacctaggcctctctc	P13599
CAT1-SWI1 rev	gctctagtcaagacttacaattaaaactagtcgaaacgatgggcggtttccacaatgttt	P13600
AOX1TT-SNF1 fwd	tggcattctgacatcctcttgaggcgcgccctaaccttgactattcacggcaagttc	P13601
CAT1-SNF1 rev	agtcaagacttacaattaaaactagtcgaaacgatggccgcagaacaatcgaaag	P13602
AOX1TT-Cat8 vfwd	gcaaatggcattctgacatcctcttgaggcgcgccctaaagtccgaataaactcccagcag	P13603
CAT1-Cat8 rev	agtcaagacttacaattaaaactagtcgaaacgatgatgccggaggaacaagtaacc	P13604
AOX1TT-Reg1 fwd	tctgacatcctcttgaggcgcgccttacatacccatttttgaaacgatatcacttagaga	P13605
CAT1-Reg1 rev	agtcaagacttacaattaaaactagtcgaaacgatggcgtcccagaccaatttatc	P13606
AOX1TT-Gal4	tggcattctgacatcctcttgaggcgcgccttaatccattatagtgtccggaacttccaa	P13607

Primer name	Sequence 5' --> 3'	Internal Number
CAT1-Gal4 rev	agacttacaattaaaactagtcgaaacgatgcatcataaagaaagactcatagatcatat	P13608
AOX1TT-Snf2 fwd	tggcattctgacatcctcttgaggcgcgccttactctttcaagggctgtgcattagg	P13609
CAT1-Snf2 rev	agacttacaattaaaactagtcgaaacgatggaccgtgaacaattaacctcaga	P13610
AOX1TT-Rpd3 fwd	ttctgacatcctcttgaggcgcgccttaatcttctttttttcagttcgttctcaagttctt	P13611
CAT1-Rpd3 rev	agacttacaattaaaactagtcgaaacgatgacttttgagctaaagccatttgacc	P13612
AOX1TT-Hda1 fwd	ttctgacatcctcttgaggcgcgccttagttagaactctcatcttcgtattcttcgatgg	P13613
CAT1-Hda1 rev	agacttacaattaaaactagtcgaaacgatggaaattttaaaagaagaagcgacggaaa	P13614
GAP-Swi1 rev	acaactatcaaaacacaactagtcgaaacgatgggcggtttccacaatgttt	P13615
GAP-Snf1 rev	acaactatcaaaacacaactagtcgaaacgatggccgcagaacaatcgaaag	P13616
GAP-Cat8 rev	acaactatcaaaacacaactagtcgaaacgatgatgccggaggaacaagtaac	P13617
GAP-Reg1 rev	acaactatcaaaacacaactagtcgaaacgatggcgtcccagaccaatttatc	P13618
GAP-Gal4 rev	actatcaaaacacaactagtcgaaacgatgcatcataaagaaagactcatagatcatatt	P13619
GAP-Snf2 rev	actatcaaaacacaactagtcgaaacgatggaccgtgaacaattaacctcaga	P13620
GAP-Rpd3 rev	actatcaaaacacaactagtcgaaacgatgacttttgagctaaagccatttgacc	P13621
GAP-Hda1 rev	actatcaaaacacaactagtcgaaacgatggaaattttaaaagaagaagcgacggaaag	P13721
AOX1-LuHNL fwd	aaaaacaactaattattgaattccgaaacgatggcttctcttcctgttagctttg	P13622
AOX1TT-LuHNL rev	attctgacatcctcttgagcggccgctcaataatcgttcaacttgatcacgagtttg	P13623
URA3-pAdr1 fwd	ttgttccgtttgaacttcctgcaggaaaattgtggatcttatctatagcaaggctatcaa	P13624
sTomato-Adr1 rev	tcacccttagaaaccatcgtttcggaattctgtgcgtgggataaagtcatcaaac	P13625
URA3-pPRM1 fwd	ttgttccgtttgaacttcctgcaggtcgtaatccttgagttttttctccgtttcc	P13626
sTomato-pPRM1 rev	cttagaaaccatcgtttcggaattctttagttataaagaagggagattaatacagggcaa	P13627
URA3-pMpp1 fwd	ttgttccgtttgaacttcctgcaggaacagaatctggaggtgtaaacaccg	P13628
sTomato-pMpp1 rev	cttagaaaccatcgtttcggaattctggctgttatatagaatctttgagaggaggg	P13629
AOX1TT-ILV5 fwd	gattaagtgagaccttcgtttgtgcggatccttcagtaatgtcttgtttctttttgttgc	P13630
pUCOri-Kan rev	gacacgacttatcgccactggcagcagccactggtaacaggattagcagag	P13631
pCAT1-pPEX5 fwd	tttattatatatatgattacacactagatcttccaaaccaaacggtctagc	P13683
pPEX5-LuHNL rev	gaagagaagccatcgtttcggaattctacgattagttagatggttgggttgagaatag	P13684
pPEX5-LuHNL fwd	aacccaaccatctaactaatcgtagaattccgaaacgatggcttctcttc	P13685
AOX1TT-LuHNL rev	cattctgacatcctcttgagcggccgctcaataatcgttcaacttgatcacgagtttg	P13686
pCAT1-pPEX5 fwd	tcatttattatatatatgattacacactagatcttccaaaccaaacggtctagc	P13763
pPEX5-pCAT1 rev	gtttggtttggaagatctagtgtgtaatcatatatataataaatgaggaataataattga	P13764
FRT-BglII-pARG4	atactttctagagaataggaacttcagatctggaacggaacgtatcttagcatgg	P13312
PstI-Kann-pArg4 rev	atcattaacaaactcagtatactgcagttagaaaaactcatcgagcatcaaatgaaactg	P13313
seq.pUC.fwd	gccacctctgacttgagcgtcg	P12007
Primer_PGAP-fw	gaaaccaccagaatcgaatat	P12921
seqAOX1TT rev	ggcaaatggcattctgacatcct	P13372
seq-pARG4 rev	cagtctttcacagtgaatctgtcgca	P13373
seq-AOX1rev	tttcattcaacctttcgtctttggatgt	P13374
seq.fwd.EGFP.MlyIstuffer.integURA3	gcccgcgcacagtctattaac	P12090

Primer name	Sequence 5' --> 3'	Internal Number
seq.rev.pPT4.EGFP.BmrIstuffer.	ttccgtatgtagcatcaccttcac	P12014
AOX1.fromBglII.fwd	agatctaacatccaaagacgaaaggttg	P12015
aox1_fw_RT	gaagctgccctgtcttaaaccttt	P09324
seq-MxrI 1 fwd	cttcataaacttttctaaatccagatacagccgg	P13375
seq-MxrI 2 fwd	aaaattttccatcgctcaatggctgatt	P13376
seq-MxrI 3 fwd	gagttggagtgggcacacc	P13377
seq-MxrI 4 fwd	atatcaatggatcttgatcaggctgctg	P13378
seq-PrmI 1 fwd	atatctagctggtccatcatgccg	P13379
seq-PrmI 2 fwd	ggtcggtggttctatcccc	P13380
seq-PrmI 3 fwd	tcggaggtaatatgatacgtatttccctcc	P13381
seq-MppI 1 fwd	tcatatgatacagctggttactgtaacagc	P13382
seq-MppI 2 fwd	tcaagttgttggaaacggtgctagaatag	P13383
seq-MppI 3 fwd	gccgtaaccaaaactttccaatccaatt	P13384
seq CAT8 fwd	agtaagatacgtacgtcatttatgacagtttagtc	P13401
seq GAL4 fwd	tcacgcatgcgtaatttttttcagatcg	P13402
seq HDA1 fwd	ccattctttcgtaagtttgaattctgttttgc	P13403
seq MPP1 fwd	agtggtgctggctggtgtat	P13404
seq MXR1 fwd	ttgatggaatcataccgattgcaaactaat	P13405
seq PMR1 fwd	tagatccctggacgttgttgattgagttat	P13406
seq REG1 fwd	acaagatttacaactttggtgagctcatca	P13407
seq RPD3 fwd	atgactagtagaattacaccaacagtgcaa	P13408
seq SNF1 fwd	agatcatcatatacccaataaattgcacaagc	P13409
seq SNF2 fwd	aaaatatactgatagttggaatactcaaataaaacagagt	P13410
seq SWI1 fwd	aaaaatacagaaggatccgccgtca	P13411
seq.CAT1 rev	atatataagctgtagacccagcac	P13660
seq.SWI1 1	ggcaggagcgttaataaacgcc	P13661
seq.SWI1 2	gtcaactactatctggacatcagt	P13662
seq.CAT8 1	caggggtatcattttcgggtatca	P13663
seq.CAT8 2	cgcaagcgacagtttgatgttgtt	P13664
seq.CAT8 3	agtatcggagctttactttgaaac	P13665
seq.GAL4 1	gggctgcttttggtgttg	P13666
seq.GAL4 2	tccaactcggcatcattgatattg	P13667
seq.GAL4 3	ttatcaacgaagagcaaccggatt	P13668
seq.HDA1 1	ttgcctgtcaatgtcaatggaaca	P13669
seq.HDA1 2	tatttgccctgctcgtaacga	P13670
seq.REG1 1	tcttgccttgagtttctccaga	P13671
seq.REG1 2	gcctgagcccaagttctc	P13672
seq.RPD3 1	tcatcgacaggttgaaacaaccta	P13673
seq.SNF1 1	ataggtcaccgcacttcc	P13674

Primer name	Sequence 5' --> 3'	Internal Number
seq.SNF2 1	gatgacactcaagttacgaccatc	P13675
seq.SNF2 2	ctgatgaggattccaatcactgtc	P13676
seq.SNF2 3	atgcccttcatcgattatcatatg	P13677
seq.SNF2 4	tctgtttggtttgctccatctg	P13678
seq-pUC rev	ggtgtaggtcgttcgctc	P13687
seq-KANMx rev	aataatcgtaaggtgtcctagagg	P13688
seq-pILV5 rev	gaatttaatcgcggcctcga	P13689

3.4 Enzymes

Table 3.9 contains all enzymes used in this thesis. All enzymes with Thermo Fisher Scientific as manufacturer were used as FastDigest variants.

Table 3.9.: All enzymes used in the course of this thesis.

Enzyme	Type	Company
*Swa*I	RE	Thermo Fisher Scientific Inc., Waltham, MA, United States
*Pci*I	RE	Thermo Fisher Scientific Inc., Waltham, MA, United States
*Asc*I	RE	Thermo Fisher Scientific Inc., Waltham, MA, United States
*Spe*I	RE	Thermo Fisher Scientific Inc., Waltham, MA, United States
*Sbf*I	RE	Thermo Fisher Scientific Inc., Waltham, MA, United States
*Bgl*II	RE	Thermo Fisher Scientific Inc., Waltham, MA, United States
*Not*I	RE	Thermo Fisher Scientific Inc., Waltham, MA, United States
*Eco*RI	RE	Thermo Fisher Scientific Inc., Waltham, MA, United States
*Bam*HI	RE	Thermo Fisher Scientific Inc., Waltham, MA, United States
*Cai*I	RE	Thermo Fisher Scientific Inc., Waltham, MA, United States
*Sfi*I	RE	New England Biolabs, Ipswich, MA, Unites States
Taq DNA Polymerase	Polymerase	Promega GmbH, Mannheim, Germany
Phusion High Fidelity DNA Polymerase	Polymerase	Thermo Fisher Scientific Inc., Waltham, MA, United States
T4 DNA Ligase	Ligase	Thermo Fisher Scientific Inc., Waltham, MA, United States
Taq DNA Ligase	Ligase	New England Biolabs, Ipswich, MA, Unites States
T5 Exonuclease	Exonuclease	New England Biolabs, Ipswich, MA, Unites States
FastAP Thermosensitive Alkaline Phosphatase	Phosphatase	Thermo Fisher Scientific Inc., Waltham, MA, United States
Taq DNA ligase	Ligase	New England Biolabs, Ipswich, MA, Unites States

3.5 Media, Buffer and Chemicals

Table 3.10: All media used in this thesis. The amounts in this list correspond to the preparation of one liter. For BMD1% and BM_ the final volume is also one liter e.g. the difference to one liter has to be filled to one liter. In case of antibiotic selection Zeocin was added at a concentration of 100µg/mL for *P. pastoris* and 25µg/mL for *E. coli*. Kanamycin was added at 100µg/mL and Geneticin at 300µg/mL.

	component	amount
YPD	Yeast extract	10 g
	Peptone	20 g
	Agar (only for plates)	15 g
LB	Yeast extract	5 g
	Tryptone	10 g
	NaCl	5 g
BMD 1%	Glucose monohydrate	11 g
	10x YNB stock	100 mL
	1M Potassium-phosphate buffer (pH 6)	200 mL
	500x Biotin stock	2 mL
BM	10x YNB stock	100 mL
	1M Potassium-phosphate buffer (pH 6)	200 mL
	500x Biotin stock	2 mL
SOC	Glucose	3.46 g
	Tryptone	20 g
	Yeast extract	5 g
	NaCl	0.58 g
	$MgCl_2$	2 g
	KCl	0.16 g
	$MgSO_4$	2.46 g
BMM2	10x YNB stock	100 mL
	1M Potassium-phosphate buffer (pH 6)	200 mL
	500x Biotin stock	2 mL
	Methanol conc.	10mL
BMM10	10x YNB stock	100 mL
	1M Potassium-phosphate buffer (pH 6)	200 mL
	500x Biotin stock	2 mL
	Methanol conc.	50mL

Stock Solutions:

10xYNB: 134 g of yeast nitrogen base (YNB) containing ammonium sulfate were filled up to 1 L with ddH_2O and dissolved completely by stirring and slightly heating the solution. Subsequently the solution was sterilized by autoclaving.

5xK_2PO_4 buffer: 30 g of K_2HPO_4 and 118 g of KH_2PO_4 were dissolved in 800 mL ddH_2O and the pH was set by addition of 1M or conc. KOH to a pH of 6. Finally, the solution was filled up to 1 L with ddH_2O and sterilized by autoclaving.

500xBiotin: 20 mg of biotin were dissolved in 100 mL ddH_2O and the solution was filter sterilized and stored at 4°C.

10xDextrose (20%): 220 g of glucose monohydrate were weighed and filled up to 1 L with ddH_2O.

50xTAE buffer: 484 g of TRIS and 29.2 g of EDTA were dissolved in 114.2 mL acetic acid (conc.) the volume was adjusted to 2 L with ddH_2O.

BEDS: 1.632 g of Bicin was dissolved in 920 mL ddH_2O. The pH was set to 8.3 by addition of 2M NaOH and concentrated NaOH and afterwards, 30 mL Ethylenglycol, 50 mL DMSO and 182 g of Sorbitol were added to the solution. After checking the pH (8.3) the solution was filter sterilized.

1M DTT: 1.54 g of DTT was dissolved in 10 mL ddH_2O and filter-sterilized. 1 mL aliquots were stored at -20°C.

3.6 Kits and Protocols

3.6.1 Kits

Table 3.11.: Kits and solutions used in this thesis.

Kit	company
Gene JET Miniprep Kit	Thermo Fisher Scientific Inc., Waltham, MA, USA
Wizard SV Gel and PCR Clean-Up System	Promega GmbH, Mannheim, Germany
Y-Per yeast protein extraction reagent	Thermo Fisher Scientific Inc., Waltham, MA, USA
Glucose-UV Hexokinase assay kit	DIPROmed Handels GmbH, Weigelsdorf, Austria
RNAse later solution	Life Technologies Inc., Carlsbad, CA, USA
(Ambion®)RiboPure™ Kit	Life Technologies Inc., Carlsbad, CA, USA

3.6.2 Transformation Protocols

The preparation of *E. coli* TOP10F' competent cells was performed according to (Seidman 2001) and cells stored at -80°C. For the electroporation of *E. coli* TOP10F' 3µL of either ligation or Gibson preparation was directly used without desalting. 80µL of frozen cells were thawed on ice used for transformation with 2.5 kV/25 µF/200 Ω using Bio-Rad Bio-Rad Gene Pulser System (see figure 3.1). After electroporation 1mL of SOC medium was added to the transformation and cells were regenerated at 37°C for one hour. After centrifugation and resuspension in a smaller volume the cells were plated on selective LB (Luria-Broth) agar plates. The incubation was performed over night at 37°C.

The preparation of electrocompetent *P. pastoris* cells was performed according to Lin-Cereghino *et al.* 2005 "Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris". The cells were freshly prepared for every transformation and discarded if not used. By default the transformation was performed with 1µg linearized plasmid DNA and the BioRad gene pulser system (see figure 3.1) at 1.5 kV/25 µF/200 Ω. After electroporation 1mL of a 1:1 mixture of 1M sorbitol and YPD1% glucose was added to the transformation and cells were regenerated at 28°C for two hours. After centrifugation and resuspension in a smaller volume the cells were plated on selective YPD1% glucose agar plates. The plates were incubated for 3 days at 28°C.

3.6.3 DNA Isolation from *E. coli* (plasmid) and *P. pastoris* (genomic DNA)

For the isolation of plasmids from *E. coli* the Gene JET Miniprep Kit from Thermo Fisher Scientific Inc., Waltham, MA, USA was used. The cells were grown on selective LB agar plates, scraped off the plate using a round sterile wooden tooth pick and further processed according to the protocol. The DNA was eluted in 50µL ultrapure ddH_2O (Fresenius Kabi).

The genomic DNA isolation was performed according to the protocol of Hoffman and Winston 1987 and DNA was eluted in 100 µL ultrapure ddH_2O (Fresenius Kabi). The quality and quantity of isolated DNA was checked with a Nanodrop 2000c (Hoffman 1987).

3.6.4 Isolation of intracellularly expressed LuHNLp from P. pastoris

In order to test for HNL activity the intracellulary expressed LuHnlp was isolated using Yper yeast protein extraction reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). Deep-Well-Plate grown cells were pelleted at 4000 rpm and 250µL of reagent used according to the protocol accompanied with the Kit. The samples were frozen at -20°C.

3.6.5 Glucose Determination

The determination of the glucose concentration present in samples was performed with the Glucose-UV Hexokinase assay kit (DIPROmed Handels GmbH, Weigelsdorf, Austria). 198µL reaction solution and 2µL sample were incubated at RT for 20 minutes and NADH absorption was measured at 340nm wavelength in 96 well PS Microplater (Greiner Bio-One GmbH, Frickenhausen, Germany). For adsorption measurements SynergyMx Plate Reader (Biotek Inc., Winooski, United States) was used. Additionally quick measurement was done with applying a few microliters to Accutrend glucose test stripes (Roche Diagnostics International AG, Rotkreuz, Switzerland).

3.6.6 Cultivation of *P. pastoris* strains in Deep-Well-Plates (DWP)

A 96-DWP (Bel-Art Products, Wayne, NJ, United States) was filled with 250 µL of BMD1% (Basal Minimal Dextrose Medium 1%) in each well. Each well was inoculated with a single colony of a *P. pastoris* transformant with a sterile toothpick. Subsequently the

cells were cultivated in HT Infors Multitron Shaker at 28°C and 320rpm for 60 hours. In case of methanol induction 250µL of BMM2 were added to each well and put again at 28°C and 320rpm. After 12 hours reinduction with 50µL BMM10 was performed. This was repeated in 12 hour cycles for a total of 60 hours.

3.6.7 Cultivation of *P. pastoris* strains in DAS Gip fermenter system

For the cultivation of *P. pastoris* strains in the DAS Gip fermenter system 750mL of BMD1% was used. All 8 reaction vessels were filled with 525mL 1% glucose solution and pH- and O_2 -electrodes were calibrated. The function of spargers, rotors, air filters and temperature control were tested prior to autoclaving at 121°C for 21 minutes. After sterilization the vessels were allowed to cool down and 150mL 1M sterile Potassium-Phosphate-Buffer, 75mL 10x sterile YNB and 1.5 mL of 500x Biotin were added to each vessel. All strains cultivated in fermenters were prior subjected to an ONC in 50mL YPD 1% glucose at 28°C 120rpm overnight. After OD_{600} measurement the 750mL of BMD1% were inoculated to OD_{600} of 0.5. The cultivation was performed at 28°C and 500rpm stirrer speed and pH was kept constant at pH 6.00 with 12.5% Ammonia solution. The O_2 saturation was kept constant at 100% and if necessary stirrer speed was allowed to increase to meet this demand. The fermentation parameters were checked by OD_{600}, glucose concentration and fluorescence measurement. After glucose depletion the cells were allowed to remain in this stage for 6-8 hours and afterwards the 750mL medium induced with 1% methanol. Evaporation was controlled throughout the fermentation. Each vessel was further cultivated under the same conditions mentioned above and after methanol depletion (observed with oxygen and base peak) a reinduction with 1% glucose was performed. Throughout the fermentation samples of 1mL were regularly drawn at specified time points (figure 4.10-figure 4.13), centrifuged with 4000rpm and processed using Ambion RNase later Solution (Life Technologies Inc., Carlsbad, CA, United States). Samples prepared in this way were flash frozen and stored at -80°C until further processing.

3.6.8 RNA Isolation and Microarray Hybridization

The isolation of frozen and RNase later solution processed RNA was performed using Ambion® RiboPure™ Kit (Life Technologies Inc., Carlsbad, CA, United States). The isolation process was performed according to the protocol supplied by the manufacturer. RNA quality and quantity was measured using Nanodrop 2000c (Thermo Fisher Scientific

Inc., Waltham, MA, United States). During the isolation process washing of surfaces with 1%SDS was used to inactivate possible interfering RNases. The isolated DNA was stored at -80°C. The transport of all RNA samples to our partner Karin Wagner at ZMF (Zentrum für medizinische Forschung Graz, Stiftingtalstraße 24, A-8010 Graz) was executed using cooling packs at -80°C in a thermo isolated Styrofoam box. All further processing of RNA samples was done at ZMF facilities by Karin Wagner. The labeling of RNA was done with Affymetrix GeneChip 3'IVT Express Kit P/N 702646 Rev. 8; Cat.No. 901228 according to the protocol supplied by the manufacturer. For the hybridization 14 gene chips "Affymetrix Pichia Arrays PPA01a520396F" were used to perform the hybridization procedure according to "Affymetrix GeneChip® HT hybridization, Wash and Stain Kit (Cat No. 900720)" using the device "Affymetrix Genechip® fluidics station 450: protocol FS450_0007 for Cartridge Arrays". After the successful hybridization the read out was performed with "Affymetrix Scanner: GCS3000; AGCC (Command Console Software AGCC 3.2.)" and data were processed with "Affymetrix Genexpression Console (3.2) using Affymetrix default analysis settings for generation of CEL-files."

3.6.9 PCR Procedures

All PCR procedures during this thesis were performed using Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, MA, United States) or *Taq* DNA Polymerase (Promega GmbH, Mannheim, Germany) and dNTP Mix (Thermo Fisher Scientific Inc., Waltham, MA, United States). See Table 3.12 and 3.13 for a more detailed description.

Table 3.12: Master template for PCR reactions

PCR Components	Volume
template	x µL*
5x Phusion HF Buffer	10µL
dNTP mix 2mM	5µL
Primer fwd 10pmol/µL	2.5µL
Primer rev 10pmol/µL	2.5µL
ultrapure ddH2O	29.75-x µL
Phusion DNA polymerase	0.25µL
total	50µL

* Template amount varied according to DNA concentration, 5ng of plasmid and 35 ng of genomic DNA were used by default

Table 3.13: Cycle Parameters for PCR reactions

Cycle Step	Temperature	No. of Cycles	Time [sec]
Initial Denaturation	98°C	1	300
Denaturation	98°C		30
Annealing	58°C	30	30
Extension	72°C		60/kbp
Final Extension	72°C	1	420
Holding	4°C	∞	∞

For oePCR reactions a slightly different formulation was used. The first round of PCR reaction was performed without external primers, essentially the templates act as primers through their overlapping regions. 18 cycles were used to create the initial oePCR fragment. In the second round external primers together with replenished dNTPs, polymerase and buffer were added and another 30 cycles of PCR were performed. (see table 3.14)

Table 3.14: Master template for oePCR reactions

PCR Components	Volume
template 1	x_1
template 2	x_2
template 3	x_3
5x Phusion HF Buffer	10µL
dNTP mix 2mM	5µL
Primer fwd 20pmol/µL	-*
Primer rev 20pmol/µL	-*
ultrapure ddH_2O	34.75µL –x_1-x_2-x_3
Phusion DNA polymerase	0.25µL
total	50µL

*[1]PCR Components	Volume
template 1	-
template 2	-
template 3	-
5x Phusion HF Buffer	5µL
dNTP mix 2mM	2.5µL
Primer fwd 20pmol/µL	3.75µL
Primer rev 20pmol/µL	3.75µL
ultrapure ddH_2O	14.9µL
Phusion DNA polymerase	0.125µL
total	75µL (50µL+25µL)

* after the first 18 cycles of PCR reactions the components were added to the 50µL and the PCR reactions was run for another 30 cylces.
*1 fragments with their overlapping parts acts as selfprimer, as template 5ng and accordingly x_2 and x_3 in a molar ratio 1:1 were used.

3.6.10 Restriction Endocnuclease Reactions

All restriction endonuclease reactions, including linearizations for transformation were performed in this way. All enzymes were obtained from two sources, either Thermo Fisher Scientific Inc., Waltham, MA, United States and New EngIand Biolabs, Ipswich, MA, Unites States (see table 3.15). In general 1µL of enzyme is used for the digestion if 1µg of plasmid DNA. Thermo Fisher Scientific FastDigest enzymes were incubated for 1h at 37°C and regular Thermo Fisher Scientific and NEB enzymes were incubated for 6h at 37°C. Enzyme Inactivation was performed at 85°C for 20 minutes (see Table 3.15).

Table 3.15: restriction digestion of plasmids

Components	Volume
Buffer 10x	4µL
RE	1µL
template	x µL
ultrapure ddH_2O	35µL -x
total	40µL

3.6.11 Ligation Reactions and Gibson Assembly

All ligation reactions performed during this thesis were done with T4 DNA ligase (Thermo Fisher Scientific Inc., Waltham, MA, United States). 50-100ng of cut plasmid and insert DNA corresponding to a molar ratio of 1:3 were used for the ligation reaction with a final volume of 20µL. For every reaction a fresh 2µL aliquot of dATP containing T4 DNA ligase buffer was used. The reaction was either kept at 21°C for one hour or at 16°C overnight. The enzymatic reaction was inactivated by heating to 75°C for 5 minutes. 3µL of the ligation reaction were used for transformation.

For the Gibson Assembly reaction 100ng of cut vector and insert DNA in a molar ratio of 1:1 with final amount not exceeding 250ng were used. 15µL of already prepared Gibson Assembly reaction mixture (New EngIand Biolabs, Ipswich, MA, Unites States) were mixed with vector and insert DNA and filled up to a final volume of 20µL. The reaction was performed at 50°C for an hour and 3 µL of the reaction mixture used for transformation.

3.7 Screening Assays

3.7.1 Fluorescence Measurement of eGFP and sTomato expression in *P. pastoris* strains

For the measurement of fluorescence SynergyMx Plate Reader (Biotek Inc., Winooski, United States) was used in combination with Nunc™ MicroWell™ 96-Well Optical-Bottom Plates with Polymer Base (Thermo Fisher Scientific Inc., Rochester, NY, United States). 10µL of the cultivated *P. pastoris* strains were mixed with 190 µL ddH_2O and fluorescence was measured for eGFP at 488nm excitation – 507nm emission and for sTomato at 554nm excitation – 581nm emission. In the case of growth curve experiments in DWPs cells were diluted with ddH_2O to be in a range of OD_{600} of 0.2-0.7. Raw fluorescence data were subtracted with the fluorescence value of the medium blank and the resulting values divided by OD_{600} resulting in OD_{600} normalized fluorescence data.

3.7.2 Screening for hydroxynitrile lyase activity

The hydroxynitrile lyase activity was measured according to Krammer *et al* 2007, with acetoncyanohydrin acting as a substrate. For the assay itself 10µL of isolated cell extract was used and performed according to the protocol (Krammer 2007).

3.7.3 Screeening for *mxr1*Δ on BMM 1% agar plates

The *mxr1*⊿ knockout strains show a growth deficiency on methanol as sole carbon and energy source (Lin-Cereghino *et al* 2006). Therefore linearized and transformed PpPKC1-Mxr1 plasmid strains were screened by growing them according to the Cultivation of P. pastoris strains in Deep-Well-Plates (DWP) and finally diluted 1:100 with sterile ddH_2O. These diluted strains were subsequently stamped on BMM1% agar plates (Basal Minimal Medium with 1% methanol). The stamped plates were incubated for 3 days at 28°C.

4. RESULTS AND DISCUSSION

4.1 Regulated overexpression of specific transcription regulators enables methanol free induction of the *AOX1* promoter

After extensive literature research I was able compile a set of putative transcription regulators for targeted overexpression. All transcriptional regulators listed in table 4.1 regulate catabolite repression/dererepression of non-glucose carbon sources in different yeasts.

Table 4.1.: Synopsis of putative factors involved in the regulation of carbon metabolism listed with their respective function in methylotrophic yeasts. Listed in this table is condensed information of published data in methylotrophic yeasts *P. pastoris*, *H. polymorpha* and *C. boidinii*. In case we were unable to find experimental data from methylotrophic yeast species, we would reference to the homologous protein function in *S. cerevisiae.*

Protein Name	Function	Size [bp]	References
Mxr1	Master regulator of methanol and peroxisomal genes in *P. pastoris* Mxr1p and *C. boidinii* (Trm2p), the *S. cerevisiae* homolog Adr1p is also involved in activation of catabolite repressed genes	3468	**Lin Cereghino 2006 Parua 2012 Sasano 2010**
Prm1	Prm1p in *P. pastoris* and its homologue Trm1p in *C. boidinii* and Mut3p in *H. polymorpha* are positive regulators of MUT genes	2970	**Sasano 2008 Vallini 2000**
Mpp1	Mpp1 (methylotrophic peroxisomal protein1) in *H. polymorpha* important for growth on methanol, annotated as YLL054C in *P. pastoris*	2667	**van Zutphen 2010**
Swi1	ΔSwi1ΔSnf2 double knockout showed defective methanol utilization in *H. polymorpha*	2454	**Ozimek 2004**
Snf2		5009	
Snf1	*S. cerevisiae* Snf1 kinase is a master regulator of carbon catabolite-derepression	1664	**Wilson 1996**
Cat8	Zinc cluster transcriptional activator involved in *S. cerevisiae* in activation of catabolite repressed genes.	3111	**Young 2003**
Reg1	annotated as regulatory subunit of Glc7p master phosphatase in *P. pastoris*, Glc7p acts antagonistically to master kinase Snf1 by dephosphorylation	1995	**Zaman 2009**
Gal4	*S. cerevisiae* regulator of galactose metabolism, in *P. pastoris* annotated as Lac9, unknown function as *P. pastoris* is unable to grow on galactose	2988	**Traven 2006**
Rpd3	Histone deacetylases from *S. cerevisiae*, regulate transcription, silencing, autophagy and other processes by influencing chromatin remodeling, *Δrpd3* and *Δhda1* genes allowed constitutive promoter binding of Adr1 and Cat8	1516	**Rundlett 1996 Tachibana 2007**
Hda1		2226	

In Figure 4.1 the design of all overexpression plasmids employed in the following studies is shown. All plasmids are based on the T4 plasmid family (Ruth 2010) and harbor a URA3 sequence for homologous genomic integration. This integration sequence allowed the linearization of all transcription factor overexpression plasmids with *Swa*I. In order to investigate the overexpression of the respective transcription factor and its putative effect on P_{AOX1} activation a bidirectional promoter system was applied. The capability of the transcription factor to regulate P_{AOX1} activity is measured by the expression of single Tomato fluorescence protein.

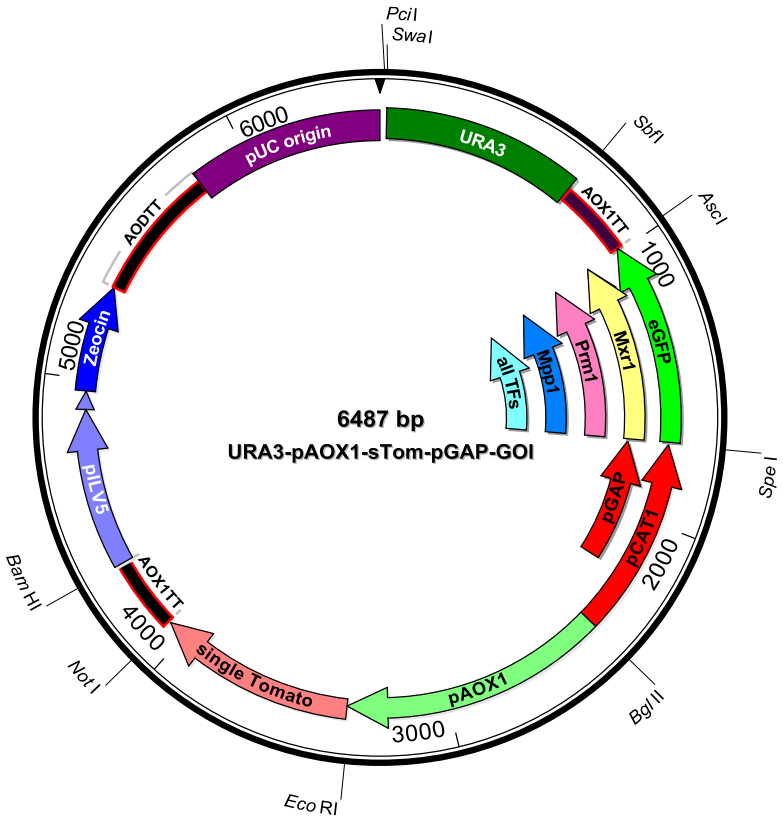

Figure 4.1.: Plasmid map of constructs used for transcriptional regulator overexpression. 11 different target genes from table 4.1 (only five shown) and *eGFP* (ctrl) were cloned downstream of P_{CAT1} and P_{GAP} resulting in 24 different plasmids. All other cassettes remained constant. For reasons of cloning convenience restriction sites were incorporated. *Eco*RI and *Spe*I restriction sites were tested for their possible effects on expression but showed no difference to the restriction site free versions of the promoters used (data not shown). As is commonly performed in the case of all in-house T4-based plasmids, I included the P_{AOX1} Kozak sequence upstream of the respective restriction site. Due to the differing insert sizes restriction enzyme uniqueness has to be evaluated for every single plasmid.

For the overexpression of the activator two differently regulated promoters were chosen (P_{GAP} and P_{CAT1}). The P_{GAP} is a constitutive promoter whereas P_{CAT1} is derepressed as can be seen in figure 4.2. The constitutive expression of activators may allow inducing P_{AOX1} expression already on glucose, however there might also be a negative effect, since strong methanol inducible *MXR1* overexpression was detrimental to cells (Lin-Cereghino 2006). Therefore constitutive overexpression of *MXR1* and similar factors might cause similar detrimental effects and we used in addition the tightly regulated P_{CAT1} promoter to overexpress these transcription factors. The P_{CAT1} is repressed on glucose as growth substrate and starts expression in the stationary phase upon glucose depletion and probably also if induced by H_2O_2 or other intracellular ROS (reactive oxygen species). Possible detrimental effects of constitutive overexpression may therefore be circumvented with P_{CAT1} while still retaining the inducibility of the system and avoiding detrimental effects at the same time.

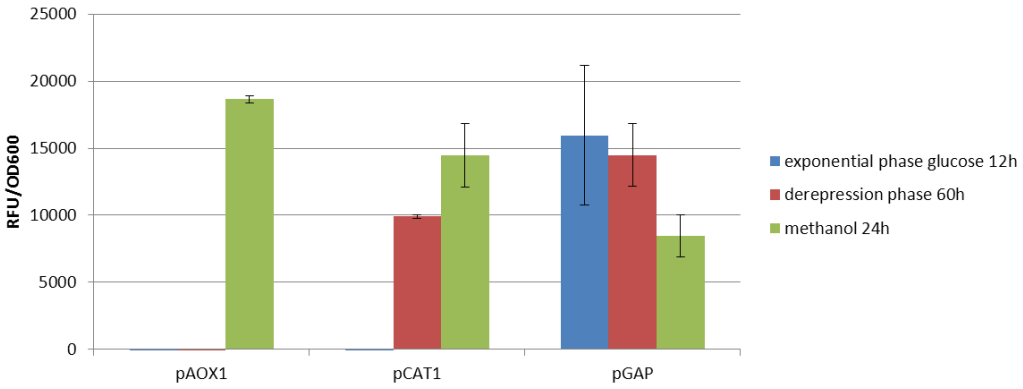

Figure 4.2.: Regulation profile of three different promoters – P_{AOX1}, P_{CAT1} and P_{GAP}. The *eGFP* CDS was cloned under the control of all three promoters, transformed in *P. pastoris* and screened in deep-well-plates (DWP) for 60h on glucose and 24h on methanol. Fluorescence measurements were performed after 12h, 60h and 84h after inoculation. This measurement correspond to exponential phase on glucose, derepression phase and methanol induction phase, respectively.

The particular transcription factors and *eGFP* as a control were cloned under control of the respective promoter. Transformant selection was achieved via conferment of zeocine resistance for all 24 plasmids. Additionally *MXR1*, *PRM1*, *MPP1* and control plasmids were cloned with geneticin resistance markers. In addition, restriction sites were incorporated between all assembled sequences to allow easy replacement of parts. Subsequently, the plasmids were assembled via Gibson-Assembly (Gibson 2009) and finally the inserted sequences and their flanking regions were sequenced by Sanger sequencing.

After transformation of *P. pastoris*, 50-100 clones were picked for cultivation in deep-well plates and screened for sTomato expression (see material and methods). In figure 4.3 the expression of the respective TF overexpression strain under glucose conditions (growth on glucose for 60h) and methanol induction (24 hours on methanol) are shown.

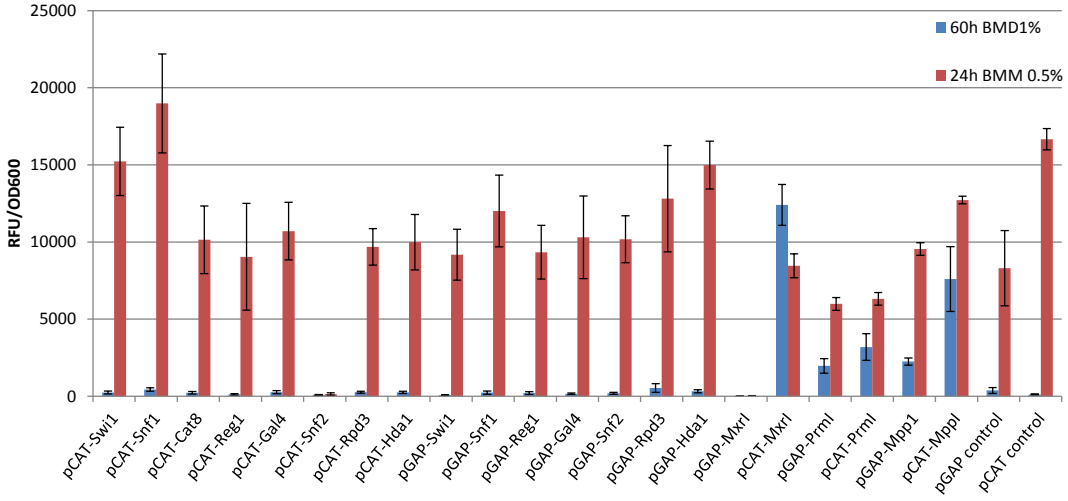

Figure 4.3.: Screening data of putative transcriptional regulator overexpression involved in catabolite repression and MUT gene activation. The factors listed in table 4.1 were cloned downstream of P_{GAP} and P_{CAT1}, linearized with *SwaI* and transformed into *P. pastoris* CBS 7435. As controls (P_{GAP} and P_{CAT1} control) eGFP was cloned instead of transcriptional regulator while P_{AOX1}-*sTomato* remained constant. Roughly 40 clones of each construct were cultivated under glucose and methanol conditions in a DWP. The arithmetic means and standard deviations shown correspond to roughly 40 transformants.

The majority of factors showed neither an effect under depressed conditions nor upon methanol induction compared to wildtype P_{AOX1}. Certain factors showed effects that can be separated into three groups.

4.1.1 Possibly improved methanol induction.

The first group shown on the far left of figure 4.3 comprises of P_{CAT1}-*SWI1* and P_{CAT1}-*SNF1*. Interestingly, neither P_{CAT1}-driven expression of *SNF1* nor *SWI1* was activating P_{AOX1} under glucose conditions but positive effects upon methanol induction were seen compared to most other constructs. In *S. cerevisiae SWI1* is described as a member of the SWI/SNF chromatin remodeling complex involved in regulation of a diverse set of genes - *ADH2*, *GAL4* and *SUC2* amongst other factors. (Peterson 1992, Peterson 1994, Prochasson 2003) Snf1p on the other hand is described to be a master regulator phosphorylating a

broad range of transcription factors and therefore causing their activation or repression. (Jiang 1996, Wilson 1996, Celenza 1984) In *P. pastoris* these two factors seem to act as positive regulators of methanol inducible genes further increasing P_{AOX1}-driven expression. Taking their function in *S. cerevisiae* into consideration and inferring similar functions in *P. pastoris* changes of chromatin structure and phosphorylation status could positively act on MUT genes. However, in this study we were not focusing on finding activators under methanol conditions but regulators already functional in glucose medium. Rescreening of *SNF1* and *SWI1* constructs needs to be completed and further testing undertaken until a final positive effect can be attributed to them.

4.1.2 Detrimental effect on methanol

The second group of response in my setting was visible for overexpression of *SNF2*. The P_{CAT1} controlled expression of *SNF2* resulted in the inability to induce P_{AOX1} expression on methanol. This is unexpected since *SNF2* in *S. cerevisiae* is part of the same SWI/SNF chromatin remodeling complex described above (Peterson 1992, Peterson 1994, Wu 1997, Ozimek 2004). Due to the fact that *H. polymorpha snf2*⊿ strains could not grow on methanol anymore we assumed that this is an activating factor and overexpression should rather have a positive effect. The massive overexpression taking place during methanol induced P_{CAT1}-controlled expression is most likely detrimental to cell viability and might destroy the cell's ability to regulate P_{AOX1} transcription in a similar fashion as previously described for *MXR1* (Lin-Cereghino et al. 2006). Since *SNF2* overexpression showed no positive effect before methanol induction the P_{CAT1} might be too strong. In addition to the analysis of effects on protein expression we also performed microscopy experiments (see Appendix).

4.1.3 Methanol free activation of P_{AOX1}

Three methanol gene activators *MXR1*, *PRM1* and *MPP1* visible on the far right of figure 4.3 activated P_{AOX1} already under derepressed conditions and did not require methanol induction. The overexpression of these three TFs employing P_{GAP} and P_{CAT1} showed different levels of activation of P_{AOX1} under derepressed conditions without addition of methanol as an inducer except for P_{GAP} -*MXR1* which showed no fluorescence at all.

Under P_{CAT1} control *MXR1*, *PRM1*, *MPP1* reached approximately 75, 25 and 75% of methanol induced P_{AOX1}, respectively. The P_{GAP} driven expression did not show any effect for

MXR1. P_{GAP} driven expression of *PRM1* and *MPP1* reached about 10% and 20 % of methanol induced P_{AOX1} reporter gene fluorescence. In general, P_{CAT1} driven overexpression showed higher activation than P_{GAP} strains.

Due to the interesting results of *MXR1*, *PRM1* and *MPP1* four clones per construct were rescreened and finally one clone picked for further cultivations. In order to reduce possible plate-to-plate variability I characterized all strains on the same deep-well plate. Simultaneously, the regulatory profile of P_{GAP} and P_{CAT1} fused to P_{AOX1} was checked by investigating the *eGFP* control constructs (shown in figure 4.4). In addition to the knowledge of the P_{GAP} / P_{CAT1} promoter expression profiles this would also allow investigating possible depression effects of P_{GAP} / P_{CAT1} fusions to P_{AOX1} under glucose depleted conditions. Fusion of a constitutive/derepressed promoter to P_{AOX1} did not interfere with its tight regulation and did not lead to a derepression effect irrespective of TF coexpression (Figure 4.4).

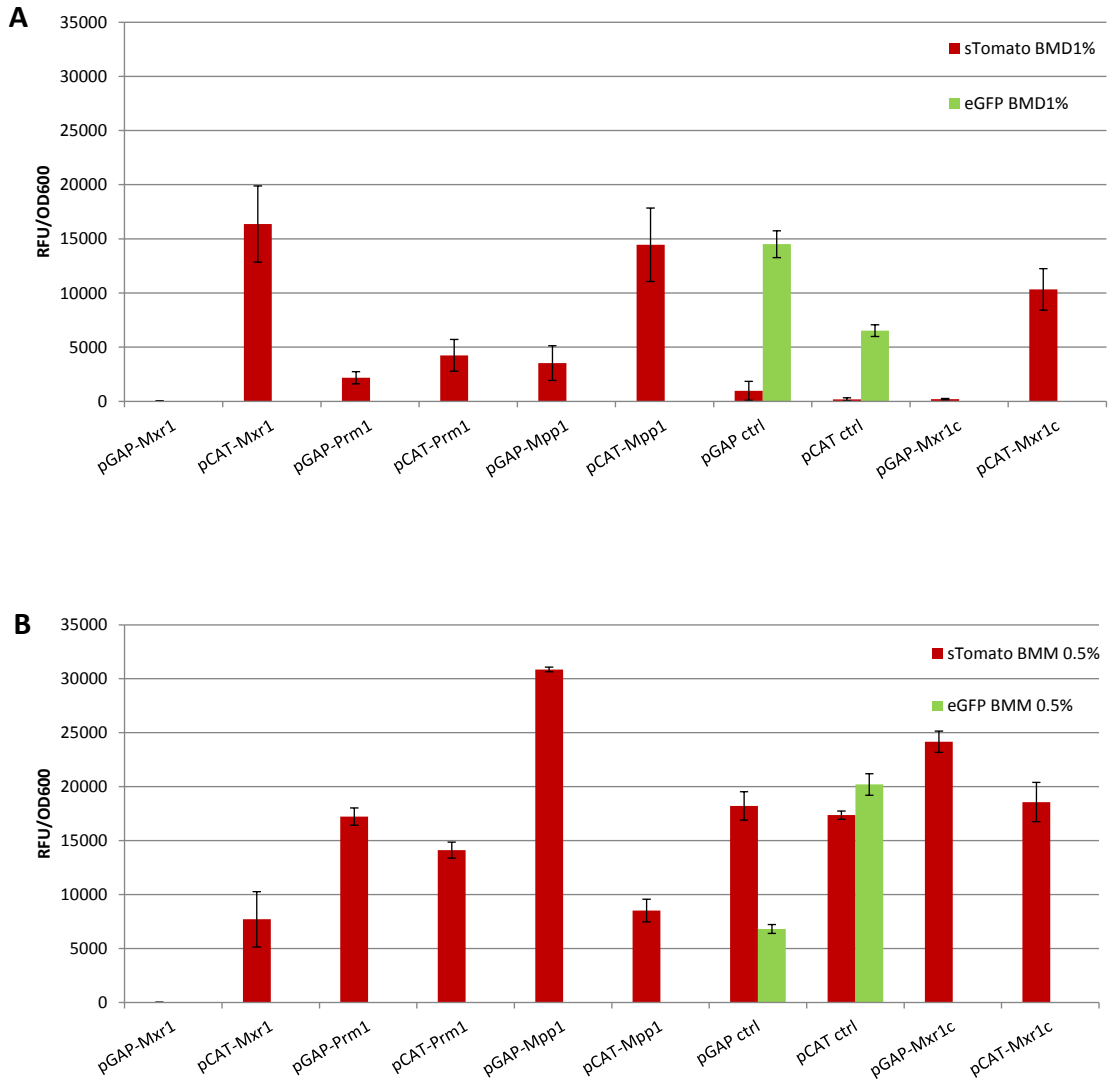

Figure 4.4.: Rescreening of methanol activator overexpression on glucose and methanol minimal medium employing differently regulated promoters. A: Overexpression of methanol activators on glucose minimal medium. **B:** Overexpression of methanol activators on methanol minimal medium. Transcriptional activators represented here were cloned in the plasmid background in figure 4.1 and screened in a single deep well plate on BMD1% (glucose minimal medium). A single transformant having shown uniform, representative expression was selected for the rescreening and grown in 7 fold replicates. After 60h on glucose deep-well-plates were induced with methanol for another 60h. Methanol was replenished in a 12h cycle. Measurement was performed after 60h on glucose and 24 hours on methanol according to material and methods. The control plasmids with P_{GAP} and P_{CAT1} harbor eGFP instead of the respective transcription factor. *MXR1*^{c} (see construction of *MXR1*^{c}) denotes the double point mutant of *MXR1*.

The P_{GAP} driven expression exerted a positive effect on P_{AOX1} activation in the case of *PRM1* and *MPP1* while we were not able to detect any positive effect of *MXR1* expression. The OD_{600} values presented in figure 4.3 and 4.4. cannot necessarily be compared since only in figure 4.4 strains were cultivated on the same plate. Additionally I observed drastically decreased transformation efficiencies for P_{GAP} constructs compared to the same plasmids harboring P_{CAT1} – while both transformations were performed with the same batch of competent cells. Taking into consideration that constitutive expression in contrast to inducible one will affect the cells permanently it would not be far-fetched to assume a possible detrimental effect already present during transformation. Therefore cells able to show growth on selection plates might not be able to functionally express transcription factor due to recombination events affecting promoter activity and gene integrity or possibly due to genomic integration in transcriptionally silenced genomic regions, although the latter possibility seems rather unlikely.

A slightly different picture emerges when we look at P_{CAT1}-controlled overexpression on glucose. Within the three methanol gene activators, overexpression of two of them namely *MXR1* and *MPP1* show a strong activating effect on P_{AOX1} activity in the absence of methanol while the response of *PRM1* expression was still observable but in the same strength as P_{GAP} -*PRM1*. Numerically speaking, *MXR1* and *MPP1* overexpression both resulted in roughly 75% of methanol induced P_{AOX1} activity while *PRM1* only was in the area of about 10% activation. It is intriguing to speculate about the possibility that the differences in the strength of activation under P_{CAT1}-regulated expression could potentially be explained by the regulation of the respective promoter in relation to the transcriptional state during the time of TF overexpression. While expression of P_{GAP} -controlled genes is constitutive, P_{CAT1} is only expressed after glucose depletion (Figure 4.2). This effect may be due to different causes: 1) P_{CAT1} could be catabolite repressed. 2) P_{CAT1} could be regulated by the nitrogen source as suggested by Rumjantsev *et al.* 2013 3) P_{CAT1} may also be regulated by ROS (radical oxygen species) stress arising in the stationary phase, as T. Kickenweiz was able to induce P_{CAT1} with H_2O_2 (personal communication). Whatever the regulatory mode of P_{CAT1} may be, this implies that any transcription factor present under conditions of glucose depletion will coincide with the cell's transcriptional machinery geared towards metabolization of non-glucose carbon sources. In this respect, the lack of signals for glucose repression under these circumstances could potentially pave the way for a beneficial effect of activators on MUT genes

Concerning the response of these three factors on methanol, an array of interesting observations could be made. During the methanol induction period of P_{CAT1}-*MXR1* I observed a sharp drop in fluorescence levels. Although the optical density at 600nm stayed roughly the same throughout the cultivation these strains had somehow lost their ability to be induced with methanol. Subsequent stamping of these cells on full medium agar plates supplemented with zeocin showed no growth whatsoever. It therefore seems that methanol induction of P_{CAT1}-controlled *MXR1* drastically decreases cell viability. As already mentioned above, strong overexpression of *MXR1* leads to a severe growth defect, possibly due to the derailment of the cell's transcriptional network. This explanation would also fit into the conceptual framework agreeing with the role of Mxr1p as a key regulator of methanol utilization genes (Lin-Cereghino 2006).

The P_{CAT1}-controlled expression of *PRM1* on methanol did not show an effect compared to the control construct. P_{CAT1}-expressed *MPP1* under methanol conditions is quite similar to P_{CAT1}-*MXR1*. Here we see again an inability to induce P_{AOX1} expression and a lack of growth on full medium agar plates supplemented with zeocin. Interestingly, in my initial screening P_{CAT1}-driven overexpression of *MPP1* did not lead to the complete inability of the *AOX1* promoter to be induced by methanol but the strength of induction was considerably weaker (data not shown). However, in further rounds of screening and rescreening P_{CAT1}-*MPP1* strains behaved very similarly compared to *MXR1* overexpression strains and resulted in no P_{AOX1} induction. Put into perspective, these findings point into the direction of a differential regulation of *MXR1* and *MPP1* on the one hand and *PRM1* on the other hand.

When methanol was added to a P_{GAP} -*MXR1* strains residing in a glucose-depleted state fluorescence did not increase at all while optical density only slightly increased, suggesting an inability of these strains to be induced with methanol. This finding may be explained with the negative effect of *MXR1* overexpression. P_{GAP} is a constitutive promoter and its activity on methanol still accounts for a third of the activity on glucose(Waterham 1997). The P_{GAP} -controlled expression of *PRM1* and *MPP1* upon methanol induction paints a very different picture. In both cases methanol induction lead to a strong activation with *MPP1* even exceeding P_{AOX1} activity of control strains P_{GAP} and P_{CAT1}. This positive effect of P_{GAP} -*MPP1* expression on methanol might indicate a transcription factor dosage effect which would make Mpp1p less affected by non-natural TF oversupply. At least some overexpression of the natural transcription factor showed positive effects – perhaps since the P_{GAP} is not extraordinarily strong in methanol medium. It would be interesting to study if a

similar effect can be observed when using a weakened P_{CAT1} or P_{DAS}. Important to mention in this context was the observation that in already available microarray data from *H. polymorpha, MPP1* was strongly up-regulated on methanol as sole carbon source (van Zutphen 2010). Also figure 4.5 shows that the native promoter of *MPP1* is strongly upregulated on methanol compared to naturally controlled p*PRM1* and P_{MXR1}. If naturally regulated Mpp1p is already available in high amounts, the addition of further molecules might possibly exert less intrusive effects on transcriptional regulation visible in the much higher level of methanol induced P_{AOX1} expression in P_{GAP} -*MPP1* strains.

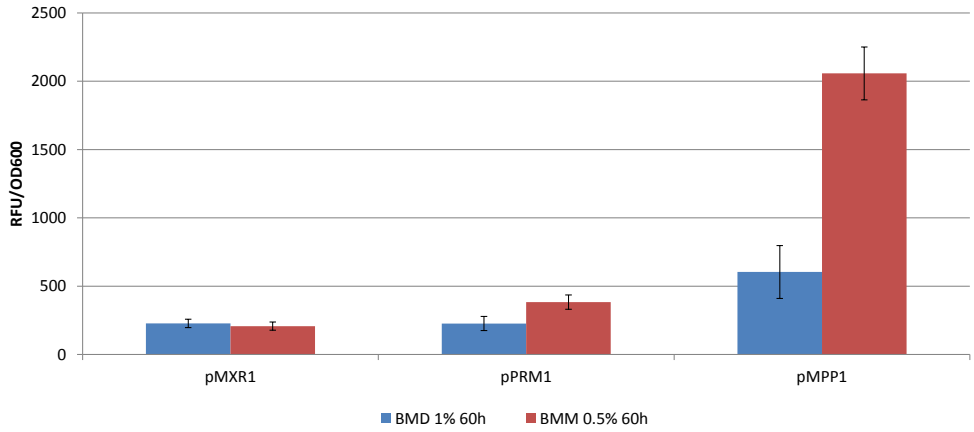

Figure 4.5.: sTomato expression level of native transcription factor promoters on glucose and methanol minimal medium. 1000bp upstream of the respective ORF of each transription factor was cloned upstream of sTomato. The cultivation of the transformed strains was done in the same manner as the overexpression strains and fluorescence was measured accordingly. Cultivation time was split in half with 60h on BMD1% and 60h on BMM 0.5% with replenishment of MeOH every 12 hours.

The effects of P_{CAT1}-regulated overexpression on methanol might reflect the importance that these factors have in regulating the expression of MUT genes. According to the severity of disruption of the natural transcriptional regulation we might be able to infer a higher degree of involvement in MUT gene regulation for *MXR1* and *MPP1*. The retained ability of *PRM1* strains to be induced by methanol points into the direction of possibly minor importance for overall regulation. It would therefore be conceivable that Prm1p regulates only a subset of genes, which will be less effected by external supply of large quantities of transcription factor. Otherwise its forceful overexpression through P_{CAT1}-conrolled overexpression would most likely also lead to the inability of methanol induction and severe growth defects.

In light of the data presented here I can conclude that excessive overexpression of some key transcription factors responsible for regulating methanol metabolism like Mxr1p and Mpp1p negatively affects the cell's ability to adapt and grow on methanol, possibly by de-railing the transcriptional machinery. It seems that for efficient protein production with this system the fine tuning of activator expression strength is crucial, since neither strong constitutive expression using P_{GAP} nor strong up-regulation under methanol induced P_{CAT1} of *MXR1* and *MPP1* allow proper function.

More importantly the findings presented here show that activation of a catabolite repressed promoter can in principle be achieved by providing the cell with a single transcriptional activator regulating that particular promoter. The moment in time at which activation takes place could theoretically be determined by choosing a promoter, which commences expression at a desired time point. We would therefore be able to trigger expression from a regulated promoter by providing the cell with a signal other than the natural trigger substance circumventing the need for its presence altogether. This allows us to combine both the positive features of the strong and tightly regulated expression of P_{AOX1} and the advantages of working in a methanol free environment. The glucose repressed nature of P_{CAT1} makes it a suitable candidate to retain inducibility of our expression system while rendering the addition of methanol redundant. Upon depletion of glucose P_{CAT1}-driven expression will commence and allow the expression of the transcription factor, which exerts its effect on the GOI promoter (gene of interest). Bioreactor cultivations may even further improve this derepressed system. After glucose depletion, cells may be further fed with a limited glucose feed, which is immediately taken up by the cells, resulting in derepressed conditions while still providing carbon for protein production. This will most likely not pose major problems since existing protocols were already developed and experimentally tested (Hartner 2008, Heyland 2010, Celik 2009).

In summary, the work presented here creates an auto-induction system for protein expression. Such concepts could further be extended to any set of promoter, associated activator and a second promoter which regulates the expression of this activator, as TAS (transcriptional amplification strategy) based systems already described in higher eukaryotes show (Liu 2008). This approach would only be limited by the availability of transcription factors regulating a promoter and the existence of a second promoter depicting the desired features. However, in the case of a lack of such parts synthetic biology tools could potentially modify or create them. Promoter sequence diversification, directed evolution and TALEN

technology all enable interesting and new possibilities to create parts for such expression systems either by modification of existing parts or by designing them from scratch (Mussolino 2012, Blount 2012, Khalil 2012, Ellis 2009).

4.2 Regulation of P_{MXR1}, P_{PRM1} and P_{MPP1}

A key experiment to interpret the effects seen more thoroughly would be the natural transcriptional regulation of *MXR1*, *PRM1* and *MPP1*. Therefore I cloned the natural promoters (1000 bp upstream of the gene) of *MXR1*, *PRM1* and *MPP1* genes upstream of an *eGFP* CDS. As can be seen in figure 4.5 and 4.6, P_{MXR1} and P_{PRM1} show similarly weak reporter fluorescence while P_{MPP1} is highly up-regulated upon methanol induction. This indicates that Mpp1p is specifically needed for methanol induction, whereas Mxr1p and Prm1p may have more general sensing/master regulator functions. These findings may also provide an explanation for the non-detrimental effect of P_{GAP} -*MPP1* overexpression. Strong overproduction of Mxr1p or Prm1p, that are naturally produced at low levels, may entail misfolding and degradation. In contrast, Mpp1p, which is naturally produced at rather high levels on methanol, might cope better with high production levels.

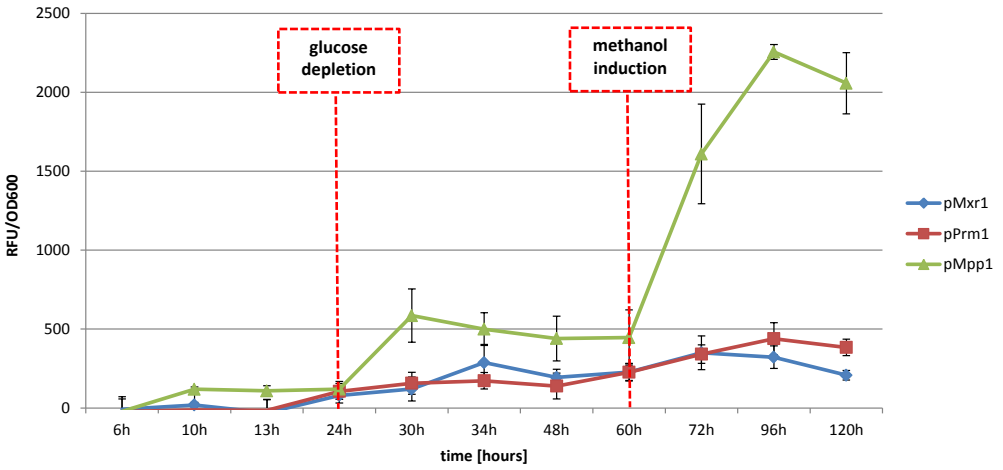

Figure 4.6.: Time-dependent regulation of methanol activator promoters on glucose and methanol minimal medium. The constructs from figure 4.5 were cultivated in the same manner as before and samples were taken at designated time points. Fluorescene, OD_{600} and glucose concentration were measured in all samples. The point of glucose depletion was reached approximately around hour 24 and cells further cultivated. Methanol induction started at hour 60 with periodical methanol replenishment (12h) and cells were further cultivated until hour 120.

In *P. pastoris* sTomato expression from native P_{MPP1} is roughly 20-fold upregulated upon methanol induction compared to its expression state on glucose (figure 4.6). Transcriptional data in the form of microarray studies by van Zutphen et al. 2010 comparing the transcriptomes on glucose vs. methanol showed a 394-fold upregulation of *MPP1* in *H. polymorpha* (van Zutphen 2010). In the P_{MXR1} screening depicted in figure 4.6 I observed that on methanol P_{MXR1} showed only 4-fold upregulation compared to glucose. Lin-Cereghino *et al.* 2006 witnessed that *MXR1* was constitutively expressed and only less than threefold upregulated on methanol with a P_{MXR1}-*BLA* reporter system (β-lactamase). In an *mxr1* knockout strain P_{MXR1}-*BLA* was less than fivefold up-regulated. According to this data the *MXR1* gene can be considered to be expressed constitutively. Comparing the data from Sasano *et al.* with sTomato expression from native P_{PRM1} in figure 4.6 we see very minor induction upon methanol similar to P_{MXR1}. Therefore P_{PRM1} can also be considered as constitutively expressed or derepressed when looking at data in figure 4.6. In *C. boidinii* Western Blot analysis of YFP tagged Trm1p (*Cb* homologue of Prm1) (expressed under control of the natural promoter) showed a constitutive expression although they saw an increase in signal on methanol.

However, transcriptional regulation is only one side of the coin. There still exists the possibility of post-translational regulation of positive methanol regulator expression, which would not be easily detected using microarray or RT-qPCR. Translated protein would in this scenario be post-translationally modified and therefore change its activity status as is the case for Mxr1. The data on transcriptional regulation of Mpp1 in *H. polymorpha* cannot completely exclude a putative seclusion/degradation mechanism. However since data gained in our reporter system seem to be in-line with published results from *H. polymorpha* this possibility seems rather unlikely.

For a transcription factor to exert its effect on promoter activation it has to be functionally expressed and be present in the nucleus of a cell (Zhai 2012). In the regulation of methanol activators it has been shown by Lin-Cereghino *et al.* that Mxr1p under glucose conditions is present in the cytosol and will be transported to the nucleus on gluconeogenic substrates like, glycerol, ethanol, oleate and methanol. (Parua 2012, Lin-Cereghino 2006) Sasano *et al.* 2008 reported that Trm1p in *C. boidinii* was localized to the nucleus regardless of the carbon source presented to the cell (Sasano 2008). The positive methanol activator Mpp1p was observed to localize to the nucleus upon methanol induction (Leao-Helder 2003).

Since all three methanol activators in *P. pastoris* are part of the zinc finger protein family and harbor DNA binding sequences, we can infer a direct binding to DNA. Although the nuclear localization of both Mxr1p and *Cb*Trm1p (homolog of *Pp*Prm1p) has been determined under diverse conditions unfortunately neither Lin-Cereghino *et al.* 2006 nor Sasano *et al.* 2008 did present any data on nuclear localization of Mxr1p and Trm1p (*Pp*Prm1p) under glucose depleted conditions. It would therefore not be unlikely that *Pp*Mxr1p is transported to the nucleus under derepressed conditions (especially as it is also localized to the nucleus on glycerol and ethanol).

On the other hand possible transport under overexpression conditions might not be directly mediated but just subject to overburdening the cellular transport machinery leading to uncontrolled nuclear import or maybe even export. Transport in this case would be unmediated and independent of the naturally regulated situation since the transport machinery is not capable of correctly regulating large amounts of transcription factor present due to overexpression. Complicating the regulatory picture is the possibility of post-translational regulation of these factors. At least in the case of *MXR1* Parua *et al.* were able to show that Mxr1p is in part regulated by phosphorylation on serine residue 215 in analogy to the *S. cerevisiae* homologue Adr1p. However, whether a similar post-translational regulation exists in either *PRM1* or *MPP1* remains to be determined.

4.3 Time-dependent P_{AOX1} activation by transcription factor overexpression

Previous results have shown that P_{CAT1} is glucose repressed and will be transcriptionally up-regulated upon glucose depletion (see figure 4.2). P_{CAT1} driven transcription factor overexpression will therefore start after glucose depletion. Also the constitutive nature of P_{GAP} controlled overexpression would theoretically mean that activation of P_{AOX1} already commences on glucose. To answer whether P_{GAP} -controlled overexpression results in P_{AOX1} activation already on glucose or whether depletion is necessary for activity I tested all strains in a time series depicted in figure 4.7

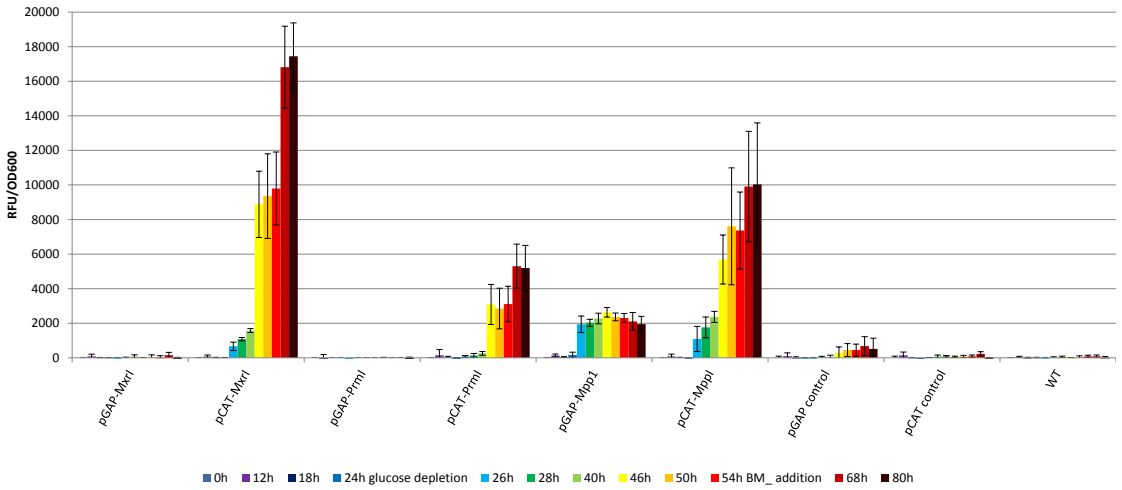

Figure 4.7.: Time-dependent overexpression of methanol activators on glucose minimal medium employing differently regulated promoters. Methanol activator strains were cultivated as a pre-culture and a defined volume used to inoculate the main culture at OD_{600} of 0.05. As shown in the graph fermentation time was 80h with addition of BM_ at time point 54h. Glucose concentration was determined via hexokinase enzymatic assay and glucose test strips.

Due to the high degree of P_{AOX1} activation and subsequent production of large amounts of sTomato in P_{CAT1}-*MXR1* and P_{CAT1}-*MPP1*, I speculated that weak leaky expression from P_{CAT1} might already be sufficient for P_{AOX1} activation and therefore expression would commence already on glucose. Taking a closer look at the time-dependent P_{AOX1} activation depicted in figure 4.7, I was able to observe that almost all sTomato protein from P_{AOX1} was produced after the point of glucose depletion. I observed that P_{AOX1} activity on glucose in all transcription factor overexpression strains is extremely low to absent but steadily rises after the time-point of glucose depletion, which was reached at hour 24. Therefore, it

seems that P_{AOX1} expression levels of up to 75% of methanol induced strains are entirely derived from carbon storage inside the cell and resources potentially liberated by autophagy since no addition of any carbon source was performed. To which extent storage molecules as for example fatty acids in the cell are degraded or recycled is not known to us, so we can only speculate which process might deliver the carbon necessary for protein production (Yurimoto 2011, Onodera 2005, Yamashita 2009). The degradation of fatty acid deposits might in part even increase P_{CAT1} driven expression due to the creation of ROS during the degradation process which in turn increase cellular stress levels (Jamieson 1998, Gurvitz 2001). However, the resources used to produce sTomato must be derived from the cells themselves. It would be conceivable that dead and lysed cells provide the carbon for still living cells but due to the OD_{600} values and growth curves obtained I consider this explanation as possible but rather less prominent compared to intracellular energy replenishment events. The fact that addition of BM_ medium (BM without any carbon source) still increases fluorescence protein expression after it has stayed constant might point into the direction of a limiting nutrient that gets replenished in BM_ medium. Since BM_ does not contain any carbon source it seems likely that carbon is most likely not the limiting substrate but rather nitrogen since a major ingredient in BM_ is YNB which contains large amounts of ammonium sulfate. Other sources present in YNB are salts, biotin and non-essential vitamins which however should normally not become limiting since they are present in excess and do not need to be replenished.

4.4 Effects of constitutive Mxr1 variant Mxr1^{c} on P_{AOX1}

Over the last two decades the research group of Prof. Elton T. Young was pivotal in advancing knowledge about the function and regulation of *ADR1*, a key transcriptional activator of *ADH2*, peroxisomal protein genes and of genes required for ethanol, glycerol, and fatty acid utilization in *S. cerevisiae* (Ratnakumar 2010, Kacherovsky 2008, Tachibana 2005, Young 2003, Blumberg 1988). They were able to show the existence of at least two, possibly even three, phosphorylation sites in Adr1p which contribute to its transcriptional activity. Even after almost three decades of research the exact mechanism by which Adr1p is activating fatty acid gene transcription remains to be elucidated. The complex regulation of Adr1p and indeed also its homologues in other methylotrophic yeasts such as *H. polymorpha, C. boidnii and P. pastoris* makes it very challenging to propose a working model of regulation possessing adequate predictive power (Parua 2012, Lin-Cereghino 2006, Kumar 2012, Kranthi 2010, Sasano 2012). While we are unable to understand the exact mechanism

at this point, we do know that phosphorylation of a serine residue in the binding motif of a regulatory 14-3-3 protein and possibly a second serine residue close to the zinc finger DNA binding domain play a crucial role in its ability to induce transcription (Parua 2012).

In *P. pastoris* the expression of Mxr1p dependent genes is regulated by the presence of different carbon sources. Parua *et al* 2012 were able to show that a 14-3-3 protein is interacting with Mxr1p via a phosphorylation at serine residue 215. They observed a carbon source dependent phosphorylation status of Mxr1p which corresponds to its activation / inactivation. Phosphorylated Mxr1p was able to bind to the 14-3-3 protein and thereby inhibit transcription of Mxr1-dependent genes (Parua 2012). According to this model restricting the binding of 14-3-3 to Mxr1p by mutating the serine residue should allow constitutive expression of Mxr1-dependent genes

I therefore proceeded to construct a double point mutant of two of these important serine residues by mutating both serine residues to alanine. The necessary information for choosing the residues was taken from Lin-Cereghino *et al.* and Parua *et al.* and personally verified by p-blasting *Sc*Adr1p with *Pp*Mxr1p. Figure 4.8 shows the multiple sequence alignment with both conserved serine residues. In figure 4.9 the Sanger-sequenced point mutations in the double mutant are shown.

```
AdrI            RENSNTFSVIQRTPDGKIITTNNNMNSKINKQLDKLPENLRLNGRTPSGK
MxrI            -----TFGSTRQSPE---------------DQSPPVPKELSFNGTTPSGK
                     **.  :::*:                .*   :*::* :** *****

AdrI            LRSFVCEVCTRAFARQEHLKRHYRSHTNEKPYPCGLCNRCFTRRDLLIRH
MxrI            LRLFVCQTCTRAFARQEHLKRHERSHTKEKPFSCGICSRKFSRRDLLLRH
                ** ***:.*************** ****:***:.**:*.* *:*****:**

AdrI            ---------------------------TRRASFSAQSASSYAL-------
MxrI            DQDPLISPRMQLFNDSNHHVNNLFDLGLRRASFSAVSGNNYAHYVNNFQQ
                                           ******* *...**
```

Figure 4.8.: Multiple sequence alignement of *S. cerevisiae* Adr1p and *P. pastoris* Mxr1p. For the multiple sequence alignement with ClustalW we used Genbank accsession number FR839631.1 for Mxr1p and U28414.1 for Adr1p. The blue highlighted stretch contains serine 34 whereas the pink highlighted stretch marks serine 215.

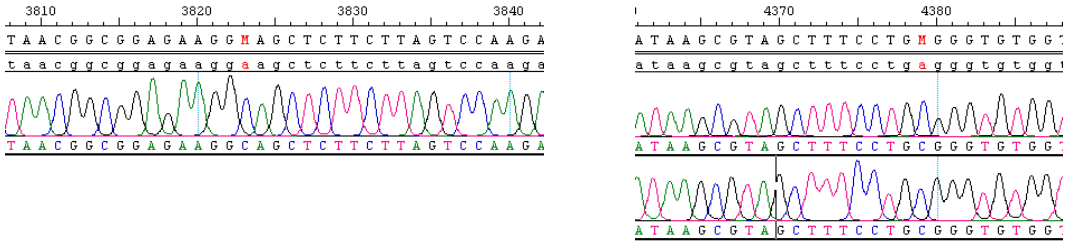

Figure 4.9.:. Sanger , ist sanger sueqening Mxr1c variant. Both exchanges from serine to alanine have been incorporated into primers and were joined together by subsequent oePCR. Both serine34 and serine215 residues have been mutated to alanine in the consitutive mutant (*MXR1^c*) by changing UCC into GCC and UCA into GCA for S215 and S34 respectively. The top row represents the wildtype *MXR1* and the bottom rows in colour the sequenced *MXR1^c* mutant.

On glucose I could observe that as with the wildtype P_{GAP} -*MXR1*, the mutant was not showing P_{AOX1} activation in presence of glucose as visible in figure 4.8. The P_{CAT1}-controlled expression of *MXR1*c did not show any difference of growth or expression behavior compared to wildtype P_{CAT1}-Mxr1.

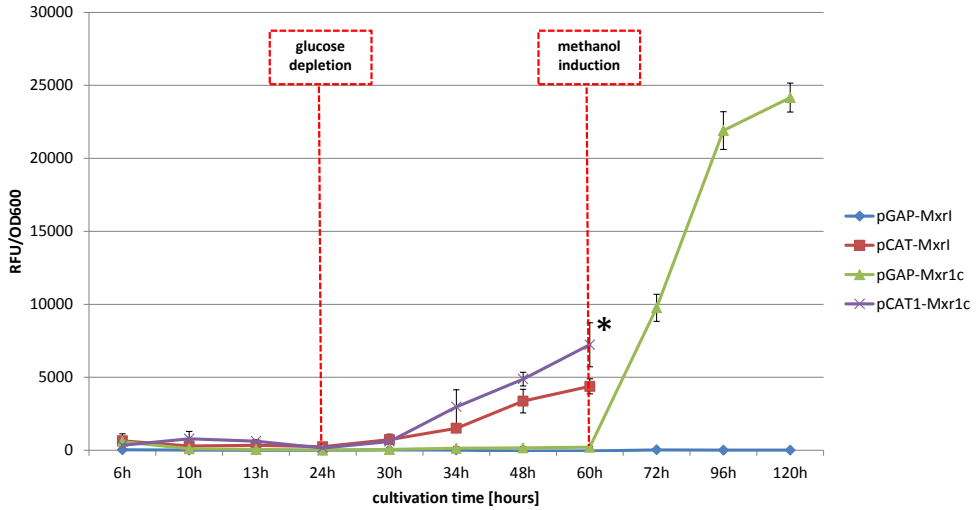

Figure 4.8.: Overexpression of native *MXR1* and double point mutant *MXR1c* on glucose and methanol employing differently regulated promoters. The cultivation was run for 60h on BMD1% and induced with methanol at hour 60. Replenishment of methanol in the form of BMM was done in 12-hour cycles and preceded to hour 120. Cells were cultivated in a single deep-well-plate with each contsruct occupying 7 wells. Arithmetic means and standard deviations of 7 wells are shown in the graph. *For P_{CAT1}-*MXR1* and P_{CAT1}-*MXR1c* values after methanol induction are not shown since growth deficiencies of these cells results in OD_{600} unsuitable for normalization.

Interestingly, if we take a closer look at the difference between methanol-induced P_{GAP} -*MXR1* and P_{GAP} -*MXR1c* we are able to observe differences in the strength of P_{AOX1} promoter activation. After methanol induction I was not able to induce P_{AOX1} expression in P_{GAP} -*MXR1* strains, while in P_{GAP} -*MXR1c* under the same circumstances P_{AOX1} could be activated to levels comparable to control strain levels. Important to mention is that during the initial screening of P_{GAP} -*MXR1c* I observed far higher P_{AOX1} activation, which was drastically weaker in subsequent screenings and rescreenings. Most likely similar phenomena as in the case of P_{GAP} -*MXR1* are present, which would explain the decrease in fluorescence.

Upon methanol induction of P_{CAT1}-*MXR1c* overexpression strains I observed essentially the same response as already described in the wildtype variant of *MXR1*. The cells were loosing OD_{600} (see figure 4.9) while fluorescence did not increase at all. The OD_{600} values fell out of the linear range and I was not able to plot fluorescence after methanol induction.

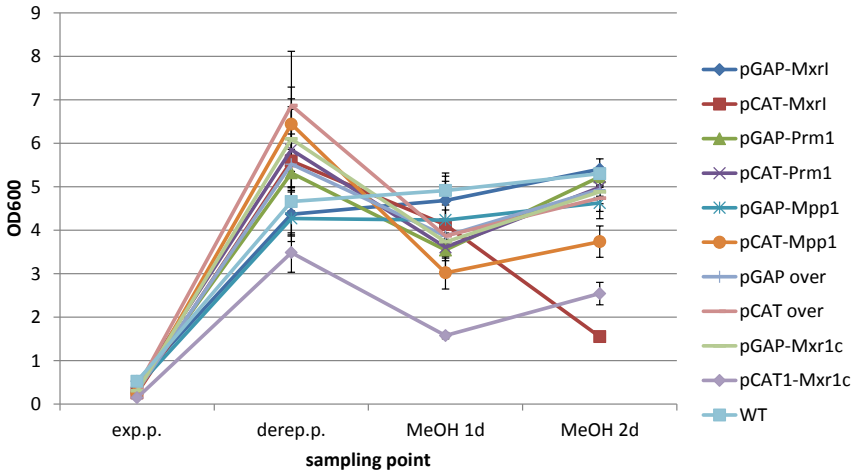

Figure 4.9.: Optical density at 600nm of different transcription factor overexoression strains in glucose and methanol minimla medium. Cells were cultivated in a single deep-well-plate with each contruct occupying 7 wells. Arithmetic means and standard deviations are shown in the graph. The cultivation was run for 60h on BMD1% and induced with methanol at hour 60. Replenishment of methanol in the form of BMM was done in 12-hour cycles and preceded to hour 120. The sampling time points for exponential phase, derepressed phase, MeOH 1d and MeOH 2d were performed at hour 12, 60, 84 and 108 respectively.

It seems therefore that the constitutive variant of *MXR1,* termed *MXR1c* is at least in part able to salvage the detrimental effect of wildtype *MXR1* overexpression observed on methanol if the applied promoter is not too strong. Therefore an experiment where *MXR1* is expressed by a weak derepressed promoter such as P_{PEX5} or a weakend P_{CAT1} would be interesting.

4.5 Microarray analysis of transcription factor overexpression strains

4.5.1 Bioreactor cultivation of methanol activator overexpression strains

The previous experiments had shown that three transcription factors Mxr1p, Prm1p and Mpp1p are single-handedly sufficient to activate P_{AOX1} under derepressed conditions. It would be highly interesting to see if these factors have overlapping or unique sets of targets genes and from this information deduct clues for further engineering strategies. Therefore I planned to cultivate the strains, isolate the RNA and perform microarray hybridization studies. For elimination of variations arising from culture conditions and additionally to test our system under different conditions, I performed bioreactor cultivations. The figures 4.10-4.13 show the growth curves and the fluorescence values obtained from fermenter cultivated strains P_{CAT1}-*MXR1,* P_{CAT1}-*PRM1,* P_{CAT1}-*MPP1* and P_{CAT1}-*eGFP* strains. The blue arrows in all figures indicate the time point of sampling for RNA isolation which corresponded to four different stages further described below.

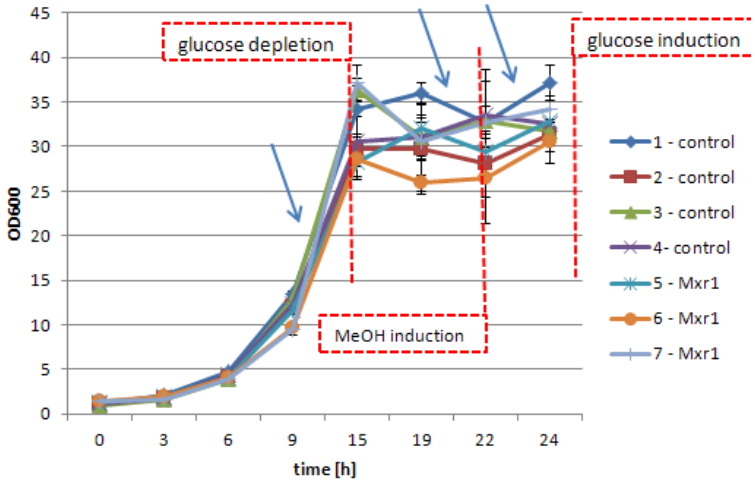

Figure 4.10.: Growth curve of fermenter cultivations of *P_{CAT1}-MXR1* and *P_{CAT1}-eGFP* (control) overexpression strains. The two strains *MXR1* and control shown here were run in four parallel bioreactors simultaneously but due to technical difficulties (pH and O2 electrode failure) only three cultivations from *P_{CAT1}-MXR1* and four from *P_{CAT1}-eGFP* (control) could be used for analysis. Glucose concentrations were measured using an hexokinase enzymatic kit and glucose control strips. The red panels indicate the time point of glucose depletion, methanol induction and glucose addition. The blue arrows correspond to the time point of RNA sampling. To assess possible derepression effects on *P_{AOX1}* a small colony of *P_{CAT1}-eGFP* control strain was used.

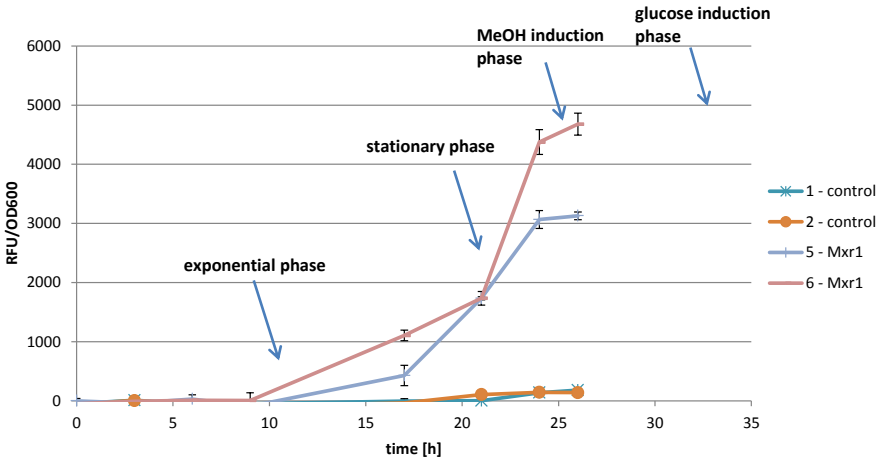

Figure 4.11.: OD_{600} normalized fluorescence of fermenter cultivated overexpression strains *P_{CAT1}-MXR1* and *P_{CAT1}-eGFP* (ctrl). The fluorescence curves shown here represent the fermenter cultivations that were chosen to isolate RNA samples for further processing. Time values for glucose depletion and methanol induction can be taken from figure 4.10. The blue arrows again correspond to the time point of RNA sampling. Arithmetic means and standard deviations shown in the graph were calculated from triplicate measurements. Due to time limitation we were unable to collect fluoresence data from the sampling point of glucose reintroduction.

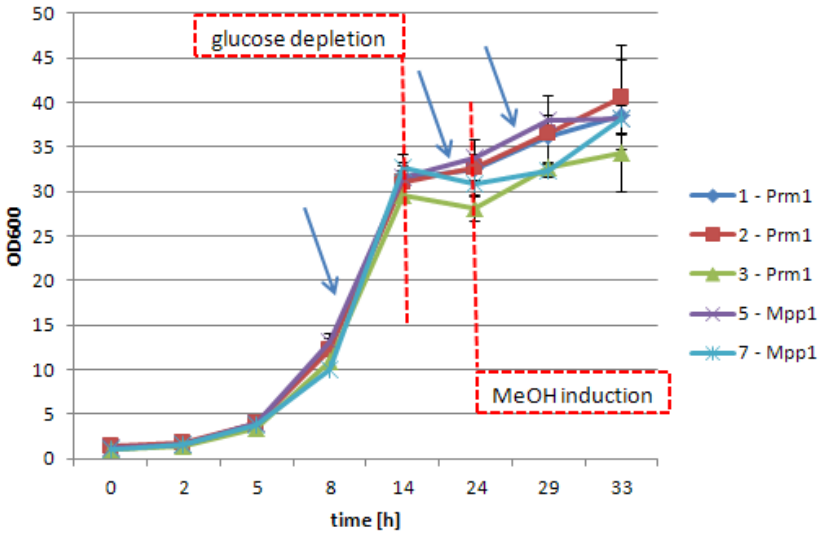

Figure 4.12. Growth curve of fermenter cultivations of *P_{CAT1}-PRM1* and *P_{CAT1}-MPP1* overexpression strains. The two strains *PRM1* and *MPP1* shown here were run in four parallel bioreactors simultaneously but due to technical difficulties (pH and O2 electrode failure) only three cultivations from *P_{CAT1}-PRM1* and two from *P_{CAT1}-MPP1* could be used for analysis. Glucose concentrations were measured using an hexokinase enzymatic kit and glucose control strips. The red panels indicate the time point of glucose depletion and methanol induction. The blue arrows correspond to the time point of RNA sampling. Arithmetic means and standard deviations shown in the graph were calculated from triplicate measurements.

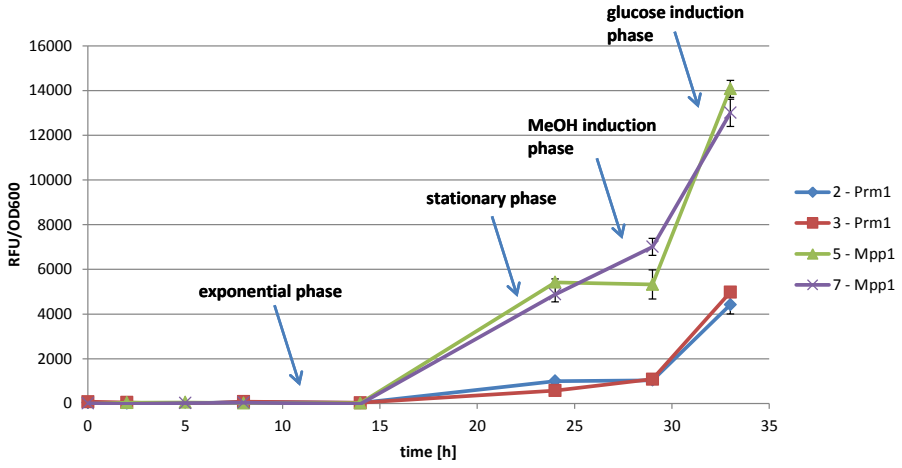

Figure 4.13.: OD_{600} normalized fluorescence of fermenter cultivated overexpression strains *P_{CAT1}-PRM1* and *P_{CAT1}-MPP1*. The fluorescence curves shown here represent the fermenter cultivations that were chosen to isolate RNA samples for further processing. Time values for glucose depletion and methanol induction can be taken from figure 4.12. The blue arrows again correspond to the time point of RNA sampling. Arithmetic means and standard deviations shown in the graph were calculated from triplicate measurements.

For the bioreactor cultivations I was using the in-house DAS Gip fermenter system with eight 1,5L bioreactors running simultaneously. Standard in-house settings for the cultivations were used (see material and methods) and RNA sampling was performed at four different time points and stored for later processing. The sampling points corresponded to four different cellular states and were taken in the exponential phase on glucose (10h), in the de-repression phase (glucose depletion – 20h), in the methanol growth phase (24h) and in the growth phase after reintroduction of glucose into the fermenter (28h). Since fermenter cultivations are laborious and time-consuming and to keep the number of arrays for hybridization at a reasonable level I decided to run fermenter cultures only of the strains showing a large effect on P_{AOX1} activation – all three transcription factors controlled by P_{CAT1} and the corresponding *eGFP* control strain. As can be seen in figures 4.10 and 4.12 growth under glucose conditions is very homogenous in all four overexpression strains while minor deviations occur upon glucose depletion. Cells reached a final OD_{600} of roughly 30 units which runs in accordance to already published data on fermenter cultivations with 1% glucose on minimal medium (Hartner 2008).

Interestingly, we saw a modest increase of OD_{600} in all strains tested indicating growth on methanol as carbon source. Also fluorescence protein production in all strains was increasing after methanol induction. This was especially intriguing since the P_{CAT1}-*MXR1* strains cultivated in deep-well plates were not able to grow on methanol observable in a decrease in optical density (see figure 4.9). Therefore it seems that the response to methanol in positive methanol activator overexpression strains is dependent on the cultivation conditions. However, cell viability via e.g. survival plating was not checked and therefore discussions can only be made on the basis of optical density and P_{AOX1} reporter fluorescence. Since completely different time-scales were applied in deep-well cultivations and fermenter cultivations we might not be able to compare overexpression strain behavior. In DWPs we examined an induction period of 60 hours in contrast to the fermentation data shown here which extend a time range of only 12 hours. Therefore it might be conceivable that initial cell response to methanol is different but when examining longer cultivation times we would be able to observe similar behavior on methanol for both settings. Examining the levels of P_{AOX1} activation obtainable by repeating the DWP cultivation protocol also on the larger fermenter scale would be interesting for the sake of comparability.

If we compare the time point of glucose depletion and the first sTomato signal we see a delay that corresponds most likely to the cell transcription adjustment and folding of sTomato. This behavior has already been observed in DWP cultivations (see figure 4.7). Simon et al 1991 showed a delay in p*CTA1* activation upon glucose depletion. A similar mechanism might also be present in *P. pastoris* P_{CAT1} (Simon 1991).

4.5.2 RNA isolation and microarray hybridization of activator overexpression strains

The RNA samples frozen in RNase-later-solution were used to isolate RNA at a later point and checked for integrity with agarose gel and capillary electrophoresis. Sample names that appear in gels and capillary electrophoresis runs in figures 4.14 and 4.15 correspond to the numbering in table 4.2. If we have a closer look at the RNA samples on the agarose gel in figure 4.14 we can observe that the 26S rRNA is roughly twice the intensity of the 18S rRNA. Absolute amounts of RNA running in the single lanes are not completely constant throughout the gel, so therefore a lane-to-lane comparison is demanding. However, the intensity of the two bands in one lane follow the above mentioned characteristic (Assessing RNA Quality by Melanie Palmer and Ellen Prediger – LifeTechnologies). Since RNA forms secondary structures under non-denaturing conditions and therefore different surface areas of EtBr interaction this 2:1 ratio might not always be perfectly apparent. The same holds true for size comparison, although this didn't pose problems in our setting – clear and distinct band can be seen. For size comparison the capillary electrophoresis runs are better suited since in this case RNA standards were available.

Table 4.2.: RNA isolations of fermenter cultivated samples with purity criteria, names and respective internal number allocation. The names appearing here refer to the four P_{CAT1} overexpression strains *MXR1*, *PRM1*, *MPP1* and control strain (*eGFP*) under glucose, glucose depletion, methanol and glucose reintroduction conditions. The numbers in the strain and condition column refer to the duplicate of the respective condition. Spectrophotometric values were obtained by Nanodrop 2000c measurements.

Number	Strain and Condition	Amount [ng/µl]	Ratio 260/280	Ratio 260/230
1	control glucose I	384.8	2.18	2.35
2	control derepression I	447.2	2.14	2.31
3	control MeOH I	293.4	2.19	2.32
4	control Glucose induction I	381	2.18	2.36
5	control glucose II	682.1	2.23	2.21
6	control derepression II	480.5	2.11	2.06
7	control MeOH II	654.8	2.21	2.24
8	control Glucose induction II	763.5	2.24	2.29
9	Mxr1 derepression I	328.9	2.19	2.32
10	Mpp1 derepression I	417.3	2.17	2.3
11	Prm1 derepression I	360.6	2.16	2.0
12	Mxr1 derepression II	580.2	2.21	2.09
13	Mpp1 derepression II	796.3	2.22	2.29
14	Prm1 derepression II	999.8	2.19	2.3

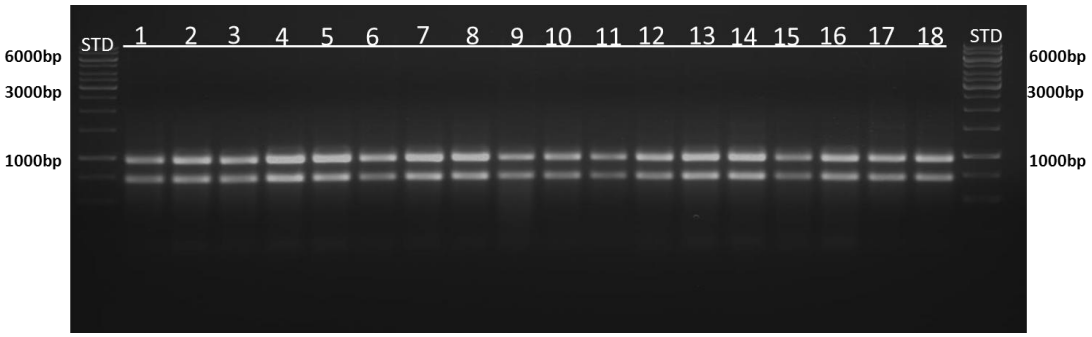

Figure 4.14.: Agarose gel showing isolated RNA samples as numbered in table 4.2. Concentration and photometric ratios 260nm/280nm and 230nm/260nm were determined and 1µg was used for assessing RNA integrity on a 1% agarose gel. The two bright bands visible correspond to 26S and 18S rRNA and at the faint band visible at the bottom of the gel most likely represents the 5.8S rRNA of *P. pastoris*. Ladder size used for referencing RNA size cannot be used due to the different nucleic acid species (DNA ladder and RNA sample). Numbers 15-18 depict additional isolations not further processed.

RNA quality can also be assessed using spectrophotometric ratios of isolated RNA samples. RNA has its absorption maximum at around 260nm and highly pure RNA shows 260nm/280nm ratios of around 2.1. Possible contaminations can be evaluated by checking 260nm/230nm ratios, which fall in the range of 2.0-2.2 for pure RNA. Spectrophotometric values lower than these values indicate contaminations with DNA, protein or phenolic compounds stemming from the extraction procedure (Assessing RNA Quality by Melanie Palmer and Ellen Prediger – LifeTechnologies). Spectrophotometric determinations aside the "gold standard" for RNA quality and integrity assessment is capillary electrophoresis analysis using for example the Agilent Bioanalyzer 2100. From the electrophoresis runs detailed information about RNA concentration, integrity and purity were gained. The outcome of this analysis is shown in figure 4.14 and 4.15. As can be seen from these figures the quality of isolated RNA was very good, visible in distinct and clear bands without the occurrence of smear. Also the capillary electrophoresis runs showed very low to zero degradation of RNA and an expected size distribution of measured RNA species as can be seen in the base line running at around zero with distinct peaks at 45sec, 40 sec and 23 sec corresponding to 26S rRNA, 18S rRNA and 5.8S rRNA respectively. Overall the quality criteria for the proceeding reverse transcription and hybridization were all fulfilled.

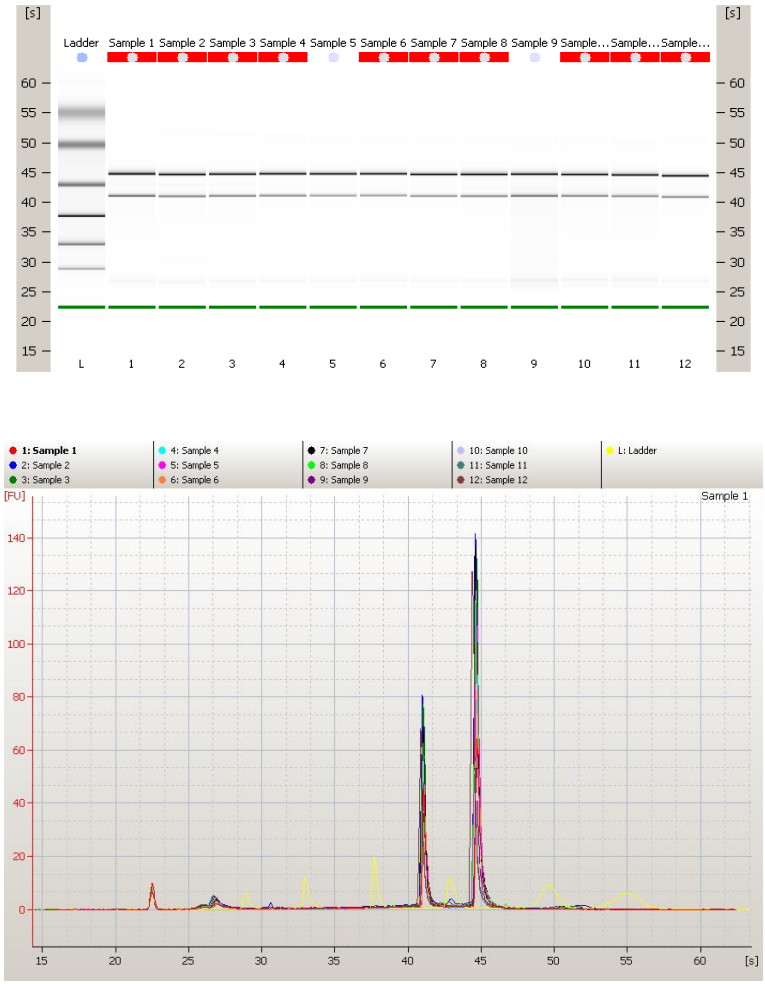

Figure 4.15.: Capillary Electrophoresis of samples listed in table 4.2 using Agilent Bioanalyzer 2100. The upper picture shows the capillary electrophoresis run imaged by Agilent Bioanalyzer 2100 expert software. The three bands clearly visible depict the 26S and 18S rRNA and the fainter band eluting at around 25 seconds shows the 5.8S rRNA. In the lower picture we see an overlay of 14 runs (colored curves) including the ladder (yellow) with corresponding elution times. Samples 13 and 14 were measured in the same manner on a second chip and showed the same behavior as sample 1-12. (Data shown in the Appendix)

Reverse transcription and microarray hybridization was performed out-of-house at a contract partner at the ZMF (Zentrum für medizinsiche Forschung) with the assistance of Karin Wagner. For reverse transcription and hybridization Affymetrix GeneChip 3'IVT Express Kit and Affymetrix Pichia Arrays, Gene Chips and fluidics station was used. Scanning and data processing was also performed by Affymetrix Scanner and Geneexpression Console (see material and methods). Table 4.3 and 4.4 show a synopsis of all array conditions and in the event of a successful hybridization the conditions selected for comparison.

Table 4.3.: Selection of transcription factor overexpression strains and conditions for microarray hybridization. Samples from figure 4.11 and 4.13 taken at different time points throughout the fermentation were used to isolate RNA. Integrity of RNA samples was checked via agarose gel and capillary electrophoresis (see table 4.14 and figure 4.15).

Array Number	Strain	Condition
1	**Control strain** (wildtype strain with control plasmid [same marker, expression cassette but no transcritption factor coexpressed])	Glucose (duplicate)
2		
3		Derepressed (duplicate)
4		
5		Methanol (duplicate)
6		
7		Glucose addition after MeOH addtion (duplicate)
8		
9	**Mxr1** overexpression (under CAT1 promoter allowing derepressed pAOX1 expression)	Derepressed (duplicate)
10		
11	**Prm1** overexpression (under CAT1 promoter allowing derepressed pAOX1 expression)	Derepressed (duplicate)
12		
13	**Mpp1** overexpression (under CAT1 promoter allowing derepressed pAOX1 expression)	Derepressed (duplicate)
14		

Table 4.4.: Microarray conditions selected for comparison. Data analysis will be performed by Dr. Gerhard Thallinger using an automated *P. pastoris* analysis pipeline.

Condition 1		Condition 2
Controls		
Ctrl glucose		Ctrl derep
Ctrl glucose		Ctrl MeOH
Ctrl glucose	<-->	Ctrl glu indu
Ctrl MeOH		Ctrl derep
Ctrl MeOH		Ctrl glu indu
Specific Overexpressors		
Ctrl derep		Mxr1 derep
Ctrl derep		Prm1 derep
Ctrl derep	<-->	Mpp1 derep
Ctrl MeOH		Mxr1 derep
Ctrl MeOH		Prm1 derep
Ctrl MeOH		Mpp1 derep
Collective Overexpressors		
Mxr1 derep		Prm1 derep
Mxr1 derep	<-->	Mpp1 derep
Prm1 derep		Mpp1 derep

Before we can proceed with microarray hybridization, the RNA samples needed to be labeled and reverse transcribed into cDNA. Transcription was performed with a poly-T primer using Affymetrix GeneChip 3'IVT Express Kit and the transcripts checked again with capillary electrophoresis. The results of this transcription can be seen in figure 4.16. What was striking to see is that although the same amount of RNA was used for all samples, two groups according to reverse transcription efficiency could be observed. The samples isolated under glucose, methanol und glucose reintroduction conditions fell into one group while de-repressed samples of the control strain, Mxr1, Prm1 and Mpp1 fell together into the second group. The major difference between these two groups was present in two respects:

On the one hand reverse transcription efficiency of derepressed samples (e.g. the total amount of labeled cDNA) was roughly half of the samples on glucose and methanol. The decreased cDNA content can be seen in the determination of total cDNA amount and in the electrospherogramm in form of the area under the curve. This observation was consistent with all samples tested. We can only speculate about the reasons for this trend; however the most striking difference between samples on glucose/methanol and derepressed conditions is the availability of carbon source enabling growth of the cells. The in principle feasibility to analyze a yeast transcriptome in near stationary phase has been already shown by Martinez 2004, Radonjic 2005, Yiu 2008 and Aragon 2006. Downregulation of transcription and translation upon glucose depletion has already been reported suggesting that the ratio of mRNA to largely abundant rRNA might change. A smaller mRNA pool would also mean a lowered rate of poly-T primer binding events and subsequently lower transcription from mRNA.

The second major difference that can clearly be seen in figure 4.16 and is marked with a dotted circle in all samples is a peak between 3000nt and 4000nt that is present in all samples but due to higher overall amounts clearly visible only in derepressed samples. The absolute amounts of this peak however are roughly the same (due to different scaling in Figure 4.16 this is not immediately apparent). According to the Affymetrix GeneChip 3'IVT Express Kit the size range of cDNA is in the range of 250nt-5500nt with the majority of cDNA in the range of 600nt-1200nt. The peak at around 200nt also present in all samples is frequently found in cDNA samples from diverse sources. However, the peak between 3000 and 4000nt is reminiscent of the 3800nt long 26S rRNA present in the yeast *S. cerevisiae* (Ribosomal RNA Sizes-Life technologies). The kit employed to reverse transcribe

RNA samples does not use RNase to eliminate remaining RNAs after transcription but selectively purifies biotinylated polyT primer transcribed cDNA with Streptavidin beads. Accidentally co-purified 26S rRNA would therefore be present in the sample and visible in the Bioanalyzer runs. If this is actually the case we would likely not see huge effects on microarray hybridization since rRNA is not taking part in sequence specific microarray binding and can only interfere through unspecific annealing events. This could potentially unfold through blocking of certain oligonucleotide probes on the chip or by binding to transcribed cDNA resulting in its inability to hybridize with the microarray.

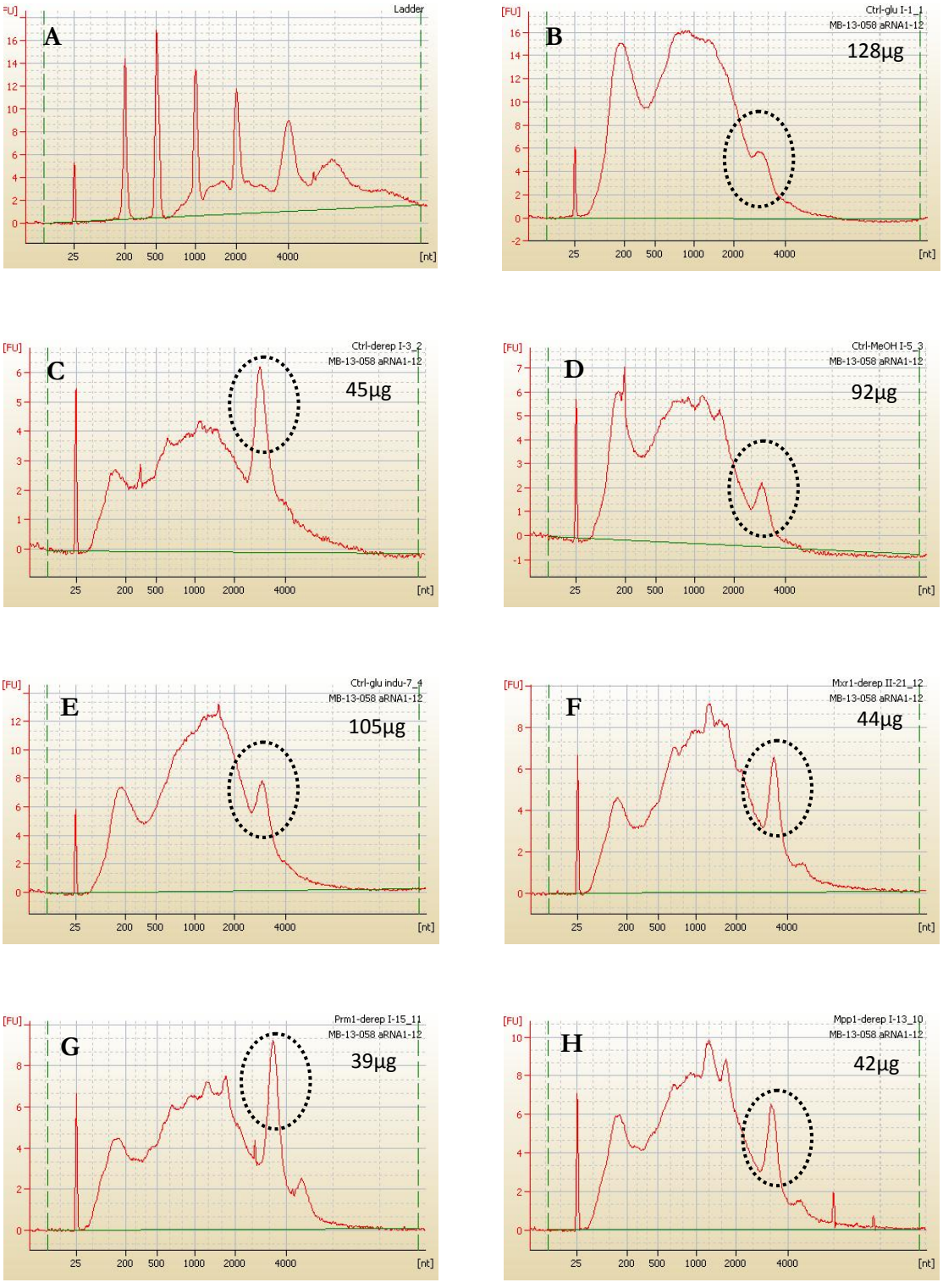

Figure 4.16.: Capillary Electrophoresis of reverse transcribed cDNA using Agilent Bioanalyzer 2100. For reverse transcription Affymetrix GeneChip 3'IVT Express Kit was used and performed according to the protocol. cDNA was subsequently applied to a gene chip and run again to check for concentration, integrity and purity. The total amount of DNA was measured and can be found in the boxes on the right side of each electropherogramm. The black doted circles indicate possible RNA contaminations in the cDNA sample. **A** is the ladder, **B-H** represent the same order as number 2-4 and 8-11 in table 4.2.

If we take a closer look at the signal distribution throughout the chip we see homogenous conditions with only slight differences in intensities in the case of derepressed samples. As the intensity box plots in figure 4.17 and figure 4.18 show the derepression group has slightly lower signal intensities. The lower box-plot on the previous page in figure 4.17 visualizes the ratio of the intensity of each probe to the median probe intensity across all arrays. Since the middle bar in the derepression samples is not zero but slightly smaller than zero this typically indicates a lower raw intensities. However through normalization these lower intensities can be somewhat minimized. Although, due to the overall lower efficiency in reverse transcription more cDNA was used for hybridization, the signal intensity still seems to be lower in the derepression group.

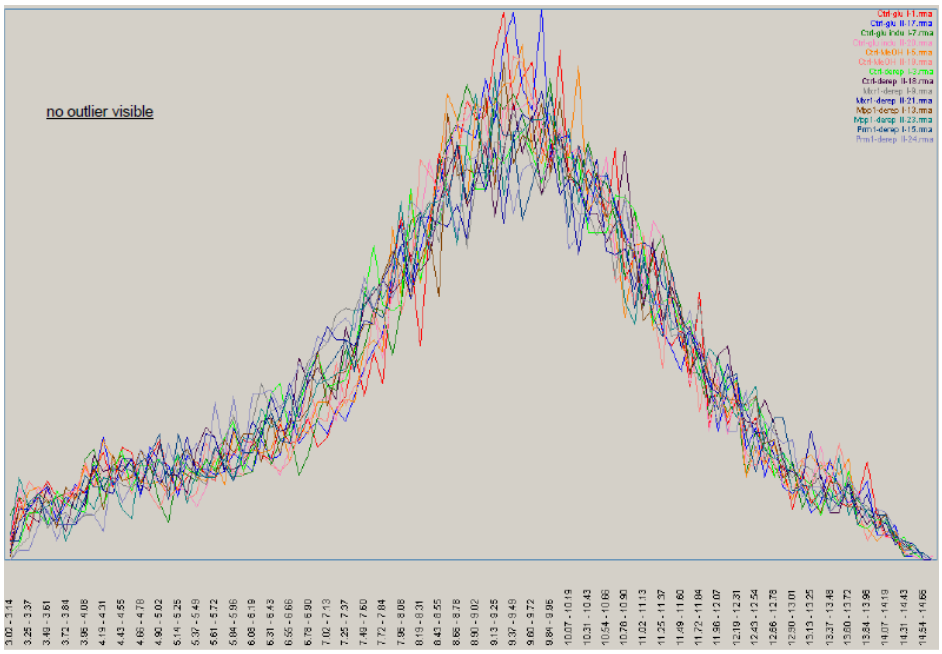

Figure 4.16.: Signal intensity histogram showing all 14 hybridizations. Distribution of signal intensities shows uniform behavior without any outliers. All arrays show a very similar homogenous pattern with no apparent phase shift.

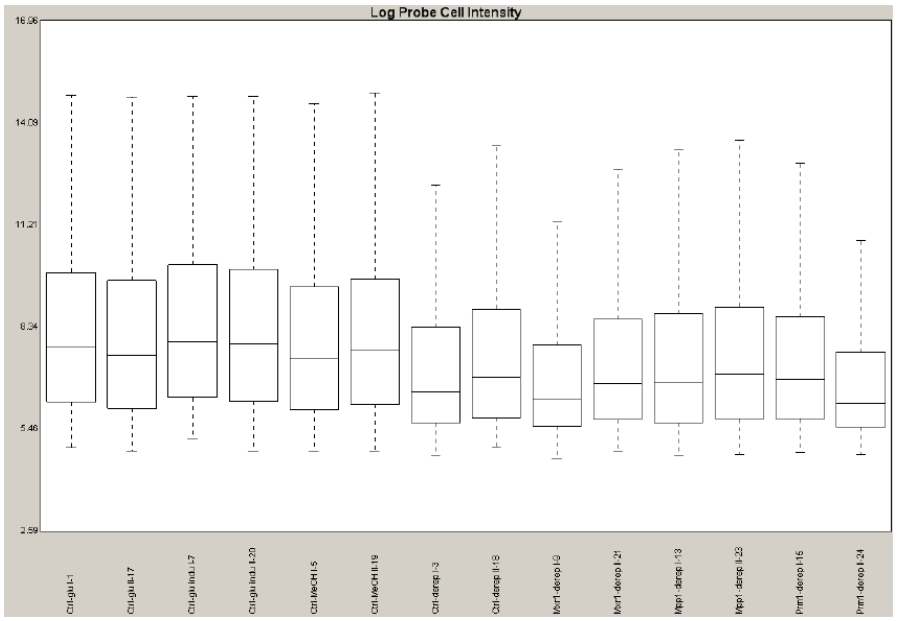

Figure 4.17.: Feature intensity Box-Plots before normalization. The median signal intensity and their respective deviation are shown for all 14 arrays hybridized.

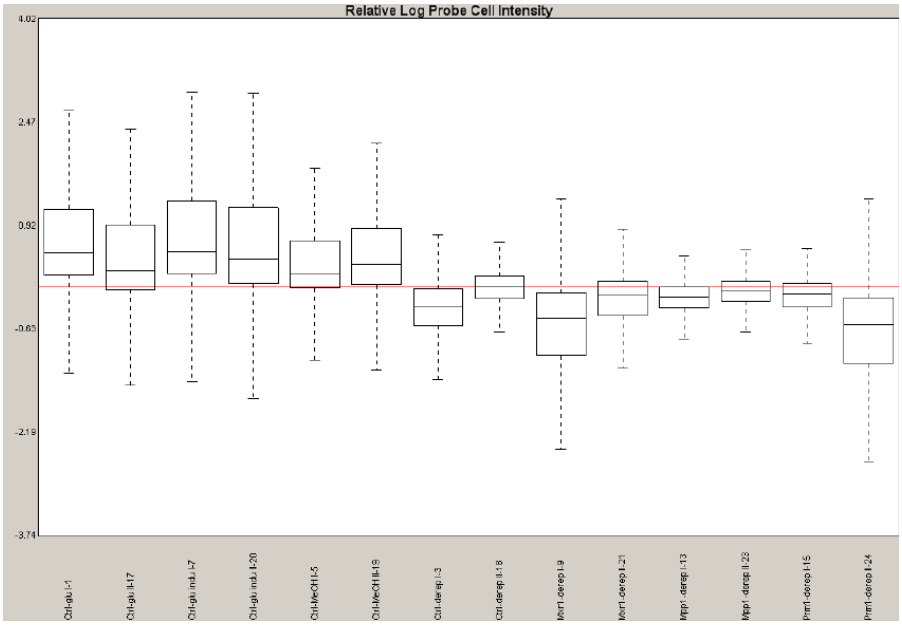

Figure 4.18.: Relative Feature Intensity Box Plot. The Relative Log Probe Cell Intensity is the ratio of the intensity of each probe to the median probe intensity across all arrays prior to summarization / normalization. It compares the distribution of intensities of each array to the others and identifies arrays with divergent probe intensity.

Figure 4.19 shows the microarray signals spiked with labeled samples to assess the accurate hybridization. Two types of spiking are used in the evaluation of hybridization. Bacterial Spikes termed CreX, BioD, BioC and BioB by Affymetrix were used and show in case of proper hybridization signal decreases in that order. Should the single spikes appear in a different order according to their signal intensity then the hybridization process was not efficient. In our case the given order of signal intensity could be achieved and therefore the criterion of proper hybridization was fulfilled.

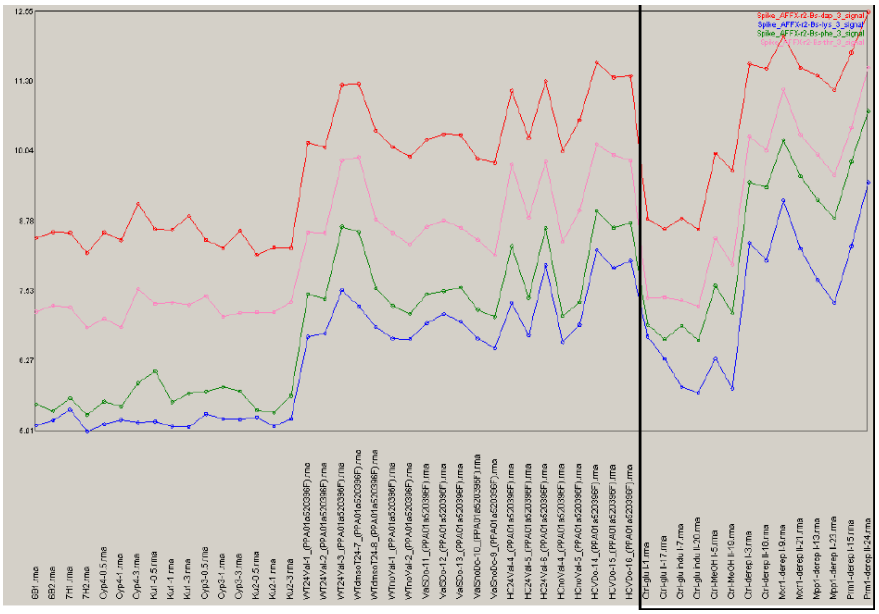

Figure 4.19.: QCcheck using Affymetrix Genexpression Console. Spike samples labeled BioB, BioC, BioD and CreX represent spike controls to monitor hybridization. Bacterial spikes showed the expected rank order which indicates a proper hybridization. CreX: 13-13,5 - BioD: 12,5-10 - BioC: 12-9,5 - BioB: 10,5-8.

A very similar approach was developed by Affymetrix to follow the efficiency of labeling. Spike samples for labeling controls are termed lys, phe, thr and dap and are subject to dilution – lys (1:100000), phe (1:50000), thr (1:25000) and dap (1:6667). Signal intensities measured should again follow in this order. If this is not the case labeling of the probes did not work efficiently. Again due to the correct order of signal measurement labeling of RNA molecules could be identified as functional as observable in figure 4.20.

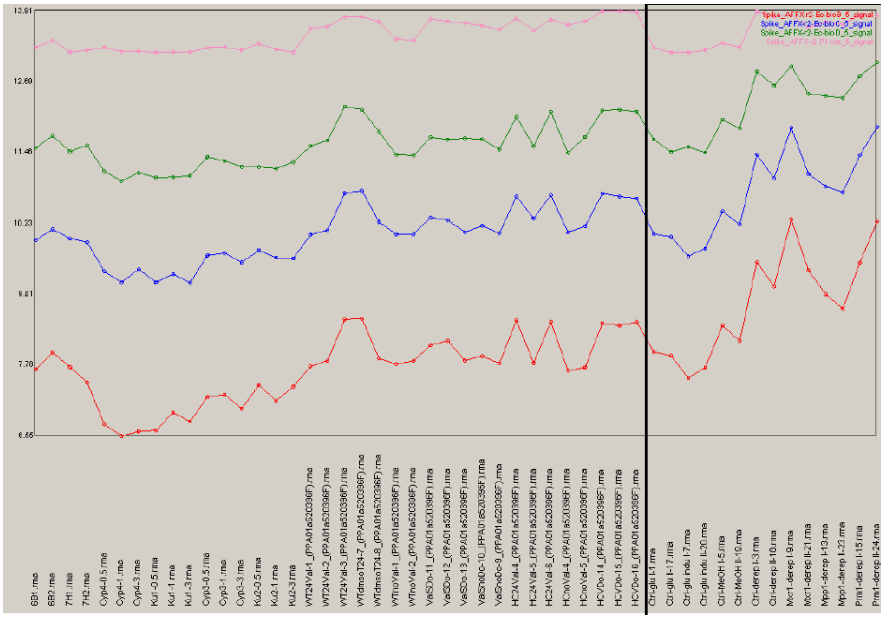

Figure 4.20.: QCcheck using Affymetrix Genexpression Console. For the labelling controls (also called PolyA controls) there is again an expected rank order. Spike samples are labeled lys (1:100000), phe (1:50000), thr (1:25000) and dap (1:6667) with lys showing the lowest signal. Measurement of these signals gives an indication about order for a proper labelling. The higher intensities of labeling controls in ‚derepression'-group are correlated with higher input of aRNA-volume of that group (‚derepression'-group showed less amplification in general).

The Pearson' correlation heat map allowed us to compare the differences in correlation coefficients among all hybridizations. From figure 4.21 we can see that the corresponding duplicates show little deviation from each other observable in the almost identical coloring within the two respective fields. While we see only very little changes in transcriptional activity in the samples on glucose and glucose reintroduction, the differences of transcription in glucose samples and methanol samples are much larger. Interesting however is that the biggest difference of glucose condition to any other array can be seen in all four derepressed conditions suggesting a unique status of the transcriptional machinery under glucose depleted status.

Taking a more detailed look at the transcriptional activity of de-repressed states compared to conditions on methanol and glucose we see very little changes on the transcriptional level for the former, but indeed large restructuring for the latter. This was somehow expected since during the shift from glucose depleted conditions to methanol most likely only genes for the activation of methanol utilization genes would be affected, while the depletion of glucose from the medium exerts enormous effects on transcriptional regulation.

Furthermore all samples under de-repressed conditions behave very similarly irrespective of which overexpression strain we look at. In the case of *MXR1* under de-repressed conditions we see a slightly larger change compared to the control strain and *PRM1* or *MPP1* under de-repressed conditions, which would fit into the picture of *MXR1* being a transcriptional master regulator.

A more detailed microarray analysis is currently processed by Dr. Gerhard Thallinger and will be available soon (provisional data are shown in the appendix). This would in turn allow us to make important contributions at least to two areas. Firstly, from the set of promoters affected by the transcription factor we might be able to find new promoters that could be used as alternative targets for protein expression, with the additional information of already knowing one major transcription activator regulating this specific promoter. Secondly, by comparing the targets regulated by all three transcription factors we might be able to construct a more refined picture of transcriptional regulation of these three methanol activating transcription factors. These data might in combination not only allow us to refine the auto-inductive expression system presented here but also find new parts for the construction of genetic circuits.

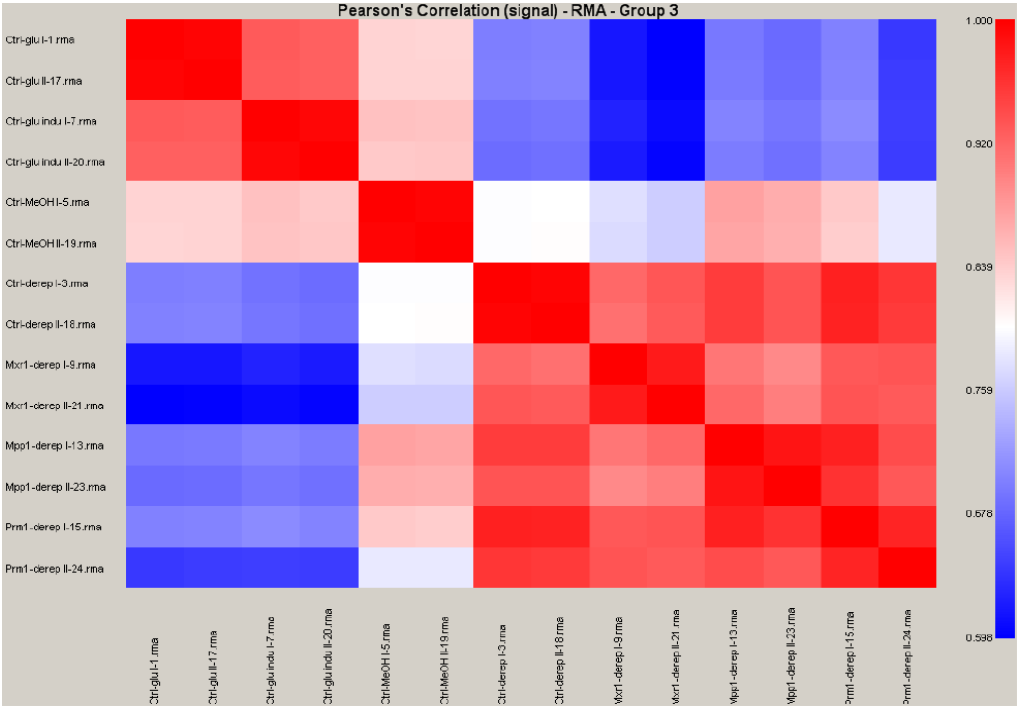

Figure 4.21.: Pearson' correlation table of all 14 hybridizations generated by QCcheck Affymetrix Geneexpression Console. This heat map shows how well each array correlates to the 13 other arrays tested.

4.6 Testing of transcription factor overexpression plasmids as possible tools for protein expression

4.6.1 Expression of *Linum usitatissimum* HNL

Since this thesis was partly set in the framework of the EU project Kyrobio - The Discovery, Development & Demonstration of Biocatalysts for use in the Industrial Synthesis of Chiral Chemical, more specifically in the work package 4 (Fermentation Science for Novel Enzymes and Improved Fermentation Strains) we were also obliged to produce high efficiency auto inductive expression systems for selected Kyrobio enzymes. The hydroxynitrilelyase (*HNL*) from *Linum usitatissimum* was chosen since it was part of the Kyrobio project and in-house screening protocols and enzyme assays were available.

In the data presented so far we see a strong activating effect on P_{AOX1} by P_{CAT1}-controlled transcription factor overexpression using sTomato as reporter gene. Therefore I chose plasmids with P_{CAT1} driven expression of *MXR1*, *PRM1* and *MPP1* to express *LuHNL*. As can be seen in figure 4.22 I exchanged sTomato with the *LuHNL* gene, to be expressed under the control of P_{AOX1} (see material and methods). Sequenced plasmids were transformed into *P. pastoris* and screened for HNL activity (Krammer 2007). I screened roughly 50 clones per construct and subjected the best clone to a rescreening. Figure 4.23 shows the outcome of this rescreening. The overall activity in all three plasmid backgrounds was rather low and only slightly higher than the buffer control. The P_{CAT1}-*MXR1* controlled P_{AOX1}-*LuHNL* expression gave the highest amount of HNL activity.

According to personal communication with Elisa Lanfranchi, 1mM of ZnSO4 was employed for the cultivation. This might lead to negative effects on our system's capability to activate P_{AOX1} expression. The fact that our system relies on the overexpression of a single transcription factor without the usage of methanol and therefore the absence of the natural transcriptional response makes it much more vulnerable to the existence of stressors. Especially taking into consideration that all three transcription factors used in this study are zinc finger proteins I speculated that a potential oversupply of zinc might interfere with transcription factor function.

Looking back at the time course of fluorescence increase we see a steady rise of protein concentration over time after glucose was depleted. The intracellular stability of *LuHNL* under glucose depleted conditions might not be comparable with fluorescence protein sta-

bility, which would in turn mean that already produced HNL will be degraded to free up carbon and energy for maintenance metabolism. A time-resolved picture of expression could potentially find the optimal fermentation time necessary to prevent excessive protein degradation of already produced *LuHNL*.

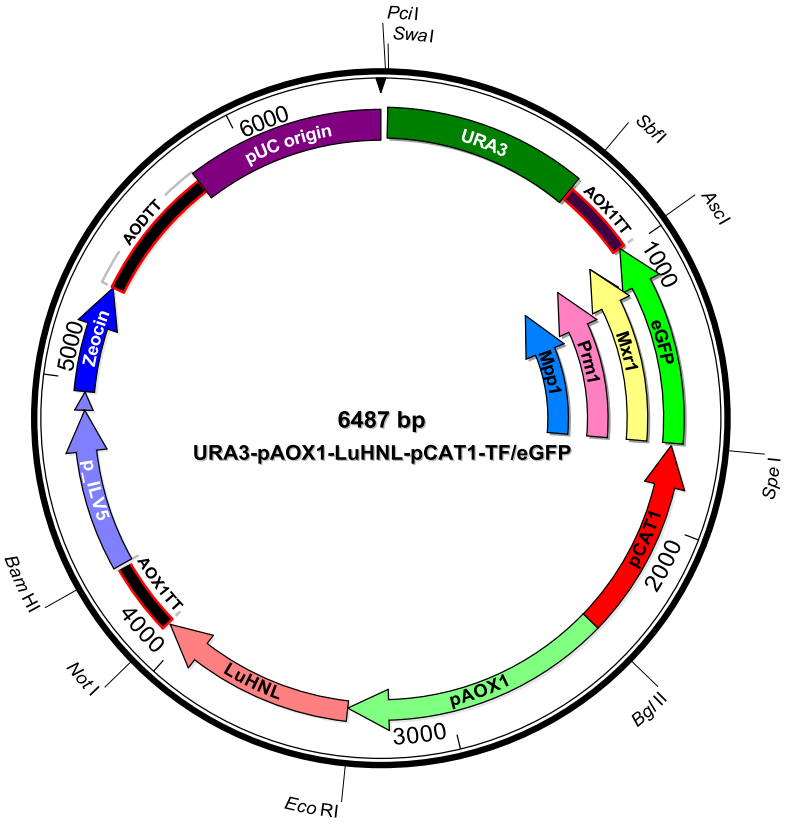

Figure 4.22.: Graphical representation of plasmids used for auto-inductive expression of *LuHNL*. Plasmids from figure 4.1 were used to constructs *LuHNL* expression plasmids by exchanging sTomato for *LuHNL* sequence. As an empty plasmid control constructs harboring *eGFP* instead of transcription factor were used.

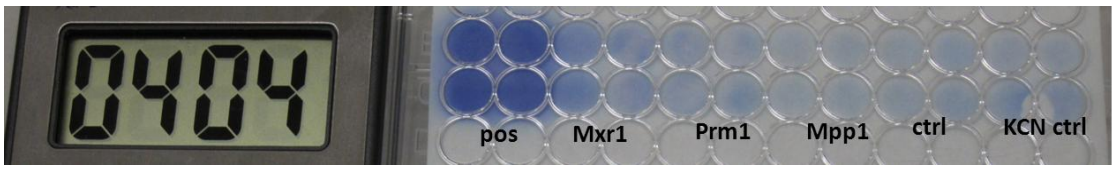

Figure 4.23.: Acetoncyanohydrin lyase assay of P_{AOX1}-*LuHNL* strains simultaneously overexpressing *MXR1*, *PRM1* and *MPP1* under P_{CAT1}. The respective transcription factor was present on the same plasmid as the *LuHNL*. As a way to monitor the chemical background reaction of acetoncyanohydrin lysis we used lysate of empty plasmid (ctrl) and 1mM KCN. As a positive control the same amount of lysate from a methanol induced single copy *P. pastoris* strain of *Lu*HNL was used (kindly provided by Lanfranchi E.). Time point of photograph taken is given in mm:ss.

4.6.2 Construction of double transcription factor overexpression plasmids for the generation of conversion plasmids

Since single overexpression gave strong activation of P_{AOX1} under glucose conditions, double overexpression of *MXR1* and *MPP1* might show an additive effect on P_{AOX1} promoter activity on glucose. Therefore I designed *MXR1*/*MPP1* double overexpression plasmids. However, the re-usage of P_{CAT1} is not an option due to the inability of combining two identical sequences via PCR so I was searching for alternative promoters with very similar regulation behavior, e.g. glucose repression and induction under derepressed conditions. The promoter of the peroxin 5 (P_{PEX5}) showed similar regulation behavior as P_{CAT1}, albeit less strong repression and upregulation upon glucose depletion and methanol induction (project lab Lukas Sturmberger). Therefore the P_{AOX1} was exchanged by P_{PEX5} and sTomato was exchanged with the respective second transcription factor, *MXR1* or *MPP1* resulting in two different plasmids harboring both transcription factors under the control of P_{CAT1} and P_{PEX5} and vice versa (see figure 4.24). In order to be able to monitor promoter activity in a time-dependent manner, *eGFP* and sTomato were used to follow expression from P_{CAT1} and P_{PEX5} respectively. The results of this experiment were not yet available at the time st which this thesis was completed.

Retransformation into already existing high efficiency expression strains could potentially result in the usage of this vector as a conversion plasmid system to drive expression from P_{AOX1} under glucose conditions without the need for methanol induction. This would allow the usage of any existing high level P_{AOX1} production strain for methanol free protein production.

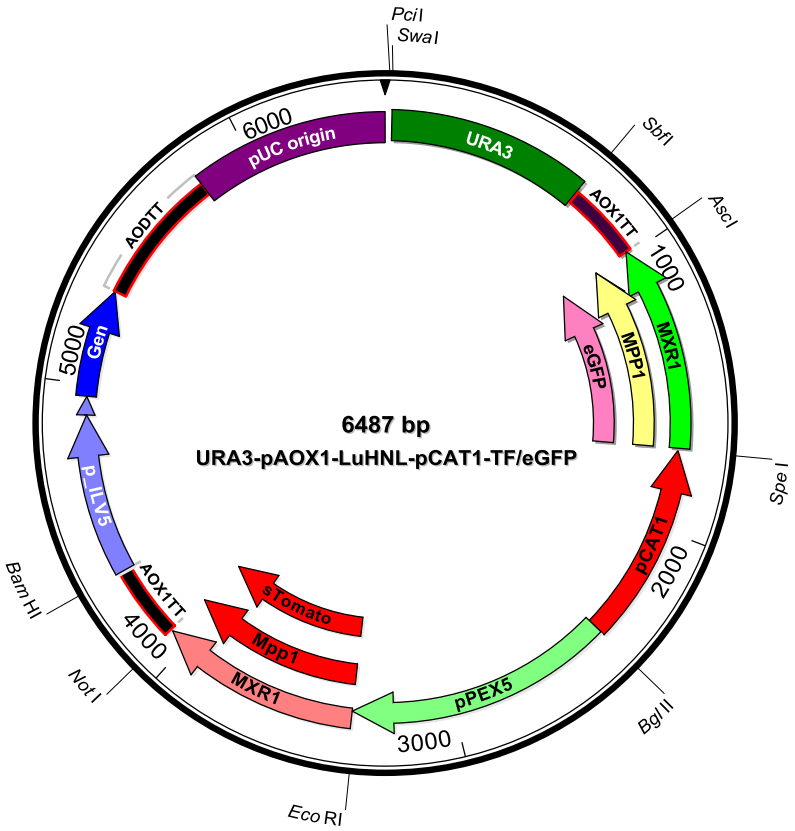

Figure 4.24.: Graphical representation of double overexpression plasmids used for testing possible additive effects on P_{AOX1} activation under glucose conditions. In these plasmid backgrounds *MXR1* and *MPP1* are expressed under P_{CAT1} and P_{PEX5} in a way that both transcription factors are overexpressed at the same time. However in one plasmid always two different transcription factors are found. As controls P_{CAT1}-*eGFP* and P_{PEX5}-*sTomato* were used.

4.7 Generation of transcription factor knock-out strains

4.7.1 Construction of plasmids for generating transcription factor knockouts

The effect of transcription factor overexpression can be contrasted with the transcriptional response that a possible knock-out strain shows. For this reason plasmids for knocking out the targets already determined for overexpression were constructed. To achieve this endeavor the flippase-based knock out system devised by Mudassar Ahmad was employed (see figure 4.25).

The plasmids for construction of targeted single gene knock-outs were generated in the same way as described in figure 4.25 below, linearized with *Swa*I and transformed into *P. pastoris*. Transformants capable of growing on selective media plates were picked and grown on minimal glucose medium. A genomic DNA isolation using the bead-bashing method (see material and methods) was performed and PCR verification of possible knock-outs was checked with primers shown in figure 4.25. By using a forward primer binding within the genome of *P. pastoris* and a reverse primer within the plasmid the existence of the positive knock-out can be shown. In case of a positive knock-out a PCR product corresponding to the size of the 5' upstream and 3' downstream region should appear. If the genomic integration didn't lead to a deletion of the targeted gene there won't be any PCR product visible. Subsequent activation of the flippase expression by methanol induction leads to flipping and therefore recycling of the resistance marker. Successful flipping is again verified via PCR by using forward and reverse primer binding in the genome. In case of a successful knockout of a methanol activator induction of the flippase would most likely not be possible, therefore a strategy to overcome this problem was designed (see section "Generation of efficient systems for resistance marker recycling").

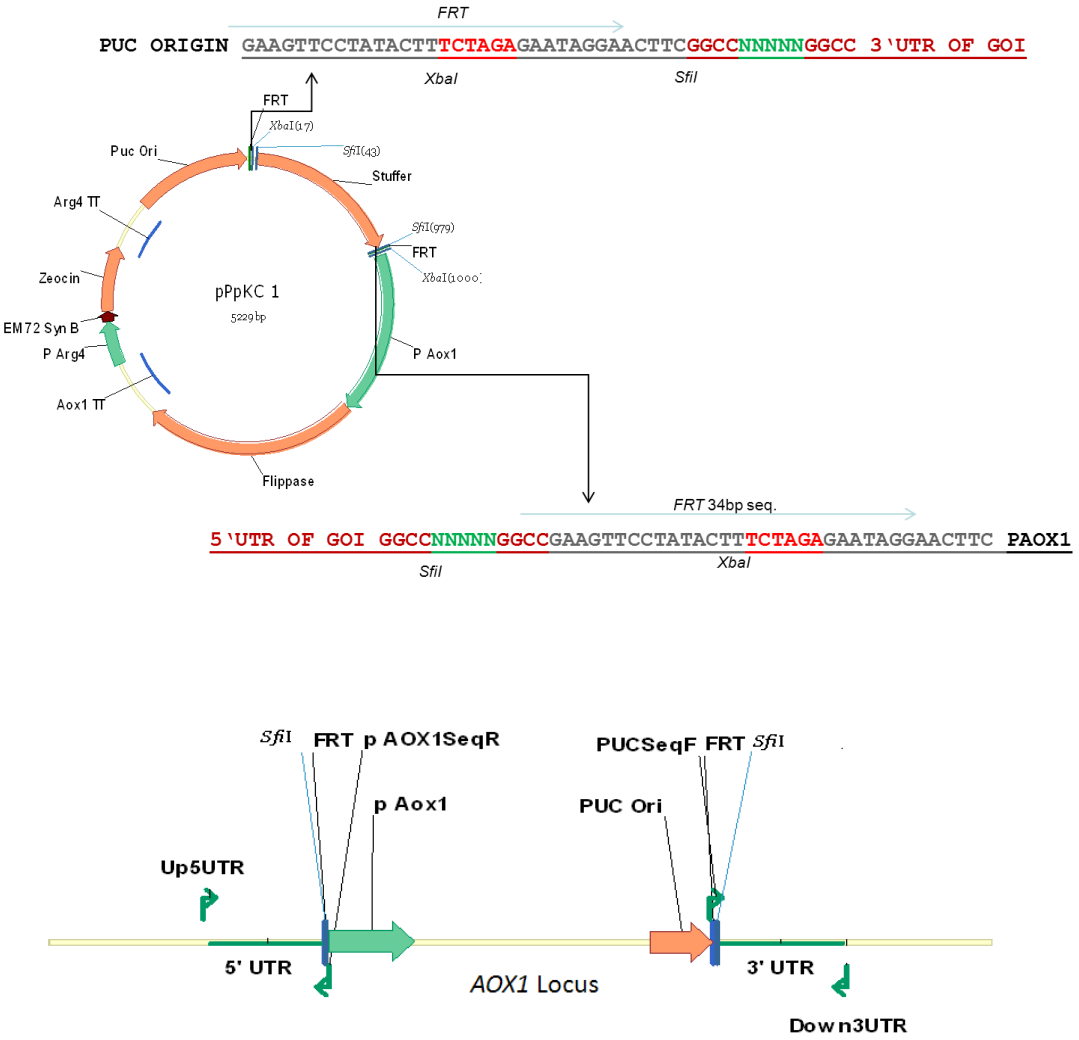

Figure 4.25.: Flippase based knock out system using plasmid pPpKC1 and subsequent flipping for resistance marker recycling. After the knock-out target has been determined, roughly 1000bp regions 5' upstream and 3' downstream are picked to complement a *Swa*I cutting site and amplified with FRT and *Sfi*I sites adjacent to the ends. Cloning into the backbone as shown above (3'UTR-*Swa*I-5'UTR) results in plasmids, that will be linearized with *Swa*I and upon transformation and homologous recombination result in a knock out of the targeted gene. Deletion of the *AOX1* gene is shown as example. The images of plasmids pPpKC1 were kindly provided by M. Ahmad.

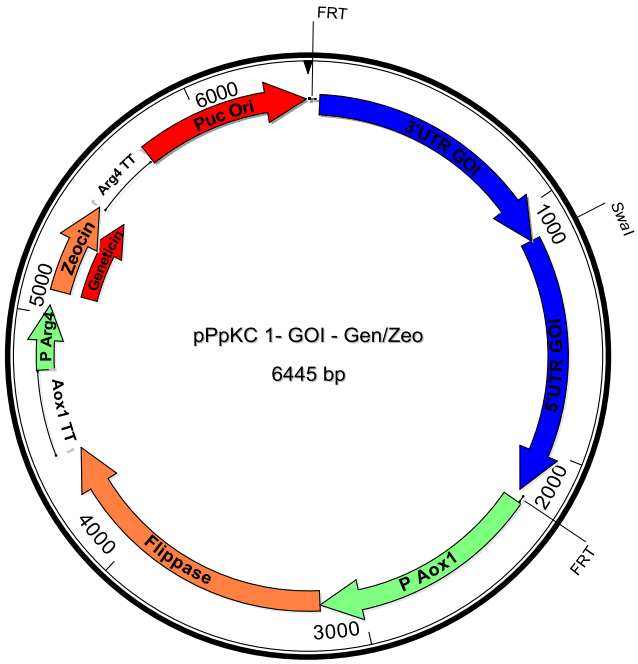

Figure 4.26.: Graphical representation of knock-plasmids with Zeo and Gen marker. Construction of knock-out plasmids listed in table 4.1 was performed by selecting a 1000-1400bp sequence 5' upstream and 3' downstream of the targeted gene by additionally taking a *SwaI* restriction site complementation into consideration. The two fragments were joined together by oePCR and FRT sites adjacent to the ends incorporated on the forward and reverse primers. All plasmids were cloned with a zeocin and geneticin/kanamycin resistance marker. After homologous recombination the correct integration is checked with primers binding in the P_{AOX1} adjacent to the 5'UTR and in the genome. A second pair binding in the pUC Ori adjacent to the 3'UTR and again in the genome of *P. pastoris* was selected.

After screening roughly 50 clones of each construct by genomic DNA isolation and PCR as described above I was unable to construct any of our knock-out targets. Since there was already a knockout phenotype described for *mxr1*Δ strains it was possible to use a functional screening assay to determine successful knock-outs. The inability of *mxr1*Δ strains to grow on methanol as sole carbon source was used to screen 350 transformation clones with wild type and mutS as positive and negative controls respectively. In addition genomic DNA of all 350 clones was isolated and checked via PCR. Neither one of these two methods however gave any positive knock-outs. In the construction process of various knock-out strains Mudassar Ahmad reported to me that a knock-out rate of 20-50% was the average value obtained with this system. Since all plasmids have been sequenced prior to transformation, the possibility of mutated sequences can be excluded. Cell viability of successfully knocked out *mxr1*Δ strains is most likely not a problem due to the existence of pub-

lished data on *mxr1*Δ strains (Lin-Cereghino 2006, Parua 2012). Even taking into consideration the very low homologous recombination frequency of wild type CBS 7435 the examination of 400 clones should result in at least one positive knock-out.

A possible factor influencing knock-out efficiency is very likely found in the selective conditions employed in the transformant selection. Mudassar Ahmad used zeocin resistance for the construction of his knock-out plasmids while I chose geneticin resistance due to the possibility of retransforming zeocin-harboring plasmids already constructed prior to this study. The usage of geneticin resistance as selection marker might influence homologous recombination efficiency or exert cellular stress to a level, at which recombination of genetic material is affected. This possibility is lent credence by the observation that buffered full medium drastically increases knock-out frequency under geneticin selection conditions (personal communication with Mudassar Ahmad). Overexpression of eGFP-tagged transcription factors in these knock-out strains might in the future shed light on their subcellular localization.

4.7.2 Generation of efficient systems for resistance marker recycling

The deletion of key transcription factors regulating methanol utilization genes (MUT) is most likely affecting the ability of P_{AOX1} induction and therefore the expression of flippase to recycle the resistance marker. In principle recycling is possible using two different approaches – a non-methanol inducible promoter or externally supplying flippase activity after transformation selection.

To nevertheless being able to recycle the resistance markers the flippase has to be put under the regulation of an inducible promoter other than P_{AOX1}. The necessity for promoter inducibility is clearly evident since constitutive expression would immediately lead to a flipping under transformation conditions and therefore to an inability to achieve any growth under selective conditions. Due to the lack of any strong and tightly regulated promoter induced by substances other than methanol we had to resort to the second possibility. The activation of P_{FLD1} with methylamine is not clear yet. To achieve recycling the flippase could be supplied only temporarily at a later date. Alternatively the usage of the thiamine repressed promoter in ncombination with minimal medium could be employed and therefore this system might be a suitable alternative to a combined "all in one" expression cassette (see diploma thesis Roland Weis 2001).

5. CONCLUSION AND FUTURE OUTLOOK

During the course of this thesis I was able to construct an auto-induction system for high level protein expression in *P. pastoris*.This was proven by expression of fluorescent reporter proteins but not yet for other proteins such as industrial enzymes. By selecting the widely employed P_{AOX1} and overexpressing single methanol activators under the control of differently regulated promoters I was able to show activation of P_{AOX1} under derepressed, methanol free conditions. The targeted P_{CAT1} driven overexpression renders the usage of methanol for induction redundant while reaching expression levels of 75% of methanol induced P_{AOX1}. Additionally, this thesis resulted in the characterization of several regulators on the protein (reporter protein) and transcriptional level (microarray) which could in the future be used for the design of genetic circuits for synthetic biology.

More broadly speaking these results indicate that the overexpression of a single transcription factor under a suitable promoter is sufficient for the activation of a carbon source regulated promoter in yeast at least in absence of a repressing carbon source. The need for the presence of the original inducing substance is therefore circumvented altogether. The possibility to divert the already existing transcriptional machinery into the direction of favourable conditions for protein expression opens up a whole new window for the application of synthetic biology. The well-considered selection of the building blocks of such genetic circuits allows the generation of completely new systems with desirable features not found in nature. Combining all of these findings might lead to the creation of genetic circuits with completely new traits – modelled according to the specific needs of single applications.

The comparative analysis of microarray hybridizations of *MXR1*, *PRM1* and *MPP1* overexpression strains under derepressed conditions will not only contribute to finding new promoters which can be used as alternative targets for protein expression but also give a more refined picture about the regulation of overlapping sets of genes. This information will in the future further assist in the characterization of new regulatory elements for the incorporation into integrated circuits.

The overexpression of eGFP tagged transcription factor variants in a knock-out background may in the future provide information about the subcellular localization of transcriptional regulators. Additionally such strains could elucidate the effect of different carbon sources on intracellular localization of transcription factors.

The retransformation of plasmids expressing one or two methanol activators under derepressed conditions might allow the usage of such vectors as conversion plasmids or further enhance methanol inducible expression. Therefore already existing P_{AOX1} based high efficiency production strains could potentially be converted into auto-induction expression systems without using the hazardous and toxic chemical methanol for induction.

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7. LIST OF FIGURES

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9. APPENDIX

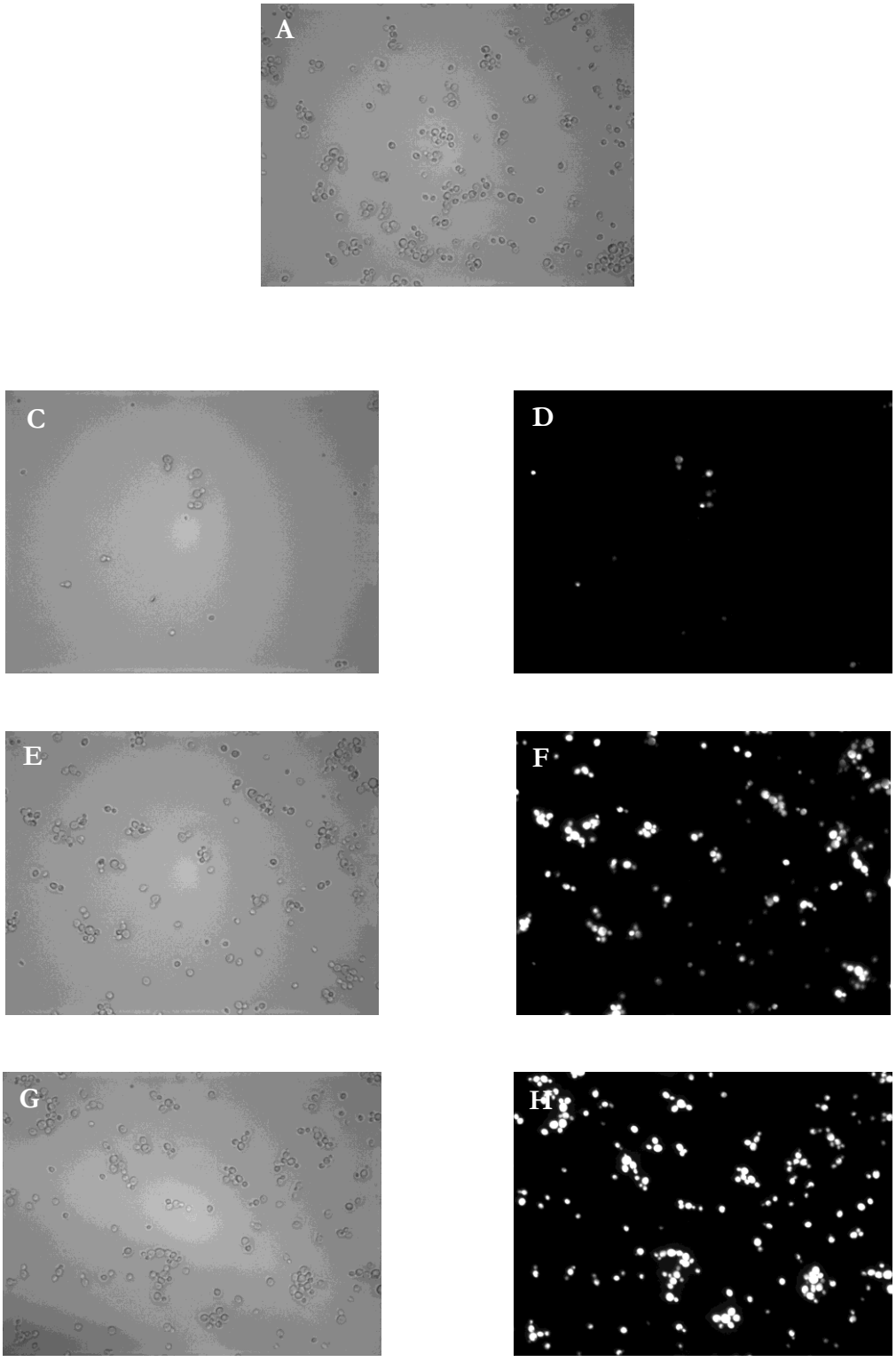

Figure 8.1.: Microscopy pictures of P_{CAT1}-controlled transcription factor overexpression strains. The left side shows phase contrast microscopy pictures while the right side shows fluorescence microscopy pictures. A: P_{GAP} *-MXR1*. C and D: P_{CAT1}*-MXR1,* E and F: P_{GAP} *-PRM1,* G and H: P_{CAT1}*-PRM1*. Sample A showed no fluorescence.

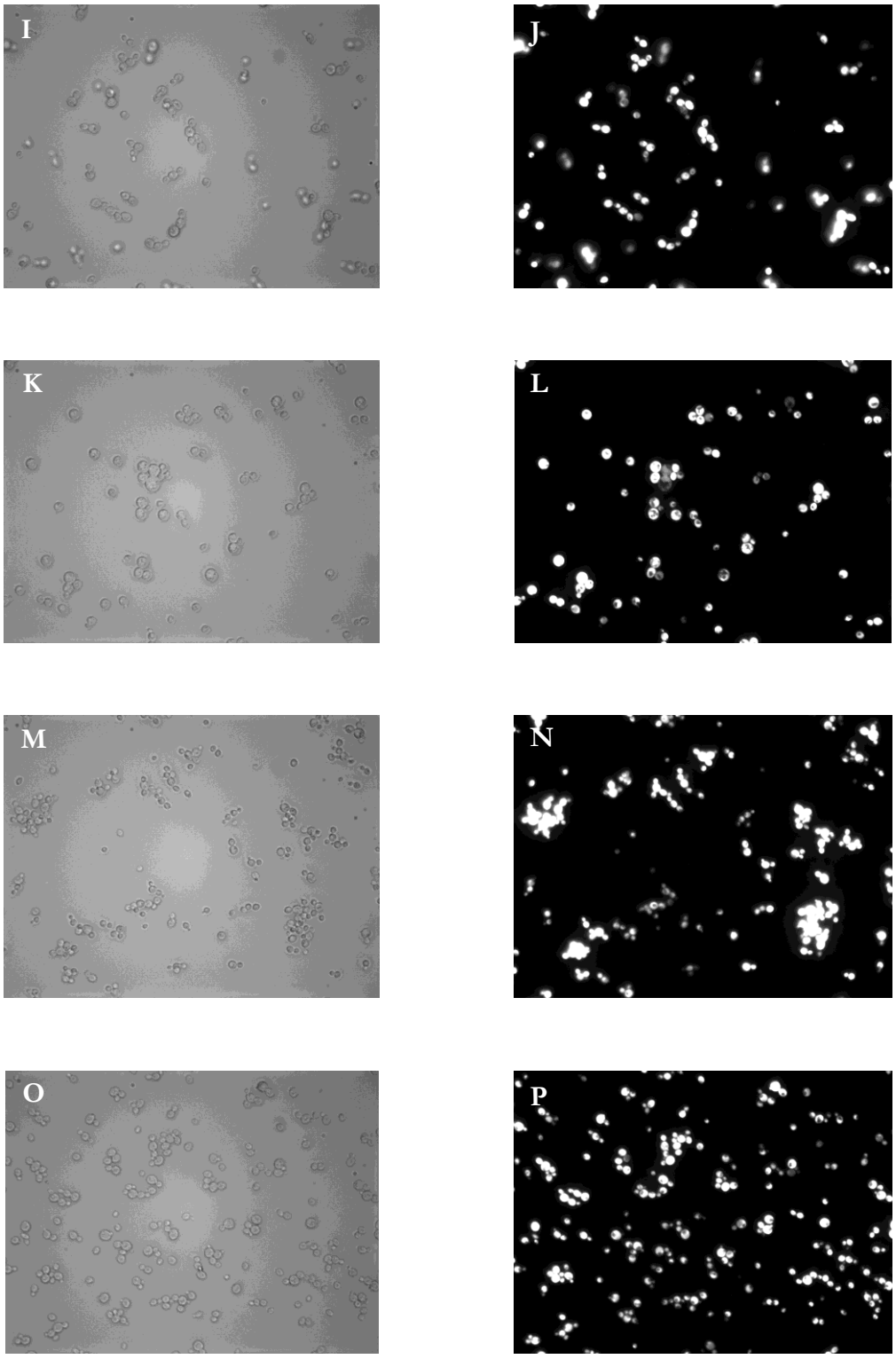

Figure 8.2.: Microscopy pictures of P_{CAT1}-controlled transcription factor overexpression strains. The left side shows phase contrast microscopy pictures while the right side shows fluorescence microscopy pictures. I and J: P_{GAP} *-MPP1.* K and L: P_{CAT1}*-MPP1,* M and N: P_{GAP} *-eGFP,* O and P: P_{CAT1}*-eGFP.*

Tab. 8.1.: BLAST results within 4 yeast species. For captions see table 1.2.

	P.pastoris	H. polymorpha	C. boidnii	P. methano-lica	S. cerevisiae	references
Mxr1	CCA40655.1		BAJ07608.1 (Trm2p)		AAA73863.1 (Adr1p)	
max score			514		140	
total score			755		232	Lin-Cereghino et al. 2006, Parua et al. 2012, Kranthi et al. 2009, Sasano et al. 2009, Denis et Young 1983, Simon et al 1991
query cover		no homologue	88%	not se-quenced	34%	
E-value			2.00E-161		2.00E-33	
ident			37%		55%	
protein size	133 kDa (F2QZ27)		170 kDa (D5MTG8)		151 kDa (P07248)	
Prm1	CCA40959.1 (Gin1p)	AAK84946.1 (Mut3p)	BAF99700.1 (Trm1p)		NP_012136.1 (Asg1p)	
max score		931	774		517	
total score		931	997	not se-quenced	645	Takagi et al. 2009, Vallini et al. 2000, Sasano et al 2008
query cover		96%	75%		56%	
E-value		0	0		1.00E-167	
ident		53%	74%		53%	
protein size	112 kDa (F2QZY1)	105 kDa (Q8NJJ7)	160 kDa (B0I4V8)		109 kDa (P40467)	
Swi1	CCA37890.1	AAQ75382.1 (Swi1p)			NP_015309.1 (Swi1p)	
max score		293			207	
total score		293		not se-quenced	249	van der Klei et al. 2006, Yurimoto et al. 2011, Peterson et al. 1994
query cover		99%	not sequenced		75%	
E-value		1.00E-87			5.00E-55	
ident		29%			29%	
protein size	93 kDa (F2QR62)	97 kDa (Q6W8T0)			148 kDa (P09547)	
Snf1	CCA38457.1	AAN84785.1			NP_010765.3 (Snf1p)	
max score		290			756	
total score		290		not se-quenced	756	
query cover		51%	not sequenced		98%	Ratnakumar et al. 2010
E-value		2.00E-95			0	
ident		56%			65%	
protein size	63 kDa (F2QSS9)	32 kDa (Q6YFD1)			72 kDa (P06782)	
Cat8	CCA38204.1				NP_014007.1 (Cat8p)	
max score					397	
total score				not se-quenced	397	Young et al 2003, Tachibana et al. 2005
query cover		no homologue	not sequenced		74%	
E-value					3.00E-117	
ident					32%	
protein size	116 kDa (F2QS26)				160 kDa (P39113)	

	P.pastoris	H. polymorpha	C. boidnii	P. methanolica	S. cerevisiae	references
Reg1	CCA36537.1				NP_010311.1 (Reg1p)	
max score					141	
total score					245	
query cover		no homologue	not sequenced	not sequenced	44%	Niederacher and Entian 1987
E-value					2.00E-34	
ident					61%	
protein size	75 kDa (F2QM90)				113 kDa (Q00816)	
Gal4	CCA37633.1				NP_015076.1 (Gal4p)	
max score					16	
total score					277	Martchenko et al 2007, Griggs and Johnston 1991
query cover		no homologue	no homologue	not sequenced	55%	
E-value					1.00E-42	
ident					29%	
protein size	113 kDa (F2QQF5)				99 kDa (P04386)	
Mpp1	CCA39317.1	AAO72735.1				
max score		231				
total score		374				van Zutphen et al 2010, WO 2012102171 , van der Klei et al 2006
query cover		68%	not sequenced	not sequenced	no homologue	
E-value		1.00E-66				
ident		33%				
protein size	98 kDa (F2QV89)	78 kDa (Q7Z7X5)				
Snf2	CCA40198.1				NP_014933.3 (Snf2p)	
max score					1180	
total score					1302	
query cover		no homologue	not sequenced	not sequenced	76%	Yurimoto et al. 2011
E-value					0	
ident					54%	
protein size	189 kDa (F2QXS0)				194 kDa (P22082)	
Rpd3	CCA37028.1				NP_014069.1 (Rpd3)	
max score					811	
total score					811	
query cover		no homologue	not sequenced	not sequenced	90%	Rundlett et al. 1996
E-value					0	
ident					87%	
protein size	54 kDa (F2QN73)				49 kDa (P32561)	

	P.pastoris	H. polymorpha	C. boidnii	P. methanolica	S. cerevisiae	references
Hda1	CCA38680.1				NP_014377.1 (Hda1p)	
max score					889	
total score					889	
query cover		no homologue	not sequenced	not sequenced	92%	Rundlett et al. 1996
E-value					0	
ident					62%	
protein size	82 kDa (F2QTF2)				80 kDa (P53973)	
Zta1	CCA38647.1	ADM49192.1 (ADHp)			NP_009602.1 (Zta1p)	
max score		106			395	
total score		106			395	
query cover		99%	not sequenced	not sequenced	99%	Kranthi et al. 2006
E-value		5.00E-28			2.00E-136	
ident		28%			59%	
protein size	36kDa (F2QTB9)	37 kDa (E0YMC0)			37 kDa (P38230)	
Rop1	CCA39607.1 (Zms1p)				NP_013630.1 (Tda9p)	
max score					265	
total score					394	
query cover		no homologue	not sequenced	not sequenced	78%	Kumar et al. 2011
E-value					2.00E-76	
ident					32%	
protein size	85kDa (F2QW29)					
Mig1	CCA40819.1	CCA40819.1	BAM38481.1 (Mig1p)		CAA39084.1 (Mig1p)	
max score	119	228	135			
total score	119	228	191			
query cover	16%	82%	23%	not sequenced		Staysk et al. 2007, Zhai et al. 2012
E-value	2.00E-30	8.00E-71	2.00E-36			
ident	74%	41%	87%			
protein size	48 kDa (F2QZJ1)					
Mig2	CCA37444.1	ABU63593.1 (Mig2p)	BAM38481.1 (Mig1p)		NP_011306.1 (Mig2p)	
max score	103	109	106			
total score	103	145	130			
query cover	16%	29%	30%	not sequenced		Zhai et al. 2012
E-value	5.00E-25	4.00E-29	2.00E-27			
ident	63%	46%	66%			
protein size	50 kDa (F2QPW6)					

Figure 8.3.: Heatmamp of methanol overexpression strains and controls under different conditions

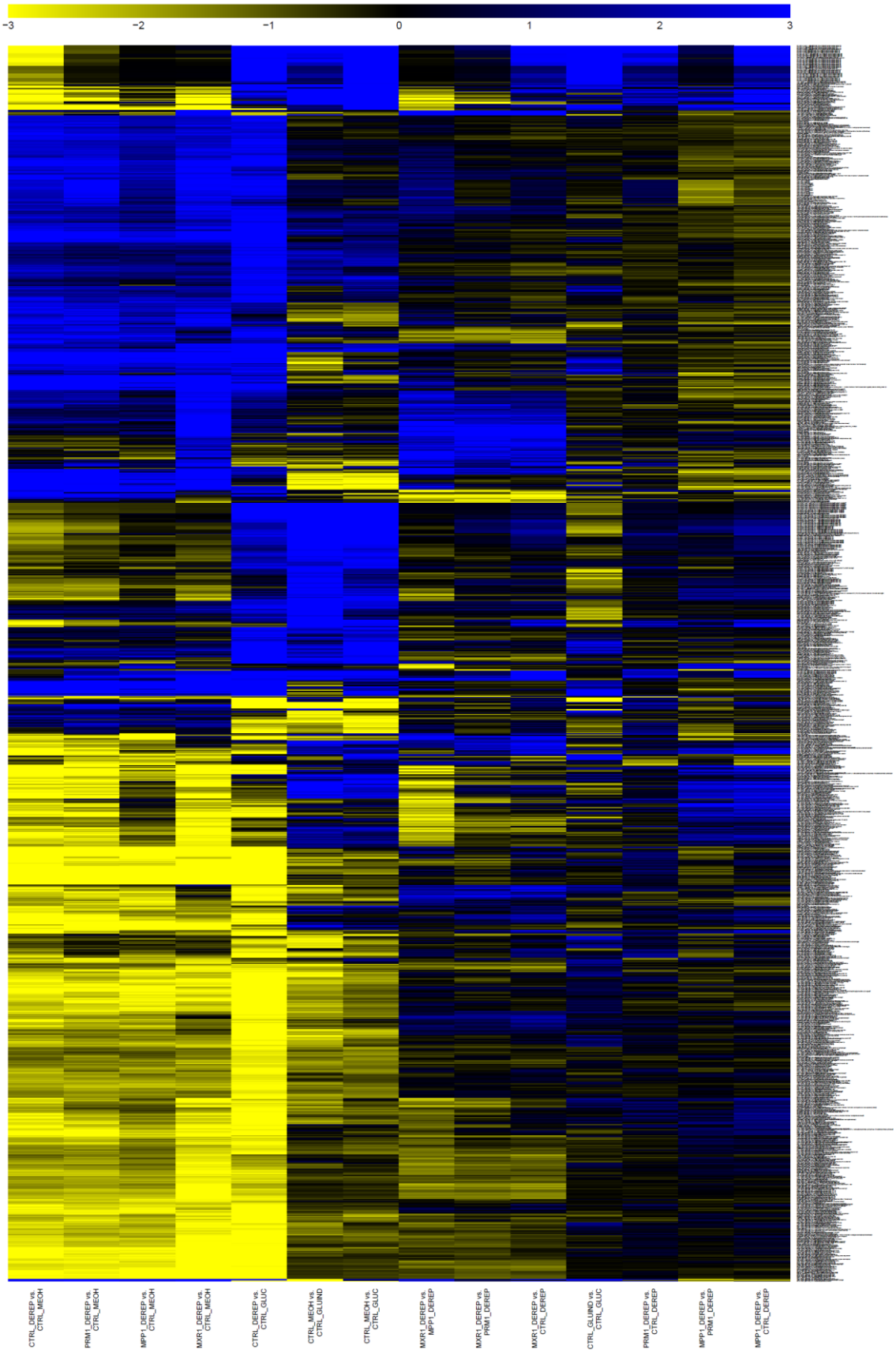

Table 8.2.: Microarray Analysis of P_{CAT1}-*MXR1* derepressed sample compared to control derepressed.

Gene Name	Description	Fold Change	adjusted P Value
undef	Cinnamyl-alcohol dehydrogenase (EC 1.1.1.195) [1.1e-72]	94.99	4.43E-08
undef	Peroxiredoxin (EC 1.11.1.15) [6.2e-14]	81.26	5.07E-06
undef	<not provided>	67.90	4.43E-08
QDR2	<not provided>	53.00	2.00E-07
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	36.39	8.55E-08
undef	16S rRNA m(5)C 967 methyltransferase (EC 2.1.1.-) [5.0e-84]	35.75	1.17E-08
undef	Glucuronyl hydrolase family protein	27.18	1.73E-08
	G10 protein [3.1e-49]	26.06	6.15E-05
DAL1	Allantoinase (EC 3.5.2.5) [2.6e-81]	25.92	4.91E-08
DapA	Dihydrodipicolinate synthase (EC 4.2.1.52)	24.95	4.43E-08
undef	<not provided>	24.45	2.85E-04
FBA2	Fructose-bisphosphate aldolase (EC 4.1.2.13) [3e-150]	24.28	2.46E-04
undef	[0.0]	21.06	6.46E-07
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	20.57	7.74E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	19.76	7.01E-08
undef	Cinnamyl-alcohol dehydrogenase (EC 1.1.1.195) [8.9e-96]	19.51	1.88E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	19.32	8.92E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	19.20	9.93E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	18.96	7.74E-08
AOX1	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	18.44	1.27E-07
undef	<not provided>	18.01	5.67E-06
	<not provided>	17.96	3.03E-05
undef	Allantoate permease [1.6e-75]	16.61	1.03E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	16.57	4.91E-08
undef	Hypothetical protein [4.2e-67]	16.33	8.05E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	16.07	8.55E-08
undef	<not provided>	15.94	3.07E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	15.55	4.43E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	15.30	4.91E-08
undef	Mandelate racemase family protein	15.21	2.36E-06
undef	<not provided>	13.80	1.17E-08
ZAS1	C2H2 ZINC FINGER PROTEIN [1.7e-76]	13.01	8.18E-07
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	12.50	5.12E-08
RHO4	GTP-binding protein [2.9e-45]	12.48	8.55E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	12.23	2.00E-08
undef	Cytochrome P450 (EC 1.14.14.1)	12.18	2.25E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	11.82	1.17E-08
undef	<not provided>	11.60	4.20E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	11.51	1.17E-08
iga	BNI4 protein [6.9e-28]	11.49	2.26E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	11.47	1.17E-08

undef	Sulfonate alpha -Ketoglutarate Dioxygenase (EC 1.14.11.17) [6.0e-86]	11.43	6.46E-07
AAT1	<not provided>	11.33	2.88E-07
undef	Transporter [7.0e-68]	10.80	1.20E-07
PDK4	[Pyruvate dehydrogenase [lipoamide]] kinase (EC 2.7.1.99) [4.3e-21]	10.24	2.52E-07
FAD1	FAD synthetase (EC 2.7.7.2)	9.85	1.30E-07
RIO1	Serine threonine-protein kinase RIO1 (EC 2.7.1.37) [3e-110]	9.63	5.63E-06
undef	Methylenetetrahydrofolate reductase (EC 1.5.1.20) [0.0]	9.50	1.88E-06
undef	Carbon-nitrogen hydrolase family protein [2.0e-63]	9.38	5.36E-06
CVT9	CVT PROTEIN 9 [8.9e-31]	9.25	1.10E-05
ACO2	Aconitate hydratase (EC 4.2.1.3) [6e-122]	9.20	5.62E-06
undef	<not provided>	9.07	1.28E-06
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	8.82	2.68E-07
undef	Cleavage and polyadenylation factor CF-IA component Pcf11 [2.6e-27]	8.72	3.66E-06
BIM1	Microtubule binding protein [1.4e-59]	8.67	4.04E-07
MIG1	DNA-binding protein creA [5.4e-21]	8.60	2.95E-04
undef	Cleavage and polyadenylation factor CF-IA component Pcf11 [2.1e-27]	8.41	6.78E-06
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	8.39	1.66E-07
SPA2	SPA2 protein [8.5e-48]	8.32	7.24E-06
RIS1	GTP-binding nuclear protein GSP1 CNR1 [2e-136]	8.24	7.74E-08
undef	Auxin efflux carrier protein [1.3e-88]	8.22	5.74E-08
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	8.17	8.20E-08
undef	Transmembrane protein, GPR1 FUN34 yaaH family [3.3e-26]	8.15	1.72E-07
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	8.14	3.30E-07
undef	Sterol carrier protein [1.0e-13]	8.04	4.77E-07
WDR4	tRNA (m(7)G46) methyltransferase subunit 2 (EC 2.1.1.33) [5.8e-62]	8.02	5.90E-06
undef	Transcriptional activator tenA [4.6e-24]	7.98	9.61E-07
ARO7	Chorismate mutase (EC 5.4.99.5) [1.1e-88]	7.97	2.62E-06
DAS2	Dihydroxy-acetone synthase (EC 2.2.1.3) [9e-104]	7.90	4.02E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	7.85	5.65E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	7.84	1.66E-07
CDA2	Chitin deacetylase (EC 3.5.1.41) [4.2e-67]	7.81	2.84E-03
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	7.70	3.75E-07
AOX1	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	7.65	8.55E-08
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	7.61	4.49E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	7.58	4.24E-07
undef	Membrane alanine aminopeptidase (EC 3.4.11.2) [5e-109]	7.53	5.34E-06
undef	<not provided>	7.53	5.92E-06
TGL3	Patatin phospholipase family protein [1.0e-68]	7.51	9.93E-08
undef	<not provided>	7.50	8.55E-08
POX2	Acyl-coenzyme A oxidase POX4 (EC 1.3.3.6) [3.2e-36]	7.50	6.31E-07
ARG3	Ornithine carbamoyltransferase (EC 2.1.3.3) [1.2e-84]	7.46	1.21E-04
RTM1	RTM1 protein [3.3e-28]	7.45	6.84E-07
ERG6	Sterol 24-C-methyltransferase (EC 2.1.1.41) [2e-146]	7.40	3.22E-03

undef	<not provided>	7.34	1.74E-05
SEF1	Suppressor protein SEF1 [6e-165]	7.23	8.55E-08
HCM1	Transcription factor HCM1 [6.7e-30]	7.16	1.49E-07
undef	Mandelate racemase (EC 5.1.2.2)	7.14	3.07E-08
undef	<not provided>	7.09	2.70E-06

Table 8.2.: Microarray Analysis of P_{CAT1}-*PRM1* derepressed sample compared to control derepressed.

Gene Name	Description	Fold Change	adjusted P. Value
undef	Peroxiredoxin (EC 1.11.1.15) [6.2e-14]	27.40	2.12E-04
DAS2	Dihydroxy-acetone synthase (EC 2.2.1.3)	21.31	4.34E-07
undef	2-deoxyglucose-6-phosphate phosphatase (EC 3.1.3.68) [4.2e-44]	12.74	6.35E-03
DAS2	Dihydroxy-acetone synthase (EC 2.2.1.3) [9e-104]	12.45	3.90E-07
GIN1	Transcriptional regulator [4e-158]	12.01	1.66E-07
RKI1	Ribose 5-phosphate isomerase (EC 5.3.1.6) [5.7e-74]	11.26	4.30E-04
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	9.91	4.34E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	9.45	3.90E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	9.41	1.33E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	9.27	4.34E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	8.72	1.79E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	8.56	2.99E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	8.36	1.44E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	8.36	1.97E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	8.27	2.99E-06
undef	<not provided>	8.22	6.00E-05
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	8.04	8.58E-08
undef	<not provided>	7.96	2.65E-02
AOX1	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	7.90	4.77E-06
undef	Sterol carrier protein [1.0e-13]	7.90	1.78E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	7.84	8.58E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	7.72	8.58E-08
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	7.59	1.70E-05
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	6.66	5.89E-06
undef	<not provided>	6.64	6.08E-05
	<not provided>	6.45	1.47E-02
undef	<not provided>	6.43	8.43E-05
undef	<not provided>	6.40	1.66E-07
FET4	Low-affinity Fe(II) transport protein [6e-115]	6.16	6.28E-03
ARO7	Chorismate mutase (EC 5.4.99.5) [1.1e-88]	5.68	3.88E-05
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	5.47	1.95E-06
FBA2	Fructose-bisphosphate aldolase (EC 4.1.2.13) [3e-150]	5.44	2.80E-02
RIB3	3,4-dihydroxy-2-butanone 4-phosphate synthase (EC 4.1.2.-) [7.2e-61]	5.26	4.52E-04
DAS1	Dihydroxy-acetone synthase (EC 2.2.1.3)	5.15	3.58E-03
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	5.13	2.86E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	5.10	7.10E-07

AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	5.05	4.34E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	5.05	4.34E-07
AOX1	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	5.03	1.42E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	5.02	6.79E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	5.02	7.60E-06
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	5.01	3.90E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	4.98	3.50E-07
MIG1	DNA-binding protein creA [5.4e-21]	4.81	8.51E-03
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	4.53	1.31E-05
undef	<not provided>	4.51	4.73E-02
undef	Membrane alanine aminopeptidase (EC 3.4.11.2) [5e-109]	4.43	2.49E-04
undef	Sin3p interacting protein	4.40	4.52E-04
undef	Oxidoreductase (EC 1.-.-.-)	4.36	4.27E-03
	<not provided>	4.31	4.46E-03
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	4.24	9.25E-06
undef	<not provided>	4.21	7.63E-07
undef	<not provided>	4.13	8.27E-04
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	4.12	3.57E-06
DAS1	Dihydroxy-acetone synthase (EC 2.2.1.3) [7e-152]	4.10	4.53E-04
SFU1	[3.2e-15]	4.10	1.92E-02
YEX1	ThiF MoeB family protein [2e-138]	4.07	5.03E-04
PSP1	DNA polymerase alpha mutation suppressor PSP1 [5.2e-37]	4.07	1.21E-03
undef	<not provided>	3.99	1.58E-02
DAS2	Dihydroxy-acetone synthase (EC 2.2.1.3) [9e-104]	3.96	1.44E-06
undef	<not provided>	3.95	7.46E-03
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	3.92	2.85E-05
DAS2	Dihydroxy-acetone synthase (EC 2.2.1.3) [7e-152]	3.90	1.71E-06
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	3.79	3.88E-05
YOX1	Homeobox protein YOX1 [3.2e-28]	3.78	3.71E-05
undef	6-phosphofructo-2-kinase (EC 2.7.1.105) [3.8e-30]	3.73	6.35E-03
undef	Cell cycle protein kinase DBF2 (EC 2.7.1.-) [4e-151]	3.69	9.14E-03
AAT1	<not provided>	3.69	1.00E-04
	<not provided>	3.68	4.44E-02
undef	CoA pyrophosphatase (EC 3.6.1.-) [4.0e-52]	3.67	2.62E-05
undef	<not provided>	3.65	2.21E-04
undef	<not provided>	3.62	2.47E-06
TAL2	Transaldolase (EC 2.2.1.2) [6e-126]	3.58	1.18E-02
undef	<not provided>	3.57	5.55E-03
ACE2	Transcriptional factor SWI5 [1.9e-33]	3.56	2.46E-02
HGT1	High-affinity glucose transporter [2.4e-25]	3.47	5.52E-03
undef	<not provided>	3.46	7.86E-03
undef	Sulfite sensitivity protein SSU1 [6.0e-30]	3.44	2.48E-05
undef	Molybdopterin biosynthesis MoeB protein [7e-138]	3.38	1.75E-03
undef	Hexose transporter [0.0]	3.35	4.83E-02
undef	Hypothetical protein	3.34	1.27E-03
QDR2	Quinidine resistance protein [5.4e-83]	3.34	4.47E-02
XDJ1	Chaperone protein dnaJ [1.3e-48]	3.32	3.75E-04
undef	<not provided>	3.27	3.74E-04
ITR2	Myo-inositol transporter [0.0]	3.26	3.35E-02

CLB2	G2 mitotic-specific cyclin [5.6e-94]	3.18	3.09E-05
FAD1	FAD synthetase (EC 2.7.7.2)	3.14	8.23E-05
KRE1	KRE1 protein precursor	3.12	1.12E-02
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	3.12	4.32E-06
undef	Peroxisomal membrane protein PMP47B [1.1e-19]	3.12	1.75E-03
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	3.11	3.98E-06
KIP2	Kinesin-like protein KIP2 [7.3e-87]	3.07	1.53E-03
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	3.05	1.85E-05
CDC5	Cell cycle protein kinase CDC5 MSD2 (EC 2.7.1.-) [0.0]	3.03	1.67E-03
undef	<not provided>	3.01	3.72E-03
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	2.97	1.53E-05
CYB2	Cytochrome B2, mitochondrial precursor (EC 1.1.2.3) [2e-154]	2.97	7.40E-04
	<not provided>	2.95	1.14E-02
CAM1	Protein Translation Elongation Factor 1B subunit beta (EF-1Bb) [2.9e-31]	2.95	2.22E-02
SER2	Phosphoserine phosphatase (EC 3.1.3.3) [2.3e-65]	2.94	3.88E-05
ACO2	Aconitate hydratase (EC 4.2.1.3) [6e-122]	2.92	3.72E-03
AOX1	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	2.92	3.35E-06
undef	<not provided>	2.90	5.13E-03
RAX2	Bud determination protein RAX2	2.89	1.21E-03
undef	Isochorismatase family protein	2.88	5.24E-03
undef	Hypoxia induced protein family [5.3e-50]	2.87	4.60E-02
undef	Tubulin beta chain [0.0]	2.87	1.65E-03
undef	<not provided>	2.85	4.53E-04
MOB1	Maintenance of ploidy protein MOB1 [2.2e-63]	2.85	7.61E-04
TOS1	Hypothetical protein	2.83	2.79E-03
undef	Cytokinesis 2 protein	2.79	3.29E-02
undef	Acetyl esterase (EC 3.1.1.-)	2.78	2.74E-04
undef	<not provided>	2.78	9.61E-03
oxc	<not provided>	2.73	1.83E-04
undef	Hypothetical protein [1.3e-32]	2.70	2.00E-02
KIN3	Serine threonine-protein kinase KIN3 (EC 2.7.1.-) [3.2e-76]	2.70	2.84E-02
undef	Methionine aminopeptidase (EC 3.4.11.18) [2e-123]	2.69	3.67E-03
undef	Cinnamyl-alcohol dehydrogenase (EC 1.1.1.195) [1.1e-72]	2.69	3.64E-03
undef	Alanine aminotransferase (EC 2.6.1.2) [1e-157]	2.69	3.96E-03
PCNA	Proliferating cell nuclear antigen [4.4e-72]	2.67	8.37E-05
undef	Glutathione S-transferase family protein	2.63	2.60E-04
aceB	Malate synthase (EC 2.3.3.9) [3.9e-23]	2.63	8.51E-03
undef	Tubulin alpha chain [0.0]	2.61	1.05E-03
AAT1	Aspartate aminotransferase (EC 2.6.1.1) [6.3e-70]	2.60	2.49E-04
TOS1	Hypothetical protein	2.57	6.15E-03
	<not provided>	2.56	2.30E-02
ARG3	Ornithine carbamoyltransferase (EC 2.1.3.3) [1.2e-84]	2.56	3.02E-02
DapA	Dihydrodipicolinate synthase (EC 4.2.1.52)	2.56	6.60E-04
BIM1	Microtubule binding protein [1.4e-59]	2.55	6.83E-04
undef	<not provided>	2.54	1.18E-02
undef	SKT5 homolog [6.2e-37]	2.54	2.14E-03
HEM3	Porphobilinogen deaminase (EC 2.5.1.61) [1.1e-91]	2.53	1.53E-02
AGC1	Calcium-binding mitochondrial carrier protein Aralar2 [6e-102]	2.51	6.36E-03
undef	M-phase inducer phosphatase (EC 3.1.3.48) [6.1e-41]	2.50	3.35E-03

FEN4	Biotin transporter [2e-168]	2.50	4.59E-02
undef	<not provided>	2.49	7.86E-05
	<not provided>	2.49	2.76E-02
MYO1	Myosin-1 isoform [0.0]	2.48	5.46E-03
undef	Covalently-linked cell wall protein [1.6e-61]	2.47	2.87E-02
undef	<not provided>	2.47	2.55E-02
undef	<not provided>	2.46	7.19E-04
SUN4	SIM1 protein [3e-116]	2.45	1.10E-02
undef	<not provided>	2.44	1.28E-02
RLR1	RLR1 protein [1.6e-99]	2.43	3.51E-02
THI4	Thiazole biosynthetic enzyme [2e-101]	2.41	2.74E-04
CLA4	Serine threonine-protein kinase SKM1 (EC 2.7.1.-) [8e-159]	2.40	3.64E-04
undef	Hypothetical protein [1.0e-45]	2.39	5.00E-04

Table 8.3.: Microarray Analysis of P_{CAT1}-*MPP1* derepressed sample compared to control derepressed.

GeneName	Description	FoldChange	adjusted P. Value
undef	Peroxiredoxin (EC 1.11.1.15) [6.2e-14]	129.82	4.17E-06
RKI1	Ribose 5-phosphate isomerase (EC 5.3.1.6) [5.7e-74]	63.52	2.91E-06
FBA2	Fructose-bisphosphate aldolase (EC 4.1.2.13) [3e-150]	61.77	7.82E-05
PSP1	DNA polymerase alpha mutation suppressor PSP1 [5.2e-37]	40.70	4.46E-07
undef	FARNESYL PYROPHOSPHATE SYNTHETASE (EC 2.5.1.1) GERANYLTRANSTRANSFERASE (EC 2.5.1.10) [2e-138]	39.35	1.98E-09
undef	Phosphomethylpyrimidine kinase (EC 2.7.4.7) Hydroxymethylpy-rimidine kinase (EC 2.7.1.49) Transcriptional activator tenA [1.1e-88]	30.94	9.56E-05
undef	2-deoxyglucose-6-phosphate phosphatase (EC 3.1.3.68) [4.2e-44]	30.10	4.34E-04
DAS1	Dihydroxy-acetone synthase (EC 2.2.1.3)	29.48	8.44E-06
undef	<not provided>	27.99	7.20E-07
AAT1	<not provided>	22.40	9.49E-08
DUR8	LSU ribosomal protein L30E Sodium pantothenate symporter	22.21	1.03E-05
OSM1	Fumarate reductase flavoprotein subunit (EC 1.3.99.1) [1.1e-70]	20.99	2.87E-07
undef	<not provided>	20.23	4.72E-09
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	19.40	5.32E-08
TAL2	Transaldolase (EC 2.2.1.2) [6e-126]	18.97	2.43E-05
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	18.88	9.79E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	18.45	4.61E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	18.22	5.32E-08
DAS2	Dihydroxy-acetone synthase (EC 2.2.1.3)	17.19	3.76E-07
MCM7	DNA replication licensing factor MCM7 [0.0]	15.64	8.17E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	15.42	2.58E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	15.34	1.87E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	15.26	1.87E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	15.22	2.87E-07

AOX1	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	14.47	3.76E-07
CYB2	Cytochrome B2, mitochondrial precursor (EC 1.1.2.3) [2e-154]	14.14	4.76E-07
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	14.01	9.65E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	13.06	1.80E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	12.72	8.76E-09
AGC1	Calcium-binding mitochondrial carrier protein Aralar2 [6e-102]	12.72	2.52E-06
undef	Thiamin-phosphate pyrophosphorylase (EC 2.5.1.3) Hydroxyethyl-thiazole kinase (EC 2.7.1.50) [1.9e-90]	12.63	2.87E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	12.58	8.76E-09
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	12.56	3.71E-07
RIB3	3,4-dihydroxy-2-butanone 4-phosphate synthase (EC 4.1.2.-) [7.2e-61]	12.46	7.37E-06
	<not provided>	12.45	3.56E-06
NAG4	Vacuolar spermine uptake protein [2.7e-88]	12.32	3.96E-05
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	12.32	8.76E-09
undef	Hypothetical protein	11.81	2.86E-06
AAT1	Aspartate aminotransferase (EC 2.6.1.1) [6.3e-70]	11.75	1.11E-07
undef	Oxidoreductase (EC 1.-.-.-)	11.59	5.66E-05
undef	<not provided>	11.41	3.47E-06
undef	<not provided>	10.79	4.93E-06
GPM2	Phosphoglycerate mutase family protein [6.6e-67]	10.54	4.59E-05
FNX1	Multidrug resistance protein fnx1 [9.9e-43]	10.54	3.76E-07
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	10.35	1.11E-07
undef	Ribulose-phosphate 3-epimerase (EC 5.1.3.1) [1.9e-92]	10.25	1.52E-05
Tdp1	Tyrosyl-DNA phosphodiesterase [5.1e-30]	10.23	1.18E-07
undef	Transcriptional activator tenA [4.6e-24]	10.03	6.33E-07
DAS2	Dihydroxy-acetone synthase (EC 2.2.1.3) [9e-104]	9.82	3.24E-07
undef	Urea active transporter [1.6e-81]	9.58	3.90E-07
undef	<not provided>	9.55	9.79E-08
HIS5	Histidinol-phosphate aminotransferase (EC 2.6.1.9) [3e-112]	9.53	5.46E-06
undef	Membrane alanine aminopeptidase (EC 3.4.11.2) [5e-109]	9.37	4.30E-06
undef	<not provided>	9.26	2.52E-06
DAS1	Dihydroxy-acetone synthase (EC 2.2.1.3) [7e-152]	9.09	6.21E-06
GSH1	Glutamate--cysteine ligase (EC 6.3.2.2) [7e-151]	8.50	9.65E-07
undef	Acetyl esterase (EC 3.1.1.-)	8.11	5.33E-07
CAM1	Protein Translation Elongation Factor 1B subunit beta (EF-1Bb) [2.9e-31]	7.82	2.55E-04
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	7.69	5.37E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	7.58	3.07E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	7.51	6.49E-07
undef	Purine-cytosine permease [1.1e-90]	7.50	3.76E-06
AOX1	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	7.48	1.49E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	7.46	9.49E-08
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	7.44	6.50E-07
FAD1	FAD synthetase (EC 2.7.7.2)	7.41	5.15E-07
undef	Sin3p interacting protein	7.38	1.84E-05
undef	<not provided>	7.34	3.60E-05
undef	Transporter, MFS superfamily [1.6e-33]	7.28	1.60E-04

undef	Serine--pyruvate aminotransferase (EC 2.6.1.51) [5.0e-64]	7.13	6.01E-05
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	7.13	4.46E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	6.94	8.17E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	6.89	5.32E-08
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	6.88	2.20E-07
undef	Phosphomannomutase (EC 5.4.2.8) [5e-155]	6.83	2.07E-05
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	6.77	8.60E-08
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	6.77	6.18E-08
ARO7	Chorismate mutase (EC 5.4.99.5) [1.1e-88]	6.70	7.18E-06
GSC2	1,3-beta-glucan synthase component (EC 2.4.1.34) [0.0]	6.69	2.64E-04
KRE1	KRE1 protein precursor	6.58	2.18E-04
undef	Phosphomethylpyrimidine kinase (EC 2.7.4.7) Hydroxymethylpyrimidine kinase (EC 2.7.1.49) Transcriptional activator tenA [2.9e-72]	6.44	1.67E-06
AOX2	PPA-AOX2 Alcohol oxidase (EC 1.1.3.13)	6.42	1.08E-06
undef	<not provided>	6.33	1.80E-05
AOX2	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	6.25	1.37E-06
undef	Sugar-proton symporter [7.6e-29]	6.20	2.71E-07
undef	Phospholipase D1 (EC 3.1.4.4) [0.0]	6.18	2.52E-07
undef	Purine-cytosine permease [5e-132]	6.08	1.28E-04
undef	Hypothetical protein	6.02	4.46E-02
undef	Zinc finger protein [1.2e-87]	5.96	2.46E-05
ENG1	Endo-1,3(4)-beta-glucanase (EC 3.2.1.6) [0.0]	5.95	7.20E-07
undef	Serine--pyruvate aminotransferase (EC 2.6.1.51) [1.8e-15]	5.77	3.41E-05
ARG3	Ornithine carbamoyltransferase (EC 2.1.3.3) [1.2e-84]	5.75	5.58E-04
ANT1	ADP,ATP carrier protein [6.0e-31]	5.72	1.67E-06
THI4	Thiazole biosynthetic enzyme [2e-101]	5.61	7.03E-07
CPA1	Carbamoyl-phosphate synthase small chain (EC 6.3.5.5) [3e-150]	5.58	1.29E-04
MSH1	DNA mismatch repair protein mutS [5e-137]	5.56	2.52E-06
undef	[9.5e-23]	5.51	1.67E-06
aceB	Malate synthase (EC 2.3.3.9) [3.9e-23]	5.45	9.93E-05
CLA4	Serine threonine-protein kinase SKM1 (EC 2.7.1.-) [8e-159]	5.44	9.65E-07
SMC2	Chromosome segregation protein SMC2 [4e-111]	5.39	6.48E-06
SAC2	[1.2e-70]	5.34	4.52E-06
undef	DNA polymerase epsilon, catalytic subunit A (EC 2.7.7.7) [0.0]	5.34	1.77E-05
undef	Methyltransferase (EC 2.1.1.-) [3.3e-71]	5.29	7.90E-04
undef	Nedd-specific protease (EC 3.4.22.-)	5.26	1.41E-04
MCH1	Permease [1.3e-41]	5.24	3.77E-06
FBP1	Fructose-1,6-bisphosphatase (EC 3.1.3.11) [6e-132]	5.19	2.38E-06
ERK1	Mitogen-activated protein kinase FUS3 (EC 2.7.1.37) [1e-120]	5.19	4.81E-04
DCD1	Deoxycytidylate deaminase (EC 3.5.4.12) [1.4e-24]	5.17	4.81E-05
XPA1	Xaa-Pro aminopeptidase (EC 3.4.11.9) [1e-131]	5.13	3.84E-04
undef	DNA damage checkpoint control protein RAD17 [4.2e-21]	4.93	9.82E-05
undef	[4.9e-12]	4.87	6.26E-04
LTP1	Protein tyrosine phosphatase (EC 3.1.3.48) [2.9e-44]	4.81	2.86E-06
TMT1	Trans-aconitate 5-methyltransferase (EC 2.1.1.-) [2.3e-42]	4.81	2.11E-03
undef	<not provided>	4.78	7.08E-05

undef	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	4.78	1.55E-02
undef	Sterol carrier protein [1.0e-13]	4.75	5.76E-06
undef	<not provided>	4.62	2.46E-05
	<not provided>	4.61	6.42E-04
Rpa1	Replication factor-A protein 1 [9e-156]	4.58	3.84E-04
undef	Peroxisomal membrane protein PMP47B [1.1e-19]	4.55	8.85E-05
undef	LSU ribosomal protein MRPL3 [1.5e-60]	4.53	1.84E-03
undef	Methionine aminopeptidase (EC 3.4.11.18) [2e-123]	4.47	8.72E-05
undef	Lipase 3 precursor (EC 3.1.1.-) [1.6e-72]	4.46	7.92E-06
RFA2	Replication factor-A protein 2 [2.0e-35]	4.41	1.01E-03
IAH1	Isoamyl acetate-hydrolyzing esterase (EC 3.1.-.-) [2.1e-39]	4.38	1.25E-05
THI73	Transporter, MFS superfamily [8e-133]	4.30	1.41E-04
PAP1	<not provided>	4.27	1.19E-06
undef	Phosphate system positive regulatory protein PHO81 [2e-102]	4.22	7.18E-06
undef	<not provided>	4.20	8.89E-06
undef	Putative serine threonine protein phosphatase	4.20	9.25E-05
undef	<not provided>	4.19	3.60E-05
CYK2	Cysteine synthase family protein [1.8e-59]	4.15	1.42E-06
undef	RNA export factor GLE1 Monofunctional riboflavin kinase (EC 2.7.1.26) [4.4e-71]	4.13	1.52E-05
undef	<not provided>	4.12	5.37E-07
undef	<not provided>	4.12	8.75E-04
RFC3	Replication factor C subunit [2e-120]	4.10	5.03E-05
undef	Transmembrane protein, GPR1 FUN34 yaaH family [3.3e-26]	4.04	4.69E-06
RBS1	CREB-binding protein [3.1e-17]	4.00	2.86E-05
ASP1	L-asparaginase I (EC 3.5.1.1) [8e-103]	3.95	7.94E-04
undef	<not provided>	3.95	4.89E-07
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	3.91	5.35E-07
undef	ATP-binding cassette multidrug transporter [0.0]	3.91	6.01E-05
FET4	Low-affinity Fe(II) transport protein [6e-115]	3.89	1.12E-02
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	3.86	5.33E-07
LCB5	SPHINGOSINE KINASE (EC 2.7.1.-) [9e-105]	3.84	1.94E-06
AXL2	AXL2 protein precursor [4.1e-86]	3.83	2.04E-04
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	3.83	1.78E-06
fthS	Methylenetetrahydrofolate dehydrogenase (NADP+) (EC 1.5.1.5) Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) Formate--tetrahydrofolate ligase (EC 6.3.4.3) [0.0]	3.83	1.07E-04
undef	<not provided>	3.79	7.96E-04
undef	Sulfite sensitivity protein SSU1 [6.0e-30]	3.79	5.20E-06
PEX6	Peroxisome biosynthesis protein PAS5 [8e-180]	3.77	6.42E-06
TOS1	Hypothetical protein	3.75	2.00E-04
undef	Peroxisome assembly protein PEX10 [2.6e-31]	3.71	3.75E-05
DAS2	Dihydroxy-acetone synthase (EC 2.2.1.3) [9e-104]	3.70	8.71E-07
BET4	Protein farnesyltransferase alpha subunit (EC 2.5.1.-) [1.1e-15]	3.70	3.60E-05
undef	Peroxisome assembly protein PEX10 [2.6e-31]	3.69	1.87E-05
AOX1	PP_AOX1 Alcohol oxidase (EC 1.1.3.13)	3.69	1.58E-06
PEX6	Peroxisome biosynthesis protein PAS5 [8e-180]	3.68	6.01E-06

PEX6	Peroxisome biosynthesis protein PAS5 [8e-180]	3.67	1.28E-06
PEX6	Peroxisome biosynthesis protein PAS5 [8e-180]	3.66	1.26E-06
undef	<not provided>	3.65	2.87E-04
AOX1	PP_AOX2 Alcohol oxidase (EC 1.1.3.13)	3.65	4.15E-07
HST2	Farnesyl pyrophosphate synthetase [6.0e-73]	3.65	7.18E-06
undef	Peroxisome assembly protein PEX10 [2.6e-31]	3.65	3.25E-05
DAS2	Dihydroxy-acetone synthase (EC 2.2.1.3) [7e-152]	3.64	9.65E-07
undef	Peroxisome assembly protein PEX10 [2.6e-31]	3.60	2.73E-05