Genetics of scleroglucan production by *Sclerotium rolfsii*

Vorgelegt von Dipl.-Ing.

Jochen Schmid

Von der Fakultät III – Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades
Doktor der Ingenieurswissenschaften
-Dr.-Ing.-

genehmigte Dissertation

Promotionsausschuss:

Vorsitzender: Prof. Dr. rer.nat. Roland Lauster

Gutachter: Prof. Dipl.-Ing. Dr. Ulf Stahl

Gutachter: Prof. Dr. Johannes Wöstemeyer

Tag der wissenschaftlichen Aussprache: 02.12.2008

Berlin 2008

D83

Danksagung

Die vorliegende Arbeit wurde im Zeitraum von April 2005 bis März 2008 im Fachgebiet Mikrobiologie und Genetik des Institutes für Biotechnologie der TU Berlin erstellt.

Mein besonderer Dank gilt Herrn Prof. Dr. Ulf Stahl für die Bereitstellung des Themas, seine großzügige, unermüdliche Unterstützung und die stete Bereitschaft zu konstruktiven Diskussionen.

Prof. Dr. Johannes Wöstemeyer danke ich sehr herzlich für die Übernahme des Gutachtens dieser Arbeit.

Mein weiterer Dank gilt Frau Dr. habil. Vera Meyer, in deren Arbeitsgruppe diese Arbeit angefertigt wurde. Sie stand mir als direkte Ansprechpartnerin auch aus der Ferne stets hilfreich zur Seite und hat viel zum Gelingen dieser Arbeit beigetragen. Vera ich wünsche Dir alles Gute.

Bei Frau Laura Funk bedanke ich mich sehr herzlich für die tatkräftige Unterstützung, sowie für ihr Interesse und Engagement in diese Arbeit und die stets gute Zeit im Labor.

Herrn Dr. Udo Schmidt, Frau Dr. Anja Spielvogel, Frau Dr. Cornelia Luban, Frau Eva Graf, Herrn Sean Patrick Riechers, danke ich ganz herzlich für die viele anregende Diskussion, nicht nur wissenschaftlicher Art. Bei Frau Birgit Baumann und Frau Susanne Engelhardt bedanke ich mich für die äußerst gute und erheiternde Atmosphäre im Institut und die sehr geselligen Mittagspausen.

Bei Herrn Thomas Bekel bedanke ich mich für die hilfreiche Unterstützung bei der Sequenzanalyse und für die Bereitstellung des von ihm entwickelten SAMS Systems, welches die Arbeit mit der Unmenge an Daten unglaublich erleichterte.

Für die finanzielle Unterstützung danke ich dem BMBF. Besonderer Dank gilt auch Herrn Dr. Volker Sieber welcher als Projektleiter fungierte und stets mit sehr guten Ideen und Hinweisen zum gelingen dieser Arbeit beigetragen hat.

Des Weiteren bedanke ich mich ganz herzlich bei Dr. Dirk Müller-Hagen für seine Unterstützung auch nach seinem Weggang, ohne Ihn wäre diese Arbeit nie so gelungen.
Ein weiteres Dankeschön auch an seine Frau Dr. Silke Müller-Hagen, für das sicherlich nicht immer einfache Korrekturlesen der Arbeit.
Ganz besonderer Dank gilt auch Frau Rita Waggad, für Ihren unermüdlichen Einsatz in der Bereitstellung von Dingen aller Art, ohne die manch ein Versuch nicht so schnell hätte durchgeführt werden können und auch für die guten Gespräche in den Freiräumen. Danke Rita.

Allen weiteren Mitarbeiterinnen und Mitarbeitern des Fachgebietes Mikrobiologie danke ich für die nette und kooperative Zusammenarbeit, insbesondere, Frau Roslin Bensmann (für die Korrekturen des Englischen) und Frau Sonja Leberecht, welche sich immer großartig für mich einsetzten.

Abschließender und überaus herzlicher Dank gebührt meinen Eltern, meiner Schwester Birgit und vor allem meiner Freundin Anja für deren stete Unterstützung und das unendliche Verständnis auch in schwierigen Zeiten. Danke!

Contents

List of Abbreviations

aa	Amino acids
AMT	*Agrobacterium tumefaciens* mediated transformation
AS	Acetosyringone
BDM	Bio dry mass
bp	Base pairs
CFU	Colonie forming unit
CPB	Citrate phosphate buffer
CTAB	Cetyltrimethylammonium bromide
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotidtriphosphate
dUTC	Desoxy-cytocine-5´-triphosphate
dUTP	Desoxy-uridine-5´-triphosphate
EDTA	Diaminoethanetetraacetic acid
EST	Expressed sequence tags
Fig	Figure
GFP	Green fluorescent protein
Glc	Glucose
GLOX	Glyoxalate cycle
GOX	Glycolate oxidase
gpd	Glyceraldehyde-3-phosphate dehydrogenase gene
hph	Hygromycin phosphotransferase gene
HPLC	High performance liquid chromatography
IPTG	Isopropyl-1-thio-ß-D-galactopyranosid
kb	Kilo bases
kDa	Kilo dalton
MOPS	3-[*N*-morpholino] propane-sulfonic acid
mRNA	messenger-RNA
nt	Nucleotide
OA	Oxalic acid
Oxdc	Oxalate Decarboxylase
OxoX	Oxalate oxidase
PCR	Polymerase chain reaction
PD	Potato Dextrose
PEG	Polyethylene glycol
PMT	PEG-mediated transformation of protoplasts
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rz	Ribozyme
siRNA	small interfering RNA
SSC	Saline sodium citrate
SSH	Suppression subtractive hybridisation
ssRNA	single stranded RNA
Tab	Table
TCA	Tricarboxylic acid cycle
tRNA	transfer RNA
X-Gal	5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid
Y	Pyrimidine
YE	Yeast extract

Coding of Nucleotides and Amino Acids

Nucleotides

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil
R	G or T
Y	T or C
K	G or T
M	A or C
S	G or C
W	A or T
B	G or T or C
D	G or A or T
H	A or C or T
V	G or C or A
N	A or G or C or T

Amino acids

A	Ala	Alanine
B	Asx	Aspartate or Asparagine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
Z	Glx	Glutamate or Glutamine
X		each amino acid

List of Figures

List of Tables and Supplemental Data

Supplemental Data

Excel sheets

Sheet A: Sequnces and tblastx hit results for all genes obtained by the SSH approach
Sheet B: List of clustered contigs obtained via KOG classification
Sheet C: Annotated contigs of *S. rolfsii* EST library, based on automatique (SAMS) and manual curation.
Sheet D: List of annotated contigs, differentially expressed in the various conditions with corresponding FunCat classification.

Text files

File A: Sequences of the 60-mer primers used for microarray hybridisation

I Introduction

The groundwork for modern fungal biotechnology was laid in the beginning of the twentieth century which was accompanied by advances in microbiology, biochemistry and fermentation technology. The pioneering works of Jokichi Takamine (production of amylase from koji mold *Aspergillus oryzae*, 1894), James Currie (development of fungal fermentation for citric acid production, 1917) and Alexander Fleming (discovery of penicillin production by *Penicillium notatum*, 1928) stimulated scientists to further explore fungal metabolic capacities and, moreover, prompted engineers to develop large-scale and controlled production processes for filamentous fungi. Improvements of fungal capacities to produce metabolites of interest were, however, mainly restricted to classical mutagenesis techniques. The development of recombinant DNA technologies for filamentous fungi, shown for the first time in 1979 for *Neurospora crassa*, was a milestone in obtaining insights into the molecular basis of product formation and to improve traditional fungal fermentations by genetic engineering.

The industrial relevance of filamentous fungi is based on their high capacity to produce primary and secondary metabolites as well as for secreting proteins, having a wide spectrum of activity such as hydrolases and proteases (Conesa 2001; Punt et al. 2002). Additionally, the fungal glycosylation machinery is capable of providing a more "mammalian-like" glycosylation pattern to proteins compared to the commonly used yeast hosts (Ward et al. 2004; Nevalainen et al. 2005; Karnaukhova et al. 2007), making filamentous fungi very attractive for the production of proteins used in medical applications. Secretion is, in particular, related to fungal morphology as it is thought to take place at the growing fungal tip (Torralba et al. 1998; Khalaj et al. 2001; Fischer et al. 2008). One focus of the current research is thus on fungal morphology to improve the secretory capacity of industrially used fungi (Papagianni et al. 2003; Grimm et al. 2005; Meyer et al. 2008).

The following three chapters are devoted to three important areas of research of genetic engineering in filamentous fungi, all aiming at the improved application of these organisms in biotechnology.

Chapter II summarizes current genomic approaches for filamentous fungi and discusses their benefit for the identification of new commercially interesting products.

Chapter III highlights the progress made in the post-genomic era, concerning new omic techniques as well as challenges and future perspectives related.

Chapter IV deals with genetic and metabolic engineering tools applicable nowadays to filamentous fungi and highlights the progress made for different transformation techniques, gene knockout or gene knockdown approaches.

II Fungal genomics: Advances in exploring sequence data

Identification of putative new and industrial applicable enzymes or secondary metabolites e.g. of medical interest, requires the evaluation of the genetic potential of a given organism. Sequence data has to be screened for genes coding for desired enzymes involved in the pathway of interest. To meet the demand of sequence information, more and more fungal genomes have been sequenced since the first genome of the yeast model organism *Saccharomyces cerevisiae* was published in 1996 (Goffeau et al.).

Generally, genome sequencing projects continue to unravel the genetic capabilities of many organisms, resulting in more than 800 fully sequenced genomes currently being available, 25 of these are fungal genomes. More than 2,500 sequencing projects are ongoing including more than 300 fungal projects (http://www.genomesonline.org/goldstatistics.htm), whereby industrial and medically interesting fungi are mainly in the focus of genome sequencing projects (Table 1).

> These genomes have been partially or completely sequenced, or they are currently being re-sequenced. However, even fully sequenced genomes such as for *S. cerevisiae* are still undergoing correction in regard of sequence details, splicing or improved annotation (Jones 2007). Comparative genomics is a very useful tool to compare less well-studied species with better understood model organisms and to clarify hypothetical genes by computational annotation. Furthermore, they help to identify differences between closely related species as regards pathogenicity, secondary metabolism or other properties (reviewed by Jones 2007). For example, the genome of *Trichoderma reesei* is considered to be a promising candidate for biofuel production from cellulose fibres. It should eventually become a monosaccharide producing factory through the use of rational metabolic engineering (Martinez et al. 2008).

'Gene mining' as a tool for unravelling new proteins masked in fungal genomes is an approach which focuses on industrial exploitation of currently unknown proteins and enzymes. For example, over 200 new proteases have been identified during annotation of the *A. niger* genome, of which two have already been commercialized - a protease preventing chill-haze in beer (Lopez and Edens 2005) and a protease used for the production of sport drinks (Edens et al. 2005).

> Detailed analysis of the *A. niger* genome combined with the reconstruction of the metabolic networks identified many gene duplications, transporters and intra- and extracellular enzymes providing new insights into the efficient citric acid production by *A. niger* (Pel et al. 2007). The *A oryzae* genome was found to be extremely rich in genes involved in biomass degradation and primary and secondary metabolism (Abe et al. 2006; Kobayashi et al. 2007). New α-amylases

and α-glucosidases have been found. The genome sequence has revealed 134 peptidase genes, in contrast to 18 peptidases hitherto known (Kobayashi et al. 2007). Comparative genomics lead to improved functional annotation of the genome of *A. nidulans* and deeper insights in pathway modelling (David et al. 2008).

Furthermore, the so called 'genome based strain reconstruction', based on comparison of high producing strains with the wild type strain, lead to reconstructed strains superior to former production strains (Ohnishi et al. 2002; Adrio and Demain 2006).

An absolute novelty decrypted by genome data and first metabolic approaches was the identification of (silent) gene clusters for mycotoxin production in fungal strains which have been regarded to be safe for many years. Species as *A. oryzae*, *A. niger* and *A. sojae* were shown to produce or own the opportunity of mycotoxin synthesis dozing in the genome (Jones 2007; Pel et al. 2007). Dependent on sequencing results, these facts indicate that using these fungi (and other species) could be more risky than initially thought, and might thus require new risk assessments (Abe et al. 2006).

'Metagenomics' or 'environmental sequencing' involves sequencing of whole ecosystems, independent of specific organisms. This boosted the amount of sequence data available (Raes et al. 2007). Metagenomics can be used to identify and analyze fungal communities (O'Brien et al. 2005), explicitly chosen proteins, mostly polysaccharide-modifying enzymes as well as proteases and nitrilases (Streit and Schmitz 2004; Blackwood et al. 2007), or to sequence fungi which cannot yet be grown in culture and therefore cannot be analyzed by traditional means. Environmental sequencing will play a major role in the decryption of complex communities and synergisms among different organisms in their natural habitats.

'Functional sequencing' allows insight into the real natural behaviour over and above the limiting conditions of cultivating strains under laboratory conditions and provides excellent opportunities to identify new and as yet unknown proteins and enzymes.

III Post-genomic approaches to unravel the metabolism of filamentous fungi

New omic-tools allow fast and easy decryption of fungal genomes and the metabolites produced. These tools can be used to explore new commercially interesting products and to improve metabolic fluxes by rational design. The main characteristic of the so-called 'post-genomic' era is the need for methods and tools to analyze the huge amount of data produced. The suffix 'omics' was created in the 1990's in the field of bioinformatics and marks the realization of the importance of information processing in biology; omics is a general term for a broad discipline of science and engineering for analyzing the interaction of biological

information in various 'omes'. These include the genome, transcriptome, proteome, metabolome, expressome, interactome and many more fields which have been defined such as glycome, ionome, lipidome or even physiome and reactome.

> In most cases, genomics, transcriptomics and proteomics together with metabolomics are used to identify theoretical knockout events or metabolic fluxes in pathways of interest. The main focus is on gathering information for engineering metabolic networks to manipulate the regulatory mechanisms of the entirety of biological processes.

The term 'systems biology' is often used in combination with the omics and is the 'biology' that focuses on complex systems in life. It is a holistic approach allowing the analysis of the topology of biochemical and signalling networks involved in cellular responses also in addition to being able to capture the dynamics of the response. While different omics deliver only a piece of the puzzle, it is hoped that systems biology will eventually map the complete picture (Siliang Zhang 2006).

Transcriptomics

RNA based technologies have been developed enormously in the last few years whereby two different strategies have emerged: i) strategies based on the knowledge of DNA sequences (microarrays), and ii) strategies which do not require any sequence information such as suppression subtractive hybridisation (SSH) and serial analysis of gene expression (SAGE) (Breakspear and Momany 2007). The first fifty fungal microarrays were reviewed by Breakspear and Momany (2007), describing the development of fungal array approaches. Interestingly, they highlight the lack of basidiomycete microarray experiments (only three have been performed to date).

Comparative transcriptional analysis of *A. oryzae* using DNA microarrays has indicated the potential of new proteins identified, and is as a tool which can be used to develop industrial systems (Abe et al. 2006). Anderson et al (2008b) used a comparative transcriptomic approach where one Gene Chip was developed for transcriptome analysis of triplicate batch cultivations of *A. nidulans*, *A. niger* and *A. oryzae*.

> They were able to identify 23 genes conserved across *Aspergillus sp.* (mainly sugar transporters and enzymes) and 365 genes which were differently expressed in only two of the *Aspergilli.* Thus, such a cross-species study based on transcriptome analysis can be used for the identification of new enzymes or even strains. In a former publication the authors described the use of transcriptome data to perform metabolic network modelling in *A. niger*, which will be explained in detail in the metabolomics section of this chapter (Andersen et al. 2008).

SSH and SAGE approaches also helped to understand the infection process of plant pathogen fungi and mycoparasitic fungi. (Schulze et al. 2004; Carpenter et al. 2005; Larraya et al. 2005; Matsumura et al. 2005; Gowda et al. 2006; Salvianti et al. 2008; Yu et al. 2008). Furthermore, transcriptomic analysis of different culture conditions will help to identify genes involved in product formation and may lead to overproducing strains 'debugged' from bottlenecks or unnecessary genes (Foreman et al. 2003). Also, insights in energy and primary metabolism has been achieved by transcriptional analysis (Maeda et al. 2004). The method of transcriptional profiling has also been used to reveal previously unknown, but relevant pathways (van der Werf 2005; Rautio et al. 2006).

Proteomics

Transcriptomic approaches are mostly based on the straight forward development of microarray chips, whereas the proteomic fields require analytical techniques such as mass spectrometry (MS) or nuclear magnetic field resonance. The latest review of *Aspergillus* proteomics provides a summary of all information on this technique in the last years (Kim et al. 2008b).

> In 10 different studies using *Aspergillus* proteomics, a total of 28 cell surface proteins, 102 secreted and 139 intracellular proteins have been identified. A proteome map highlighting proteins identified in major metabolic pathway has been summarized by Kim et al. (2008b).

For *Phanerochaete chrysosporium*, more than 1,100 intracellular and 300 mitochondrial proteins were resolved in 2D gels and the metabolic flux shift was determined by comparative proteomics for growth on vanillin. By using vanillin, which is the decomposition product of lignin, the lignin degradation by white rot fungi can be studied. Many more proteomic approaches have been performed using fungi to identify new products for industrial applications as recently reviewed by Kim et al (2007).

Metabolomics

It is assumed that around 10,000 secondary metabolites may be produced by fungi, examples of which are found in *Aspergillus* and *Penicillium*; however, less than 10% are known (Smedsgaard and Nielsen 2005). Much understanding of the central metabolism and regulation in less-studied filamentous fungi can be learned from comparative metabolite profiling and metabolomics of yeast and filamentous fungi (Smedsgaard and Nielsen 2005). Metabolomics is considered to be more sensitive than transcriptomics or proteomics due to the measuring of metabolite concentrations caused by environmental perturbations that may

not affect transcription or protein level, an example being enzyme activity levels (Oliver et al. 1998). The use of metabolomics means that methods are available that can simultaneously determine over 1,000 charged metabolites using capillary electrophoresis – MS (Soga 2007). Even techniques which simulate the whole cell metabolism have been developed (Ishii et al. 2004).

> *Aspergillus nidulans* represents an important model organism for studies of cell biology and gene regulation. Kouskoumvekaki et al. (2008) initiated a metabolomics approach in recombinant *Aspergilli* for clustering and classification. More than 450 detected metabolites were analysed and resulted in the identification of seven putative biomarkers by which classification into genotype was possible. Thus, metabolite profiling is a powerful tool for the classification of filamentous fungi as well as for the identification of targets for metabolic engineering (Kouskoumvekaki et al. 2008).

A metabolic model integration of the bibliome, genome, metabolome and reactome was constructed for *A. niger* (Andersen et al. 2008). A complete metabolic network was presented which shows great potential for expanding the use of *A. niger* as a high-yield production platform. By the multi omic approach, the use of precursors was identified to increase productivity. In order to combine the knowledge of the underlying metabolic system and the analysis of the data, novel computational approaches and methods that go beyond commonly used statistical techniques are required (Van Dien and Schilling 2006).

> There has been progress made using methods for analyzing quantitative metabolomics data in the context of the entire network. Methods based on Gibbs energies of formation, the second law of thermodynamics and on known direction reactions within the cell allow dynamic analysis of metabolites in the cell (Kummel et al. 2006).

Mainly, the metabolic flux analysis (MFA) has become a fundamental tool in metabolic engineering to elucidate the metabolic state of the cell and has been applied to various biotechnological processes (Spegel et al. 2007). The “conventional MFA” is based on mass balances using stoichiometric constraints coupled to extracellular product formation rates and is a widely used technique (Vallino and Stephanopoulos 2000).

Applying the full range of omics technologies for strain improvement, it would be desirable if the ‘concept of sample’ was shared among these technologies. In particular, to focus on a biological sample that is prepared for use in a specific omics assay (Morrison et al. 2006). A common data file format should be found to enable fast and secure file sharing and to reduce redundant data and the potential for errors. The main issue as regards the combined analysis of the metabolomics data with other omics results is the data integration problem (Mendes 2006).

There is also the call for standards in sample collection and processing to obtain reliable results and to avoid significant error in omics data. It is essential to use the same biological sample when confronted with diverse omic approaches (Weckwerth et al. 2004; Martins et al. 2007).

This has resulted in many omics standardization initiatives aiming at the development of new concepts to overcome the problems of data confusion and sample correlation (Morrison et al. 2006; Jones et al. 2007). Also claimed has been the necessity to develop common databases for storage of metabolomics data (Kouskoumvekaki et al. 2008).

IV Metabolic engineering: Finding the optimum genetic strategy

For successful optimization of a fungal metabolism, several issues of engineering strategy have to be taken into account. Every approach depends on the organism of choice, the target to be engineered and the aim of the intervention. From various genetic engineering tools available nowadays for filamentous fungi, the most suitable has to be chosen in order to specifically and efficiently improve the strain of interest.

Choosing the right transformation technique

Highly sophisticated genetic methods are available nowadays for filamentous fungi; however, safe and suitable transformation techniques are fundamental prerequisites for genetic engineering approaches. Different transformation techniques enable scientists to design and develop rational metabolic engineering strategies for industrially important fungal species. Linear DNA or plasmids are transferred into the fungal cell by chemical treatment of protoplasts using Ca^{2+} and polyethylene glycol (Spegel et al. 2007), by physical treatment such as electroporation and biolistic procedures or by using *Agrobacterium tumefaciens* mediated transformation (reviewed by Fincham 1989; Ruiz-Diez 2002; Casas-Flores et al. 2004; Michielse et al. 2005b; Meyer 2008).

Historically, PEG mediated transformation of protoplasts was the first transformation technique established in 1978 for the budding yeast *S. cerevisiae* (Hinnen et al. 1978). This method has afterwards been used in filamentous fungi (Punt et al. 1987; Stahl et al. 1987; Fincham 1989; Ruiz-Diez 2002). Progress in the last few decades has been achieved in establishing alternative transformation methods to overcome the limits related to protoplast formation, which is highly dependent on the quality of the cell wall degrading enzyme preparation and often only leads to low transformation rates (Michielse et al. 2005b).
In most cases, asexual spores such as conidia or sporangiospores are used as they are considered to be the most favourable constituent for transformation. However, if these are not available whole mycelium has to be transformed, making the procedure more difficult and laborious. In addition, the multinuclear state of some conidia, mycelia and protoplasts hampers the selection of transformants and makes the selection process tedious (Deed and Seviour 1989; Farina et al. 2004). When comparing the transformation rates of yeast and filamentous fungi, it becomes evident that transformation of yeast is usually much more efficient (Ruiz-Diez 2002).

New systems such as electroporation have been developed. This is actually a method often used for transformation of filamentous fungi (reviewed by Ruiz-Diez 2002). There are three possible fungal structures that can be transformed by means of electroporation: protoplasts, conidia or young germlings (Chakraborty et al. 1991; Ruiz-Diez 2002).

Biolistic transformation was introduced in 1987 (Sanford 1987; Klein et al. 1992) and has been applied to a number of filamentous fungi (Ruiz-Diez 2002). This method is especially beneficial for those organisms which cannot be transformed by *Agrobacterium*-mediated transformation (Cramton et al.). The use of *A. tumefaciens* as a mediator of foreign DNA into fungal cells is a tool which has been adapted for many different fungal organisms in the last few years (Michielse et al. 2005b). The transformation rate is up to 100–1,000 times higher than with protoplastation and can be used for most fungi which cannot be transformed by other methods or where protoplasts do not regenerate sufficiently (Meyer et al. 2003b; Casas-Flores et al. 2004; MacKenzie 2004). However, even this method is not suitable for all fungi. There are also reports on less successful attempts, for example in *A. niger* (Michielse et al. 2005b).

> AMT and all other transformation techniques cannot be used for every fungal organism without first being adapted. This may be explained by major differences found in fungal genomes such as *A. nidulans* (Galagan et al. 2005), *A. oryzae* (Machida et al. 2005) and *A. fumigatus* (Nierman et al. 2005). Analysis of their genomes has shown that there is only 68% of amino acids identity shared by all three species (Galagan et al. 2005), an evolutionary distance comparable to that between human and fish (Dujon et al. 2004; Fedorova et al. 2008). Roughly 70% of *A. nidulans* genes could be mapped to a syntenic block with either *A. fumigatus* or *A. oryzae*, with about 50% of *A. nidulans* in conserved synteny across all three species (Galagan et al. 2005), suggesting that this could be one reason for the observation that each transformation method has to be adapted and optimized for every single species and even strain.

Apart from transformation approaches, sexual crossing is a useful tool to improve the phenotype of filamentous fungi. Unfortunately, most medical or industrial interesting fungi lack a sexual cycle, precluding them from classical genetics. However, insights into the genome of *A. fumigatus* and *A. oryzae* revealed that both are potentially capable for sexual reproduction (Dyer and Paoletti 2005; Galagan et al. 2005; Paoletti et al. 2005), suggesting that classical genetic tools can also be established for theses fungi. These findings challenge the whole taxon of *fungi imperfecti* and even highlight the power of genome analysis for taxonomical (re)classification.

A very important and interesting question as regard to the different transformation systems is the fate of the introduced DNA. DNA which has been introduced will be either maintained autonomously (occurs rarely) or is integrated into the genome via homologous or

heterologous recombination. Homologous recombination targets the foreign DNA to regions showing sufficient homology, whereby the DNA becomes integrated into the genome as single or tandem copy. In contrast, heterologous recombination events occur randomly, resulting in single or multicopy integrations (de Groot et al. 1998; Malonek and Meinhardt 2001; Mullins and Kang 2001).

> In the case of AMT, mainly single-copy integration events occur, whereas mainly multi-copy integrations are observed after transformation by PMT. This observation can have an important impact on the choice of a suitable transformation system for the design of a metabolic engineering strategy. AMT can be the method of choice for targeted integration into the genome and PMT can be used for ectopic and multi-copy integration to improve protein expression in the strain of interest (Meyer 2008). The latter strategy has indeed been shown to be a powerful tool for protein over-expression in *Aspergillus* and *Trichoderma* (Verdoes 1995; Lee et al. 1998; Askolin et al. 2001).

In addition, it has been found that AMT can increase homologous recombination frequency for example in *A. awamori* (Michielse et al. 2005a). This observation pointed to new application opportunities of AMT as a suitable method for directed and insertional mutagenesis (Michielse et al. 2005b; Sugui et al. 2005; Lee and Bostock 2006; Betts et al. 2007).

V Enhancing gene targeting efficiency

Metabolic engineering can be used to delete genes of unwanted side pathways, thereby redirecting the metabolic flux to the required product forming pathway. In addition, targeted integration of genes to a genomic locus known to strengthen transcription is one strategy to enhance protein expression and thereby to improve productivity of an industrial process. Besides, gene targeting is also the method used for functional genomics. The mode of integration of foreign DNA is determined by two competing processes – homologous recombination (HR) and non-homologous end joining pathway (NHEJ) (Dudasova et al. 2004; Krogh and Symington 2004). The low rates of HR events usually observed can render some filamentous fungi unattractive for many industrial applications.

However, advances in fungal gene targeting have recently been achieved by suppressing the NHEJ-pathway (for review see Meyer, 2008). As summarised in Table 2, a dramatic increase in HR efficiency has been reported when strains were used in which the NHEJ-pathway was inactivated (Kooistra et al. 2004; Ninomiya et al. 2004; Goins et al. 2006; Ishibashi et al. 2006; Krappmann et al. 2006; Nayak et al. 2006; Poggeler and Kuck 2006; Takahashi et al. 2006; Meyer et al. 2007).

Phenotypic analysis of defective NHEJ strains revealed that these strains showed higher sensitivity to various toxins and irradiation (Ninomiya et al. 2004; da Silva Ferreira et al. 2006; Meyer et al. 2007). Therefore, some unexpected growth behaviour could appear, and the more elegant solution would be a transient silencing of the NHEJ-pathway as recently reported for *Candida glabrata* and *A. nidulans* (Ueno et al. 2007; Nielsen et al. 2008).

RNA-based tools for metabolic engineering

RNA technology is an attractive alternative to DNA-based methods to silence gene expression post-transcriptionally and thereby to control unwanted metabolic pathways. Different RNA techniques such as antisense RNA, RNAi and hammerhead ribozymes have been shown to be valuable tools for filamentous fungi (Table 2). These methods provide the advantage of not deleting a gene, thereby bypassing the possibility of lethal or other unwanted effects on the organism.

RNA based methods are especially valuable when i) gene targeting approaches fail, ii) multiple copies of a gene of interest are present in the genome or iii) isogenes might compensate for the knockout of the deleted gene (Akashi et al. 2005). For example, silencing of a whole gene family using only a single antisense RNA construct has been described for *A. oryzae* (Yamada et al. 2007). Another advantage of the RNAi mechanism is its locus independence due to its mediation by a mobile *trans*-acting signal in the cytoplasm. Consequently, this mechanisms can be used in fungi which have multinuclear hyphae or a low targeting efficiency, even in heterokaryotic fungal strains, RNA-based downregulation of genes is possible (Nakayashiki 2005; de Jong et al. 2006).

Antisense RNA gene silencing is performed by using single stranded RNA which is complementary to an mRNA strand transcribed within the cell. Formation of a complementary mRNA hybrid physically blocks the translation machinery and thereby stops translation of the endogenous mRNA.

> Successful gene silencing using artificial antisense constructs have been reported for different filamentous fungi (Zheng et al. 1998; Kitamoto et al. 1999; Bautista et al. 2000; Ngiam et al. 2000; Lombrana et al. 2004; Blanco and Judelson 2005). For example, antisense silencing of the protease aspergillopepsin B resulted in a reduction of 10–70% of protease levels and in a 30% increase in heterologous thaumatin production in *A. awamori* (Moralejo et al. 2002).

However, antisense-mediated reduction of gene expression to zero levels has not been reported to date. Nevertheless, exactly this phenomenon can be used for knocking down gene expression instead of knocking it out.

For instance, the wide-domain transcription factor CreA, the key component of carbon catabolite repression in *Aspergillus* (Dowzer and Kelly 1991; Ruijter and Visser 1997; Shroff et al. 1997), negatively regulates a number of industrially important enzymes. Baustia et al (2000) have reported partial suppression of *creA* expression in *A. nidulans* by its antisense molecule (about 50% reduced expression was estimated) yielding in partial alleviation of glucose repression and thereby in a substantial increase of the productivities of intra- and extracellular glucose-repressible enzymes.

Another RNA-based technology for filamentous fungi is the use of catalytic RNA molecules, termed ribozymes. The spliceosome and ribosomes are two examples for naturally occurring ribozymes. The so-called hammerhead ribozyme is the smallest and best studied class of catalytic RNAs (Akashi et al. 2005; Mueller et al. 2006). The hammerhead ribozyme and other ribozymes are antisense RNA molecules. They function by binding to the target RNA moiety through Watson-Crick base pairing and inactivate it by cleaving the phosphodiester backbone at a specific cutting site. The substrate-recognition arms of hammerhead ribozymes are engineered so that the arms are rendered complementary to any chosen RNA, enabling the ribozyme to bind to its target. The functionality of hammerhead ribozymes as a tool for RNA-based technology on gene expression has been shown for bacterial, yeast, plant and mammalian systems (Bussiere et al. 2003; Akashi et al. 2005; Isaacs et al. 2006). Mueller et al. (2006) have recently provided a proof of principle for filamentous fungi.

Congruent to other systems, it has been shown that ribozymes, targeting the 5'-region of a substrate mRNA can lead to complete gene silencing in A. giganteus, whereas ribozymes targeting the 3'-region only lead to a partial reduction (about 20–50%).

RNA interference (RNAi) is a naturally occurring post-transcriptional gene silencing phenomenon, first described in *Caenorhabditi elegans* and thereafter in other organisms such as *N. crassa* (quelling, Romano and Macino 1992), plants ('co-suppression', Napoli et al. 1990) and animals ('RNA interference', Elbashir et al. 2001a). The concept of RNAi can be used for artificial gene silencing in nearly all organisms, even in filamentous fungi. By this method, double stranded RNA (dsRNA) trans-genetically delivered to the fungal interior, is cleaved by Dicer (Typ-III-Ribonuclease) into 2–26 nt long small interfering RNAs (siRNA). Dicer is always associated with Argonaute proteins (binds targeted si/miRNA) and acts by generating siRNA molecules which in turn target mRNAs to be silenced. Dicer cleaving products get incorporated into the ribonucleoprotein complex (RISC) (Hammond et al. 2000; Bernstein et al. 2001). Homologous mRNAs are subsequently recognized and degraded via complementary base pairing by means of incorporated siRNA in the RISC (Zamore et al. 2000; Elbashir et al. 2001b). In some organisms, a RNA-dependent RNA polymerase (RdRP)

can use the antisense siRNA to prime the conversion of endogenous mRNA into dsRNA amplifying the silencing signal (Forrest 2004).

The most effective way of posttranscriptional gene silencing in filamentous fungi can be achieved by using ectopically integrated RNAi constructs which usually code for 'double stranded RNA' molecules. These molecules are self-complementary hairpin RNAs, formed by an inverted repeat which is interrupted by a spacer sequence, and are identical to part of the endogenous sequence being targeted (Mouyna et al. 2004). Insertion of an intron in the spacer sequence greatly increased silencing efficiency in *N. crassa*, possibly due to an enhanced export of the hairpin from the nucleus during splicing (Goldoni et al. 2004). Remarkably, some fungal strains, e.g. *Ustilago maydis*, *Candida albicans* and *S. cerevisiae* lack components of the RNAi silencing machinery, indicating that this tool is not applicable for these organisms (Nakayashiki 2005). As summarized in Table 2, specific inhibition of gene expression by RNAi has been shown to be suitable for a multitude of filamentous fungi such as *A. nidulans* (Hammond and Keller 2005; Khatri and Rajam 2007), *A. fumigatus* (Bromley et al. 2006; Henry et al. 2007), *A. oryzae* (Yamada et al. 2007), *Coprinus cinereus* (Namekawa et al. 2005; Walti et al. 2006), *Fusarium solani* (Ha et al. 2006), *Magnaportae oryzae* (Kadotani et al. 2003,. 2004; Nakayashiki et al. 2005; Caracuel-Rios and Talbot 2008), *N. crassa* (Goldoni et al. 2004) and *Schizophyllum commune* (de Jong et al. 2006). Similar to antisense strategies, RNAi-induced silencing of fungal gene expression was most often found to be incomplete (maximal reduction up to 10% of wild type level) and full knockout phenotypes were seldom observed.

> A striking disadvantage of RNAi-based gene silencing is the instability of the silencing construct and the possibility of co-silencing unwanted genes ('off targets'), showing partial sequence homology to the target gene. Many transformants loose the RNAi construct after prolonged cultivation.

Chimeric constructs with two genes in tandem can result in very different silencing efficacy (Goldoni et al. 2004; Nakayashiki et al. 2005; Henry et al. 2007). In the case of *A. fumigatus,* it has been reported that approximately 50% of the transformants lack the complete RNAi construct or part of it after prolonged cultivation (Henry et al. 2007). One possible explanation for this phenomenon might be the loss of one of the inverted repeats after the first mitotic event (Henry et al. 2007).

Still, advances in using RNAi approaches have been achieved during the last years. For example, success has been reported by using inducible RNAi constructs in *A. fumigatus* (Khalaj et al. 2007) or by using new up take methods of artificial siRNA constructs in *A. nidulans* (Khatri and Rajam 2007). Nevertheless, the possibility of off target effects can

form an obstacle and future work on the approach has to address the question what is the optimum sequence length and the minimum homology to avoid any unwanted co-silencing.

VI Concluding remarks and prospects

The fungal post-genomic era is still in its infancy, however, the real power of the omic approaches is the possibility to analyze cellular processes and responses on different levels, including DNA, RNA, protein and metabolite levels (Shulaev et al. 2008).

By integrated analysis of these levels several important features of metabolic regulation has been and will be identified (Le Lay et al. 2006; Shulaev et al. 2008). Future strategies will combine all fields of omics, and will allow unravelling the dynamics of cellular metabolic activities in various filamentous fungi. The identification of new proteins, enzymes, pathways and their involvement in metabolic networks as well as ‘classic genetic’ techniques and new metabolic engineering approaches will eventually enable scientist to develop optimum production strains. The use of the new and highly sophisticated omic methods developed in the fungal post-genomic era will open the floodgates towards high-throughput analysis and efficient rational metabolic engineering approaches. The knowledge gained leads to the improvement of industrial biotechnological processes, and will help to meet the increasing need for sustainable fungal bio-products. In order to avoid bottlenecks in the post-genomic era, standardised methods and protocols for sampling, sample processing, sample collection, sample handling and integration into databanks, have to be established.

Table 1: Genome sequencing status of selected filamentous fungi

Organism	Strain	Remark	Relevance	URL
Aspergillus clavatus	NRRL 1	annotated	medical	http://www.tigr.org/tdb/e2k1/acla1/
Aspergillus flavus	NRRL 3357	annotated	medical	http://www.Aspergillusflavus.org/genomics/
Aspergillus fumigatus	Af293	annotated	medical	http://www.tigr.org/tdb/e2k1/afu1/intro.shtml
Aspergillus nidulans	FGSC A4	annotated	model organism	http://www.broad.mit.edu/annotation/genome/Aspergillus_nidulans/Home.html
Aspergillus niger	ATCC 1015	annotated	industrial	http://genome.jgi-psf.org/Aspni1/Aspni1.home.html
Aspergillus niger	CBS 513.88	annotated	industrial	http://www.dsm.com/en_US/html/dfs/genomics_aniger.htm
Aspergillus oryzae	RIB40	annotated	industrial	http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao
Aspergillus terreus	NIH 2624	annotated	medical	http://www.broad.mit.edu/annotation/genome/Aspergillus_terreus/Home.html
Coprinus cinereus	7#130	annotated	model organism	http://www.broad.mit.edu/annotation/genome/Coprinus_cinereus/Home.html
Fusarium oxysporum	FGSC 4286	annotated	plant pathogen	http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html
Laccaria bicolor	S238N-H82	annotated	symbiotic	http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html
Magnaporthe grisea	70-15	annotated	plant pathogen	http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/Home.html
Neosatorya fisheri	NRRL 181	annotated	medical	http://www.tigr.org/tdb/e2k1/nfa1/
Neurospora crassa	OR74A	annotated	model organism	http://www.broad.mit.edu/annotation/genome/Neurospora/Home.html
Phanerochaete chrysosporium	RP-78	draft assembly	industrial	http://genome.jgi-psf.org/whiterot1/whiterot1.home.html
Sclerotinia sclerotiorum	1980	annotated	plant pathogen	http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html
Trichoderma reesei	QM6a	annotated	industrial	http://genome.jgi-psf.org/Trire2/Trire2.home.html
Ustilago maydis	521	annotated	plant pathogen	http://www.broad.mit.edu/annotation/genome/ustilago_maydis/Home.html

Table 2: Genetic tools applicable to filamentous fungi for gene targeting and silencing

Organism	Reference	Efficiency
Aspergillus awamori		
antisense RNA	Moralejo et al. 2002; Lombrana et al. 2004	**80%**
Aspergillus flavus		
RNAi	McDonald et al. 2005	**<~90%**
improved gene targeting	Chang 2008	**96%**
Aspergillus fumigatus		
RNAi	Bromley et al. 2006; Henry et al. 2007	**~80%**
improved gene targeting	da Silva Ferreira et al. 2006; Krappmann et al. 2006	**75–96%**
Aspergillus giganteus		
Ribozyme	Mueller et al. 2006	**20–100%**
Aspergillus nidulans		
RNAi	Khatri and Rajam 2007	**~40%**
antisense RNA	Bautista et al. 2000; Hoffmann et al. 2000; Herrmann et al. 2006	**>50–90%**
improved gene targeting	Hammond and Keller 2005	**~90%**
Aspergillus niger		
RNAi	Nayak et al. 2006) (Barnes et al. 2008	**21–82%**
antisense RNA	Ngiam et al. 2000	**60–70%**
improved gene targeting	Meyer et al. 2007; Barnes et al. 2008	**~80%**
Aspergillus oryzae		
RNAi	Yamada et al. 2007	**90%**
antisense RNA	Zheng et al. 1998; Kitamoto et al. 1999	**75–80%**
improved gene targeting		**~63–87%**
Aspergillus parasiticus		
RNAi	McDonald et al. 2005	**~90%**
improved gene targeting	Chang; Chang 2008	**96%**
Aspergillus sojae		
improved gene targeting	Kadotani et al. 2003; Nakayashiki 2005	**64–75%**
Coprinopsis cinerea		
RNAi	Walti et al. 2006	**90%**
Cryphonectria parasitica		
improved gene targeting	Lan et al. 2008	**80–96%**
Fusarium solani		
RNAi	Ha et al. 2006	**>98%**
Magnaporthe oryzae		
RNAi	Kadotani et al. 2003; Nakayashiki et al. 2005; Caracuel-Rios and Talbot 2008	**70–90%**
antisense RNA	Kadotani et al. 2003; Nguyen et al. 2008	
improved gene targeting	Villalba et al. 2008	**>80%**
Neurospora crassa		
RNAi	Cogoni et al. 1996; Goldoni et al. 2004; Choudhary et al. 2007	**11–49%**
antisense RNA	Tentler et al. 1997; Kramer et al. 2003; W. Fecke 2008	**50–80%**
improved gene targeting	Ishibashi et al. 2006	**91-100%**
Penicillium expansum		
RNAi	Schumann and Hertweck 2007	**~95%**
antisense RNA	Garcia-Rico et al. 2007	**~95%**
Podospora anserina		
RNAi	El-Khoury et al. 2008	**100%**
Schizophyllum commune		
RNAi	de Jong et al. 2006	**80%**
Trichoderma harzianum		
RNAi	Cardoza et al. 2007	**71–98%**
Trichoderma reseei		
antisense RNA	Wang et al. 2005	**65%**

1 Scleroglucan: a versatile polysaccharide of biotechnological value

The plant pathogen basidiomycete *Sclerotium rolfsii*, which causes the so called Southern Blight in many different plants (Paintin 1928; Jem 1974; Wydra 1996), is known to produce the exopolysaccharide scleroglucan. This polysaccharide shows remarkable rheological and viscous properties rendering the substance as a multipurpose compound for many industrial applications, ranging from oil recovery over food industry to cosmetics and medical applications. Whereas the physicochemical properties of scleroglucan are well understood, only very little information is available on the biosynthesis of scleroglucan formation by *S. rolfsii* and only hypothetical approaches have been performed for describing the biochemical pathway of scleroglucan synthesis yet (Rodgers 1973). During scleroglucan production, the by-product oxalate is synthesised, which on the one hand lowers the productivity of the process and on the other hand negatively interferes with downstream processing of scleroglucan.

In the following chapter, the current state of the art with respect to scleroglucan characteristics, biosynthesis and production will be summarized.

1.1 Structure and properties of scleroglucan

Scleroglucan is a water-soluble homopolymer composed of (1→3)-ß-linked glucopyranose backbone with single (1→6)-ß-linked glucopyranosyl branches on every third subunit. The chemical structure of the four glucose repeating units of scleroglucan as established by NMR analysis (Brigand 1993) is depicted in Figure 1.

Dissolved scleroglucan chains assume a rod-like triple helical structure (Yanaki 1981; Yanaki 1983), in which the D-glucosidic side groups are on the outside and prevent the helices from coming close to each other and from aggregating.

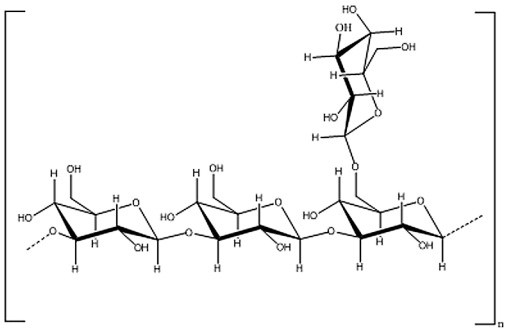

Figure 1: Chemical structure of scleroglucan.

Aqueous solutions of scleroglucan show a very high viscosity, the molecular weight is around 540,000 kDa. Scleroglucan shows remarkable rheological properties and stability over a wide range of pH (1–11), salinities and temperatures making this neutral non-ionic polysaccharide suitable for a number of various technical applications. Below 10 °C, scleroglucan solutions have a semi-colloid appearance which disappears on shaking or heating. Solutions of scleroglucan have a pseudoplastic behaviour with a high yield value, resulting in solutions of high suspension power with good pouring properties. The high yield value of scleroglucan is responsible for the extreme effectivity in holding particles in suspension, in static as well as in dynamic conditions, without the risk of sedimentation. The triple helix structure is quite thermostable. Dissolved in Dimethyl Sulphoxide (DSMO) or in solutions with a pH higher than 12.5, a random coiled state of the single chains is described (Willets 1978; Rapp 1989).

Scleroglucan disperses rather easily in water at room temperature due to the presence of (1→6)-β-D-glucopyranosyl groups which increase the solubility of the polysaccharide and decrease the ability to form gels (Sandford 1979; Cottrel 1980). Mixing, temperature, pH and concentration influence the rate at which viscosity of scleroglucan develops. The viscosity of scleroglucan solutions is affected only slightly by temperature variations. At low temperatures, close to 7 °C, solutions of scleroglucan form thermoreversible gels that may be caused by a weakly interacting triple helix cross-linking mechanism (Bluhm 1982). Hence, scleroglucan was the most stable polysaccharide among 140 polymers tested for use in polymer flooding in the North Sea oil reservoirs, as it retained more than 90% of its viscosity after 500 days at 90 °C in seawater (Brigand 1993). It forms stable gels in the presence of chromium salts and borax at pH 10–11, and can be precipitated by the addition of quaternary ammonium salts under alkaline conditions. Due to its non-ionic character, the stability of scleroglucan is not affected by alkalis and acids and by most electrolytes.

1.2 Industrial applications of scleroglucan

Scleroglucan has various industrial applications, especially in the oil industry for thickening, drilling muds and for enhanced oil recovery (Doster 1984; Pirri 1996). Other industrial uses include the preparation of adhesives, water colours, printing inks and liquid animal feed composition (Halleck 1969). In the food industry, numerous Japanese patents describe quality improvements of frozen foods (San-Ei Chemical Industries Ltd. 1982a), such as Japanese cakes (San-Ei Chemical Industries Ltd. 1982d), steamed foods (San-Ei Chemical Industries Ltd. 1982b), rice crackers (San-Ei Chemical Industries Ltd. 1982c) and bakery products (San-Ei Chemical Industries Ltd. 1982e).

Refined scleroglucan is mainly used for cosmetics as it can be applied in hair control compositions and in various skin care preparations, creams and protective lotions (Halleck 1970).

In pharmaceutical products, scleroglucan may be used as a laxative (Duc 1982), for tablet coatings (Sheth 1967) or for stabilizing suspensions in general. It also has immune stimulatory effects compared with other biopolymers, and its potential contribution to the treatment of many diseases should be taken into account in therapeutic implementation regimens (Patchen 2000; Jain et al. 2007). The use of scleroglucan as an antitumor, antiviral and antimicrobial compound has also been investigated (Singh et al. 1974; Jong and Donovick 1989; Prets 1991; Mastromarino et al. 1997).

1.3 Brand names and producers of scleroglucan

The production of scleroglucan was first reported by Halleck (1967) who observed that the fungus imperfectus *Sclerotium glucanicum* secretes this extracellular polysaccharide. The first product named Polytran® was introduced on the market by Pillsbury Co., commercialization was done by CECA S.E. under the name Biopolymer CS® in 1976. Subsequently, Satia, a division of Mero-Rousselot (France), produced scleroglucan under the trade name of Actigum CS6®. Sanofi Bio-Industries (Carentan, France), which obtained the rights from Satia and CECA, were the main scleroglucan producers, trading scleroglucan under the commercial names Polytran® and Actigum®, respectively (Sandford 1979; Ouriaghli 1992; Survase et al. 2007a).

Degussa Food Ingredients acquired Sanofi Bio-Industries in 1995 and manufactured scleroglucan under the name Actigum™ CS. Cargill acquired Degussa Food Ingredients in 2006 and continues trading scleroglucan under the same brand name. Scleroglucan is produced by Cargill in Baupte, France. The commercial product is termed scleroglucan, but it is also known with other names according to the company that produces the polysaccharide (e.g., Actigum, Clearogel® CSIl from Michel Mercier Products Inc. Mountainside, N.J. Polytetran, the Pillsbury Company (Minneapolis, USA), Polytran CECA, France, Tinocare® GL, Ciba, Switzerland).

1.4 Biosynthesis of scleroglucan

The biosynthesis of scleroglucan is nearly unknown yet. However, hypothetical pathways have been described (Brigand 1993; Survase et al. 2007a). Following the general scheme for polysaccharide production in microbial systems, three major steps have been postulated.
(i) Substrate uptake, (ii) intracellular formation, (iii) extrusion from the cell (Sutherland 1989).
A simplified scheme of the possible biosynthesis of scleroglucan is given in Figure 2, where the uptake of glucose into the cell occurs by facilitated diffusion and phosphorylation to glucose-6-phosphate via hexokinase (1). The following interconversion to glucose-1-phosphate by phosphoglucomutase (2) enables the start of polysaccharide formation:
UTP-glucose-1-phosphate uridylyltransferase synthesizes UDP-glucose from glucose-1-phosphate and UTP (3). A (1→3)-β-glucan synthase polymerizes the backbone chain (4).
The last step or steps to the accurate branching at every third glucose molecule is not clarified.
Furthermore to only hypothetical biosynthesis pathways, location and transport routes of scleroglucan within the cell remained cryptic up to now.

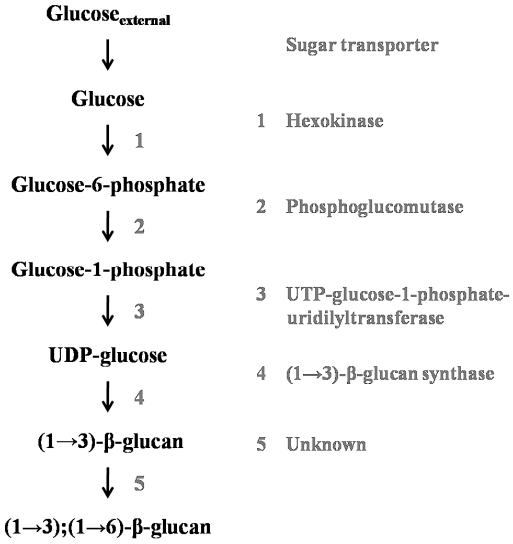

Figure 2: Hypothetical scleroglucan synthesis by *S. rolfsii*.

The biosynthesis of scleroglucan is thought to be closely linked to the synthesis of oxalate, a reducing agent and strong acid. These attributes of oxalate prevent the use of scleroglucan in many applications such as cosmetics or in food industry when not absolutely purified.
The following section highlights microbial oxalate metabolism and the role of oxalate in scleroglucan production by *S. rolfsii.*

1.5 Structure and properties of oxalate

Oxalic acid (IUPAC name: ethanedioic acid) is the simplest dicarboxylic acid. The chemical structure consist of 2 C atoms and 4 O atoms and 2 H atoms, therefore the resulting chemical formula is $H_2C_2O_4$ (Fig. 3). Because of the joining of two carboxyl groups, this is one of the strongest organic acids with pKa1 = 1.27 and pKa2 = 4.28, with strong reducing properties. The anions of oxalic acid as well as its salts and esters are known as oxalates.

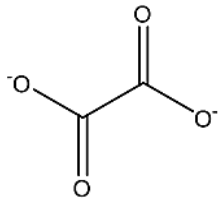

Figure 3: Chemical structure of oxalate.

The affinity of divalent metal ions is sometimes reflected in their tendency to form insoluble precipitates. Oxalic acid combines with metals ions such as Ca^{2+}, Fe^{2+}, and Mg^{2+} to deposit crystals of the corresponding oxalates, which could irritate gut and kidneys in the human body.

Due to this binding affinity of vital nutrients such as calcium, long-term consumption of foods high in oxalic acid can be problematic. Especially people with kidney disorders, certain forms of chronic vulvar pain, gout, or rheumatoid arthritis are typically advised to avoid foods high in oxalic acid or oxalates and to follow an oxalate low diet. Under certain conditions (high concentration, high pH) oxalic acid can precipitate in the kidneys as calcium oxalate crystals, forming an estimated 80% of kidney stones (Coe et al. 2005). Some *Aspergillus* species produce oxalic acid, which reacts with blood or tissue calcium to precipitate calcium oxalate (Pabuccuoglu 2005). There is some preliminary evidence that the administration of probiotics can affect oxalic acid excretion rates (Lieske et al. 2005).

1.6 Microbial oxalate metabolism

Oxalic acid is produced in the glyoxylate cycle (GLOX) a short cut of the tricarboxylic acid cycle (TCA) with circumvention of both decarboxylation reactions from isocitrate (Fig. 4). Thus, glyoxylate production involves the assembly of an acetyl-CoA to oxaloacetate, subsequently leading to the formation of isocitrate via citrate to directly yield in the production of succinate and malate. The GLOX is situated in small vesicles called peroxisomes or glyoxysomes. It provides the possibility to build carbohydrates out of fatty

acids and to circumvent both carbon atom wasting decarboxylation steps of the TCA. The glyoxysomes are localized next to mitochondria within the cell.

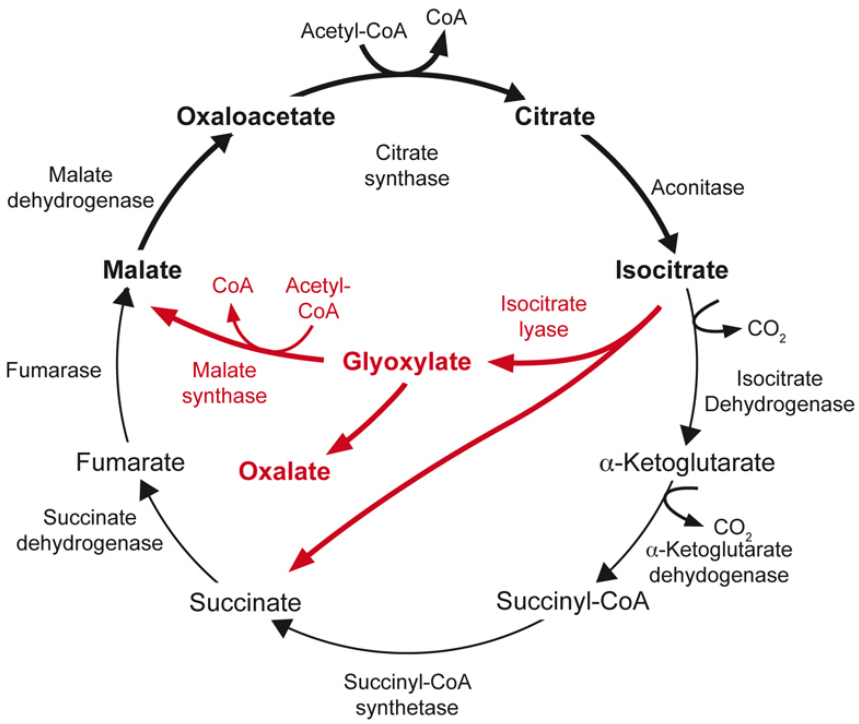

Figure 4: Schematic overview of the tricarboxylic acid cycle and the glyoxylate shunt initiated by isocitrate lyase, described to be taken by *S. rolfsii* for oxalate biosynthesis.

Only a limited number of bacteria have been described, which are able to utilize oxalate as sole carbon and energy source. *Methylobacterium extorquens* was the first described oxalate-degrading bacterium (Bousfield 1985). However, *Pseudomonas oxalaticus* is the most popular oxalate-decomposing bacterium described in the literature (Sahin et al. 2000).

Unique to these oxalate-degrading bacteria is their energy production, which apparently depends on the oxidation of formate (Blackmore 1970). In *Oxalobacter formigenes*, the proton motive force used for energy conservation is generated by an electrogenic antiport of $oxalate^{2-}$ and $formate^{1-}$ by the oxalate-formate exchanger, OxlT. The coupling of oxalate-formate exchange to the reductive decarboxylation of oxalyl CoA forms an 'indirect' proton pump (Anantharam et al. 1989; Stewart et al. 2004).

Many fungi, belonging to ascomycetes, basidiomycetes and zygomycetes, a few lichens and slime mold genera also produce calcium oxalate crystals during some phases of their life cycle.

Although its role is still not clarified (Dutton 1996), oxalic acid in brown rot fungi, for example, can be assigned to different functions like free radical formation, iron chelating and

establishment of pH gradients (Goodell 1995; Mikio Shimada 1997), Ca^{2+} precipitation as well as pectin and cellulose hydrolysis (Green 1995; F. Green III 1996). In phytopathogenic fungi, oxalate has been described as a very important factor contributing to fungal virulence. One role of oxalate is to lower the pH of the ambient environment, resulting in increased fungal polygalacturonase activity which degrades the cell wall and tissue of the infected plant (Bateman and Beer 1965; Favaron et al. 2004). Other roles include sequestration of Ca^{2+} from cell walls, hydrolysis of plant pectin, suppression of plant defence responses and as an elicitor of programmed cell death (Errakhi et al. 2008; Kim et al. 2008a).

Oxalate is also thought to be involved in the infection process of *S. rolfsii*. It acts as chelating agent and deprives the Ca^{2+} ions from the plant cell wall, making the pectic fraction more available to fungal hydrolases and provides an acidic pH necessary for maximum activity of cell wall degrading enzymes released by *S. rolfsii* (Çaliskan 2000). The pH optimum of polygalacturonase is approximately 2.5 in *S. rolfsii* (Yoshihira 1969) and around 3–4 in brown rot fungi (Clausen 1996).

1.7 Conditions that favour scleroglucan and oxalate production by *S. rolfsii*

Scleroglucan production has undergone a long-time optimization by many different approaches; most of them based on media design to increase scleroglucan yields.

Astonishingly, conditions favouring scleroglucan production have been assigned also to increase the amount of oxalate secreted into the media. Oxalic acid as the main by-product in scleroglucan production is absolutely undesirable. Its production is linked to the culture conditions and varies with the carbon and nitrogen source and the initial pH (Maxwell and Bateman 1965; Lee 1998). Oxalate accumulation is also influenced by the presence of precursors or other chemicals in the cultivation medium (Survase et al. 2007b), the influence of dissolved oxygen has been described by Schilling et al (2000). The most favourable conditions for oxalate formation include high carbohydrate concentrations and adequate aeration, limited supply of inorganic nutrients and relatively high pH (Maxwell and Bateman 1965; Wang 1994; Lee 1998).

Scleroglucan production also is markedly affected by C-source selection and concentration, showing the highest value with 150 gL^{-1} sucrose (Farina et al. 1998). The nitrogen source is the second main factor for scleroglucan production, where $NaNO_3$ shows the best effect on scleroglucan production while $(NH_4)_2SO_4$ reduces scleroglucan production up to 70% (Farina et al. 1998; Lee 1998; Survase et al. 2006). Other factors such as P-source or initial pH and addition of diverse substances are not in such an order as C- or N-source, but also influence

scleroglucan production. Higher phosphate concentrations have been described to increase scleroglucan yields. In contrast, the addition of L-threonine or ascorbic acid was shown to decrease scleroglucan production (Farina et al. 1998). Most favourable conditions for scleroglucan production are high concentrations of sucrose or glucose in combination with $NaNO_3$ as N-source.

2 Aim of the thesis

The aim of this thesis is to get molecular insight into the biosynthetic pathways of scleroglucan and oxalate production of *S. rolfsii*. Whilst both products are very well described und examined on physicochemical level, only little information on their biosynthesis is available to date.
In order to improve the biosynthesis of scleroglucan by metabolic engineering, genetic tools enabling the analysis and control of *S. rolfsii* gene expression have to be established as well.

1. A suitable transformation system for *S. rolfsii* has to be established including a stable selection system. Furthermore, suitable transformation vectors as well as appropriate expression cassettes have to be developed for genetic and metabolic approaches.

2. The biosynthesis of oxalate has to be unravelled. In this respect, a PCR approach will be used to screen the genome of *S. rolfsii* for the presence of genes putatively involved in oxalate metabolism based on a literature survey.

3. For the identification of genes involved in scleroglucan biosynthesis, a transcriptomic approach, namely SSH (Suppression Subtractive Hybridisation) has to be conducted. A prerequisite for this approach is the development of a scleroglucan producing medium and a scleroglucan non-producing medium.

4. Furthermore, a second transcriptomic approach will be followed based on the establishment of an EST library obtained from *S. rolfsii* grown in scleroglucan producing and scleroglucan non-producing medium. This library has to be sequenced and annotated.

5. Finally, lead genes obtained from approaches 2–4, predicted to function in scleroglucan and oxalate synthesis shall be identified for metabolic engineering of *S. rolfsii*, i.e. for the establishment of strains with improved/lowered scleroglucan/oxalate producing properties.

3 Materials and Methods

3.1 Equipment

Autoclave	1651; Fedegari, Italia
Centrifuge	Sorvall RC-5B; Dupont, Bad Homburg Microrapid K; Hettich, Tuttlingen
Cleanbench	uvub 1200 Uniflow
Electrophoreses chambers	Wide Mini Sub TM Cell, Mini Protean (16 x 14 x 0,15 cm); BioRad, München
Fluorescence microscope	Axioscope, Zeiss
Geiger-Müller counter	Bertold LB 1210 C; Wildbad
Incubator	Certomat IS; Sartorius, Göttingen
Incubator	Biometra OV1; Biometra, Göttingen
Orbital shaker	Duomax 1030; Heidolph Instruments, Schwabach
PCR Thermocycler	Biometra
Photographic equipment	Image Station 440 cf; Kodak, Berlin Camedia Digital Camera C-4000 Zoom, Olympus, Hamburg Olympus, OM-2 with 50 mm objective, Dunco, Berlin;
Pipetting Equipment	P10, P20, P200, P1000; Abimed
Power Supplies	Phero Stab 500; Biorec Fischer, Reiskirchen
Rotors	GSA, SS34; Dupont, Bad Homburg AH650
Spectrophotometer	Uvikon 860; Kontron Instruments, Neufahrn
Thermo Cycler	TGradient ; Biometra, Göttingen
Transilluminator	INTAS; Göttingen
UV Cross linker	UV Stratalinker ™ 1800; Stradayene, LaJolla, USA
Water bath	Grant LTD; Thermomix, Bbraun, Melsungen
X-ray cassettes	Kodak X-Omatic with intensifying screen; Kodak, Berlin

3.2 Enzymes, chemicals and kits

AccuPrep™ Plasmid Extraction kit	Bioneer, Japan
Acrylamid solution (40% Acrylamid: Bis-Acrylamid 29:1)	Amresco, Solon, Ohio
Agar-Agar, Glucose, EDTA, SDS, N,N,N',N' Tetramethylethylendiamin (TEMED)	Serva, Heidelberg
Agarose	Bethesda Research Laboratories
Ampicillin, IPTG, X-Gal, phenol	BIOMOL, Hamburg
APS, Urea, Acetosyringone	Fluka, Neu-Ulm
Cefotaxime, Hygromycin B, acetic acid, ethidium bromide, HCl, KCl, NaCl, NaOH, $MgCl_2$, $MgSO_4$, NaCl, NaOH, CH_3COOK, Na-acetate, PEG 4000	Merck, Darmstadt
DNA size marker, Taq Polymerase, T4 DNA ligase, GeneRuler™	MBI, Fermentas, St. Leon-Rot
dNTP's, RNAse	Roche Diagnostics, Mannheim
Reaction tubes, pipette tips, petri dishes	Greiner, Nürtingen
Ethanol, glycerine, isopropanol	Roth, Karlsruhe
Hybond N+ membranes, Megaprime labelling kit [α-^{32}P] dATP	GE Healthcare, Buchler
Lambda ZAP II Predigested EcoRI/CIAP-Treated Vector Kit	Stratagene
PCR-Select cDNA Subtraction Kit	Clontech, Japan
Plasmid preparation kit, QIAquick PCR purification kit	Qiagen, Düsseldorf
SeaKem LE Agarose	Biozym, Freiburg
Tris, SDS	Sigma, Deisenhofen
Tryptone	Oxoid, Hampshire
X-ray development solution	Kodak, Berlin
X-ray film NewRX	Fuji, Japan
Yeast extract	Deutsche Hefewerke, Hamburg

All chemicals not listed above were obtained from Merck, Sigma or Boehringer Mannheim and were of analytical grade or better quality.

3.3 Strains

***A. tumefaciens* Agl1**	*A. tumefaciens* AGL0 *recA*::*bla* pTi*Bo542deltaT Mop+ CbR*. Contains helper plasmid of higher virulence than LB1100. Is known to afflict more species than other *A. tumefaciens* strains, provided by Ines Schlunk, Friedrich Schiller University Jena
***A. tumefaciens* LBA1100**	*A. tumefaciens* strain containing helper plasmid pAL1100 (*Δtra*, *Δocc*, ΔT_L, ΔT_R) determining spectinomycin resistance (Beijersbergen et al. 1992), provided by Prof. Paul Hooykaas, Institute of Biology, Leiden University (Netherlands)
***E. coli* DH5α**	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1, (Gibco BRL, Berlin)
***E. coli* XL 10-Gold®**	*TetrD* (*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA*1 *supE*44 *thi-1 recA*1 *gyrA*96 *rel*A1 *lac Hte* [F' *proAB* l*acIqZ*Δ*M*15 *Tn*10 (Tetr) *Amy Camr*], (Stradayene, LaJolla, USA). Chloramphenicol resistant (Cam^R) at concentrations of <40 µg/mL, but chloramphenicol sensitive (Cam^S) at concentrations of 100 µg/mL
***E. coli* XLBlue1**	F-, ø80d*lacZ*ΔM15, Δ(l*acZYA-argF*)U169, *recA*1, *endA*1, *hsdR*17(rk-, mk+), *phoA*, *supE*44, *thi*-1, *gyrA*96, *relA*1, *gal*$^-$
Sclerotium rolfsii	ATCC 15205

3.4 Plasmids

pAN7-1	6.7 kb vector Promoter *A. nidulans gpd*A gene hygromycin phosphotransferase gene (*hph*) Terminator *A. nidulans trpC* gene terminator region (Punt et al. 1987)
pAN-NC3	Constructed on the basis of pAN7-1 by replacing the *trpC* terminator in pAN7-1 with the *Neurospora crassa* ß-tubulin terminator of pBT6 (Orbach et al. 1986)
pBGgHg	pBGgHg is 9.6 kb in size and consists of a pCAMBIA 1300 backbone containing the kanamycin resistance gene and the right border (RB) and left border (LB) sequences of *Agrobacterium* T-DNA (Fig. A). The hygromycin resistance gene and enhanced green fluorescent protein gene (EGFP) are located between the border sequences and each is joined to the *A. bisporus* glyceraldehyde 3-phosphate dehydrogenase promoter (P*gpdA*II) and cauliflower mosaic virus terminator (35S-3′). Provided by Ines Schlunk, Friedrich Schiller University Jena
pGEM-T easy	Linear cloning plasmid (Promega 1996)
pGEM-T	Linear cloning plasmid (Promega 1996)
pUC18	2.7 kb vector the pMB1 replicon *rep* responsible for the replication of plasmid, Δ*rop* gene and a single point mutation in *rep* of pMB1; *bla* gene. *E. coli* operon *lac*, promoter P_{lac}, *lac* repressor binding site and 5'-terminal part of the *lacZ* gene encoding the N-terminal fragment of beta-galactosidase

pUCDC	7.3 kb cloning vector, derivative of pUC18, *hph* expression cassette consist of *hph* gene (*E. coli*), *gpdA*II promoter of *A. bisporus* and terminator of cauliflower mosaic virus 35S-3′. Second gene is the oxalate decarboxylase gene from *Flammulina velutipes* under control of *gpd*II promoter from *A. bisporus*; terminator is the tubulin terminator from *N. crassa*
pUCOX	5.5 kb vector containing *hph* gene from *E. coli* between *gpd* promoter from *A. bisporus* and the terminator from cauliflower mosaic virus terminator (35S-3′). Additionally this vector is carrying the oxalate oxidase gene from *Hordeum vulgare* also under control of the *gpd* Promoter from *A. bisporus* and as terminator the tubulin terminator from *N. crassa*
pUR5750	15.7 kb vector, derivate of pBIN19, contains the *hph* gene between the LB and RB from pAN7.1 and *npt*II/*npt*III (de Groot et al. 1998). Kindly provided by Prof. Paul Hooykaas, Institute of Biology, Leiden University (Netherlands)
pJET1.2/blunt	Linear 2.9 kb cloning plasmid, carrying rep (pMB1), bla (ApR) and eco47IR as lethal gene. Eco47IR enables positive selection of the recombinants. 7PlacUV5 modified Plac promoter for expression of the eco47IR gene at a level sufficient to provide positive selection T7 RNA polymerase promoter for in vitro transcription, MCS (Fermentas, Ontario, Canada)

3.5 Culture media

Cove	10 g glucose, 10 mL $NaNO_3$; add ddH_2O to final volume of 980 mL and autoclave. 20 mL cove salt solution is added
Cove salt solution	26 g/L KCl, 26 g/L $MgSO_4{*}7H_2O$, 76 g/L KH_2PO_4, 20 mL/L trace elements solution, adjusted to pH 5.5 with NaOH, filter-sterilized
Cove trace elements solution	26 mg/L H_3BO_3, 400 mg/L $CuSO_4$· 5*H2O, 1.2 g/L $FeSO_4{\cdot}7{*}H_2O$, 800 mg/L $MnSO_4{\cdot}2{*}H_2O$, 8 g/L $ZnSO_4{\cdot}7{*}H_2O$, filter-sterilized
CYM	1 g/L maltose, 2 g/L glucose, 0.2 g/L YE, 0.2 g/L tryptone, 0.05 g/L $MgSO_4{\times}7{*}H_2O$, 0.46 g/L KH_2PO_4
Czapek Dox	30 g/L sucrose, 2 g/L $NaNO_3$, 1 g/L, 0.50 g/L $MgSO_4$ x 7 H_2O, 0.50 g/L KCl, 0.01 g/L $FeSO_4$ x 7 H_2O, 1 g/L K_2HPO_4. Adjust pH to 7.2
EPSmax13	40 g/L glucose, 3.0 g/L $NaNO_3$, 2 g/L K_2HPO_4, 0.5 g/L KCL, 0.5 g/L $MgSO_4{*}7H_2O$, 0.05 g/L $FeSO_4{*}7H_2O$, 1 g/L YE, 0.7 g/L citric acid $7{*}H_2O$. pH 4.5
EPSmin17	40 g/L fructose, 1.9 g/L NH_4CL, 2 g/L, K_2HPO_4, 0.5 g/L KCL, 0.5 g/L $MgSO_4{*}7H_2O$, 0.05 g/L $FeSO_4{*}7H_2O$, 1 g/L YE, 0.7 g/L citric acid $7^{*}H_2O$. pH 4.5
Induction medium (IM)	905 mL ddH_2O were autoclaved and the following filter sterilized supplements were added : 0.8 mL 1.25 M K-buffer pH 4.8, 20 mL MN-buffer, 1 mL 1% $CaCl_2{\cdot}2H_20$ (w/v), 10 mL 0.01% $FeSO_4$ (w/v), 5 mL trace elements solution, 2.5 mL 20% NH_4NO_3 (w/v), 10 mL 50%glycerol (v/v), 40 mL MES pH 5.5 and 5 mL 20% glucose (w/v) for solid medium or 10 mL 20% glucose (w/v) for liquid medium (1 mL AS solution was added when necessary)
Trace elements for IM	100 mg/L $ZnSO_4{\cdot}7{*}H_2O$, 100 mg/L $CuSO_4{\cdot}5{*}H_2O$, 100 mg/L H_3BO_3, 100 mg/L $MnSO_4{\cdot}H_2O$, 100 mg/L Na_2MoO_4 $2{*}H_2O$, filter-sterilized

LB medium:	10 g/L peptone, 5 g/L YE, 10 g/L NaCl, adjusted to pH 7.5 with NaOH For selection ampicillin (100 mg/L) was added
LB+Supplements	10 mL 1 M $MgSO_4$, 3 mL of a 2 M maltose solution or 10 mL of a 20% (w/v) maltose solution
LC-Medium:	10 g/L Tryptophan, 8 g/L NaCl, YE 5 g/L, 1.5% Agar, pH 7.5. For selection kanamycine (100 mg/L) was added
Medium B	45.5 g/L glucose, 2 g/L $NaNO_3$, 1g/L KH_2PO_4, 0.5 g/L $MgSO_4$ 7*H_2O, 1 g/L YE, pH 4.5
MGY	1 g/L YE, 1 g/L malt extract, 10 g/L glucose. pH 5.5
MGY+	1 g/L YE, 1 g/L malt extract, 10 g/L glucose, 0.6 M sucrose, pH 5.5
NZY	5 g NaCl, 2 g $MgSO_4$ 7*H_2O, 5 g YE, 10 g NZ Amine (Casein hydrolysate), pH 7.5 adjusted with NaOH
NZY Top-Agar	NZY broth with 0.7% of agar
SOB	20 g/L Tryptone, 5 g/L YE, 0.2 g/L KCl, 0.6 g/L NaCl

3.6 Buffers reagents and solutions

Antibiotics (final concentrations)

Ampicillin	100 µg/mL
Cefotaxime	95.48 µg/mL (200 µM)
Hygromycin B	75-150 µg/mL
Kanamycin	100 µg/mL
Spectinomycin	250 µg/mL

10x Ligase buffer	500 mM Tris-HCl (pH 7.5), 70 mM $MgCl_2$, 10 mM Dithiothreitol (DTT).
10x MOPS	200 mM 3-[*N*-morpholino]propane-sulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA. Adjusted to a final pH of 6.5-7 with NaOH, do not autoclave
10x TE	0.1 M Tris/HCl, 10 mM EDTA (pH 8.0)
20x SSC	175.2 g/L NaCl, 88.2 g/L sodium citrate, adjusted to pH 7.0
5x TAE	242 g/L Tris, 57.1 mL/L glacial acetic acid, 100 mL/L 0.5 M EDTA (pH 8.0)
CsCl- pad	CsCl solved in 90 mL 0.01 M EDTA (pH 7.5) and 0.1 mL DEPC. Incubation at RT (30`) Solution is filled up to 100 mL with H_2O, followed by autoclaving
Extraction buffer	4 M GuSCN, 0.1 M Tris/HCL pH 7.5, 1% β-Mercaptoethanol (0.14 M). 50 g GuSCN were solved in 10 mL of 1M Tris/HCL pH 7.5 and added to 100 mL with H_2O. Solution is filtered sterile and stored at RT. Directly before usage, sterilized and stored 1% of Mecaptoethanol is added
IPTG stock solution	Isopropyl β-D-1-thiogalactopyranoside in ddH_2O at concentration of 100 mM. Stored at -20 °C

Loading buffer	60% sucrose, 20 mM EDTA, 0.025% bromophenol-blue
RNA sample buffer	720 µL formamide, 160 µL 10x MOPS, 260 µL formaldehyde, 220 µL DEPC-H_2O, 100 µL 80% glycerine, 40 µL bromophenol-blue, 7.5 µL ethidiumbromide. Always prepared fresh prior to use
SM Buffer	5.8 g NaCl, 2.0 g $MgSO_4$ * $7H_2O$, 50 mL, 1 M Tris-HCl (pH 7.5), 5.0 mL 2% (w/v) gelatine. Adjust to 1 L with ddH_2O, autoclave
TE-SDS buffer:	0.1% SDS, 5 mM EDTA, 10 mM Tris/HCL pH 7.5
X-gal stock solution	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in N,N-dimethylformamide (DMF) at concentration of 40 mg/mL. Stored in the dark at -20 °C
***S. rolfsii* transformation**	
K-buffer pH 4.8	Add 1.25 M of K_2HPO_4 to 1.25 M KH_2PO_4 until pH 4.8 is reached
MN-buffer	30 g/L $MgSO_4$*$7H_2O$, 15 g/L NaCl
MES pH 5.5	1 M methylethylsulfonate, adjust pH with NaOH, filter-sterilized
AS solution	0.2 M acetosyringone in DMSO
Competent *E. coli* cells	
SOB	2% bacto tryptone, 0.5% YE, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$
TB	250 mM KCl, 10 mM HEPES free acid, 15 mM $CaCl_2.2H_2O$, 55 mM $MnCL_2.2H_2O$
Transformation solution	1 mM EDTA, 0.2 M NaCl and 10 mM Tris/HCl pH 7.6 in 8% PEG 2000

3.7 Selection of putative *S. rolfsii* transformants

As putative transformants were considered those colonies which showed a reinforced growth compared to the other colonies on Hygromycin selection plates. A clear classification of single colonies was often difficult due to non-uniform distribution of mycelia; therefore sometimes mycelia were taken at random which showed different morphology.

3.8 Homogeneous *S. rolfsii* suspension

Homogeny suspension of *S. rolfsii*, suitable for dilution, is achieved by milling a *S. rolfsii* culture, cultivated for 48 h in an EMF of 250 mL (28 °C, 500 rpm), under sterile conditions with the help of a custom blender. The obtained suspension was regarded as prospective homogenous after three milling steps, each for 30 seconds.

3.9 Cultivation conditions for bacteria and filamentous fungi

In general, *E. coli* strains were cultivated in LB medium at 37 °C. Recombinant *E. coli* strains were selected in LB medium with additional 100 µg/mL of ampicillin (LB amp).
A. tumefaciens strains were cultivated in LB medium at 28 °C. Recombinant cells were selected in LB medium with additional 100 µg/mL of kanamycin.
S. rolfsii was cultivated in EPSmin17 medium or EPSmax13 medium at 28 °C, 250 or 500 rpm on a magnetic stirrer.

3.10 Cryoculture

To 600 µL of a bacterial obtained from a culture in logarithmic growth phase, 400 µL glycerine (99%) was added, thoroughly mixed and stored at -70 °C.

Cryoculture of *S. rolfsii* were made by storage of harvested mycelium on the one hand and by stored sclerotia on the other hand. Highest regeneration rate was achieved by storage of sclerotia in 20% of glycerol and at -80 °C. An overview of the different storage conditions is given in the appendix (Table A + B).

3.11 Methods for DNA and RNA analysis and modification

3.11.1 Quantification of RNA and DNA by UV-spectroscopy

The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260 nm. The major disadvantage of this method is the inability to distinguish between DNA and RNA and the relative insensitivity of the assay (an OD_{260} of 0.1 corresponds to a 5 µg/mL dsDNA solution or 4 µg/mL ssRNA, referring to 1 cm path length in a cuvette).

3.11.2 Synthesis of cDNA library

As absolute prerequisite to sequence an EST library of *S. rolfsii*, there has to be synthesized a cDNA library first. This synthesis has to be performed including normalisation by putative constitutively expressed genes (glucoamylase 2 of *S. rolfsii*). First strand synthesis is conducted by use of following primer.

5'-AAGCAGTGGTATCAACGCAGAGTATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30*N-3'

The defined 5'-ending is caused by addition of the RNA Oligo to the first strand synthesis and the so called template shifting of the Reverse transcriptase. 20 µg of cDNA library from pooled mRNA are obtained with an average length from 500 to 1,000 bp.

3.11.3 Suppression Subtractive Hybridisation (SSH)

Suppression subtractive hybridisation allows subtracting similar populations of mRNA obtained by an organism cultivated under different conditions. Obtained clones carry transcripts of genes that are expressed in one population but not in the other. For subtractive hybridisation the CLONTECH PCR-Select^{TM} cDNA subtraction kit was used. This method was originally described by Diatchenko et al. (1996).

3.11.4 Clonetch PCR-Select cDNA Subtraction Kit

Differentially transcribed polyA-mRNA was enriched from the tester-cDNA (cDNA1). The mRNA from *S. rolfsii* grown in EPSmax13 was used as tester-cDNA. The polyA-mRNA from growth in EPSmin17 served as Driver-cDNA. Adapters are ligated solely to the tester-cDNA.

3.11.5 Reverse Northern Blotting for verification of SSH-clones

Colony hybridisation

Agar plates (LB + Amp) coated by Hybond-N-circularized membrane (positively charged, Amersham or Qiagen). Two coated Agar-plates were inoculated with 48 SSH-clones, and cultivated over night. The filter was drawn off and incubated successively in following solutions for 3 minutes in 1 mL 10% SDS, 5 min in 1 mL denaturation solution (0.5 M NaOH, 1.5 M NaCl), 5 min in 1 mL neutralisation solution (1.5 M NaCl, 1 M Tris/HCl pH 7.5), 5 min in 1 mL 2 x SSC (pH 7.0). Filter were cross-linked and washed (removing of cell debris) in washing solution (5 x SSC, 0.5% SDS, 1 mM EDTA, pH 7.0–8.0), eventually cell debris on filter were carefully removed using a paper towel. The filter were dried and stored at 4 °C.

Assembly of P^{32}-tagged first strand cDNA from EPSmax13 or EPSmin17

Total volume of reaction: 25 µL, with 5–10 µg total-RNA from EPSmax13 (**or** from EPSmin17) in nuclease-free ddH_2O, the solution is tempered to 70 °C for 10 min and then cooled down on ice for 2 minutes. 2.5 µL 10 x ArrayScript RT buffer (Ambion) were added, together with 2.5 µL Oligo(dT) Primer (10 pmol/µL) and 1 µL RNAse Inhibitor. Then were added 1 µL 5 mM dGTP, 1 µL 5 mM dTTP, 1 µL 5 mM dCTP and 0.2 µL 5 mM dATP. In the last step 2 µL of [α-^{32}P] dATP (Hartmann, SCP-203) and 1 µL Superscript II RT (Ambion, 200 U/µL) were added. The whole preparation was incubated at 42 °C for 1 h and then of 2 µL RNAseH (10 U/µL) were added by a following incubation for 30 min at 37 °C.

Hybridisation (Rapid-Hyb buffer; Amersham)

Pre hybridisation temperature: 65 °C, Hybridisation temperature: 65 °C
Hybridisation time: 1 h
Vector hybridisation temperature: 65 °C, Hybridisation temperature: 70 °C

Hybridisation time 1 h

1. Washing: 1 x 20 min, RT, 2 x SSC, 0.1% SDS
2. Washing: 2 x 15 min, 65 °C, 1 x SSC, 0.1% SDS

3.12 Transformation methods

3.12.1 Preparation of heat shock competent *E. coli*

Transformation of *E. coli* was performed according to Inoue et al. (1990). *E. coli* was inoculated in 10 mL SOB in a 100 mL Erlenmeyer flask and grown over night at 37 ºC. 3 mL of over night culture was diluted in 250 mL SOB in a 1 l Erlenmeyer flask and grow at 18 ºC with vigorous shaking (until OD_{600} = 0.6). After centrifugation the pellet was resuspended of ice-cold TB and incubated on ice for 10 min. The pellet of the next centrifugation step was resuspended in 1/12 of the original volume of ice-cold TB. DMSO was added with gentle mixing to a final concentration of 7%. 200 µL portions in precooled Eppendorf tubes were frozen in liquid nitrogen and stored at -80 ºC.

3.12.2 *E. coli* transformation protocol

DNA was added to 200 µL of cell suspension, mixed gently and incubated on ice for 2 min. The heat shock was applied for 30 sec. at 42 °C. Than the tube was returned on ice for 1 min.1 mL SOB was added and mixed gently by inversion and incubate at 37 ºC with shaking for 30 min. 100 µL of the resulting mixture were plated on corresponding media.

3.13 *Agrobacterium* mediated transformation (AMT)

3.13.1 Transformation of *S. rolfsii* using *A. tumefaciens*

The protocol was originally developed by de Groot et al. (1998), and performed according to Meyer et al. (2003b). *A. tumefaciens* strain LBA1100/AGL1 were grown at 30 °C over night in LC medium containing kanamycin (100 µg/mL) until an OD_{600} of ~2.5 was reached. Then 2 mL of cells from the culture medium were harvested using a tabletop centrifuge (3 min, 8,000 rpm, RT), washed once with IM (Bundock et al. 1995), centrifuged again, resuspended in 5 mL IM +/- 200 µM AS. The culture was incubated for 6 h at 30 °C, 170 rpm. Harvesting of cells was performed by centrifugation (3 min, 8,000 rpm, RT) and resuspended in 900 µL of IM (+/- AS, respectively). Subsequently, they were poured to the 1 cm^2 piece of *S. rolfsii* on solid IM media (+/- AS) covered with cellophane (BioRad). Co-cultivation was performed for 2–5 days at 24–30 °C. Then the membranes were transferred to solid MGY medium containing 200 µM cefotaxime (to inhibit the growth of *A. tumefaciens*) and 100 µg/mL hygromycin B for selection of recombinant clones. Plates were incubated for 7 days at 30 °C.

3.13.2 Protoplast mediated Transformation (PMT)

A preparatory culture (1/8 of mycelia from 5 days grown Petri dish in 50 mL EPSmin17) was used for inoculation of 4 Erlenmeyer flasks (50 mL EPSmin17, 28 °C with 250 rpm). Each flask was inoculated with 1 mL of substrate. The 4 flasks were harvested by centrifugation after 45 h. The mycelium was washed with aqua dest. (200 mL), and in a second step with citrate phosphate buffer (CPB) (200 mL, pH 4.5). The resulting pellet was divided into pieces of 1 g of biomass and given into a Greiner tube (50 mL). 30 mL of CPB with 0.6 M $MgSO_4$ were added. 0.5 g of lytic enzyme mix, Vinoflow FCE was added.
The tube was sealed with Para film and shake for 90 minutes at 70 rpm horizontally (28 °C). After 90 minutes the cell wall decomposition was controlled by microscopically analysis. The 4 Greiner tubes were centrifuged at 2,000 rpm, 20 minutes, RT. The supernatant was discarded and 10 mL of 0.6 M sorbitol buffer was added to two of the Greiner tubes. The pellets were dissolved by pipetting 20 times (backwards used 10 mL glass pipette). The resulting solution was added to the third and the fourth tubes, and the procedure was repeated. The obtained 20 mL were aliquot to 10 reaction tubes (2 mL) and centrifuged for 5 minutes 1,000 rpm. Supernatant was discarded from 9 of the 10 reaction tubes. From the last one only 1 mL was put off ant the rest was used for dissolving the pellet. This suspension was transferred to the next reaction tube and the pellet was dissolved. This procedure was repeated until nearly 2 mL of protoplast suspension was present in the last reaction tube. The number of protoplast was counted in a Thoma Chamber and usually was in the range from $1–7*10^7$ protoplasts/mL.

Transformation of *S. rolfsii* protoplasts
150 µL of the protoplast suspension were transferred to a Greiner tube (50 mL). 10 µg of plasmid were added (solved in 50 µL 1.2 M sorbitol CPB). Than at first 50 µL of PEG solution (60% PEG4000, 100 mM CaCl, 100 mM Tris HCL pH 7.5) was added. After 10 minutes at RT, 1 mL of PEG solution was added and kept at RT for exactly 5 minutes. Than 25 mL of pre warmed (42 °C) Top Agar (MGY+ with 0.75% agar) were added to the Greiner tube and mixed with the transformation solution. 5 mL were poured onto Petri Dishes (MGY +) and dispersed equally. The Petri Dishes than were incubated at 28 °C. After 18 h, additional 5 mL of soft agar (0.75% agar), containing 75 µg/mL (calculated to the total volume of agar) Hygromycin were poured onto the Petri dishes.

3.14 Analytics

3.14.1 Quantitative analysis of mycelia and scleroglucan (Degussa method)

Determination of titer
The Erlenmeyer flask (EMF) with fermentation broth is weighted (mFB). The fermentation broth is transferred into a vessel with 2 Vol of Isopropanol. Another empty EMF is weighted (mR). The Isopropanol solution along with the precipitate is filtered over a 74 µm mesh filter.
An empty crystallization vessel is weighted (mk). The precipitate is transferred to the crystallization vessel and following drying periods (48 h) under the extractor hood (removal of Isopropanol), drying for 2 h, 60 °C under vacuum, and drying of precipitate in an exsiccator are performed. The vessel wit precipitate is weighted (mPS). The titer is calculated by following formula: titer = ((mPS – mK) * 100) / (mFB – mR) [%]

Determination of mycelium mass
40 g of fermentation broth are transferred to a 100 ml beaker, a magnetic stir bar is added and both are weighed together (mB). The beaker is covered with a watch glass and stored at 4 °C for 48h. The pH is to 5 with 0.5 M NaOH. The sample is preheated to 56° C (30 min) in a water bath. 800 µL of Glucanex ((50 mg/mL, freshly scheduled) = 1 mg Glucanex/g broth) are added. An incubation step for 30 min at 56 °C with smooth shaking follows. An empty 50 mL Greiner tube (dried for 30 min, 60 °C in an exsiccator) is weighted. Glucanex is inactivated by heating to 90 °C for 20 min in a water bath. The sample is cooled down to RT and the starting weight is reconstructed by adding water up to 40g. 30g of this solution are transferred into the dried Greiner tube. A centrifugation step, 10 min, 4,000 rpm (Heraeus) at RT follows. The pellet is dried over night (60 °C under vacuum and following drying in exsiccator). Last step is weighing of the pellet and determination of the ratio mass/EPS.

3.14.2 Quantitative analysis of oxalate
Oxalate was determined via HPLC (Knaur column H^+), 70 °C, 0.05 M H_2SO_4, 0.5 mL/min. Detection was performed via UV detection unit at 210 nm.

3.14.3 Quantitative analysis of glucose and fructose
Glucose was detected with the same HPLC system and a refracometric detector. Standard curves were created and internal controls were used at every run.

3.15 Methods for DNA and RNA analysis

3.15.1 Isolation of plasmid DNA from *E. coli*
For small scale plasmid preparation, plasmid DNA was isolated from a 1 mL overnight culture according to the ´miniprep` method of Ish-Horowicz and Burk (1981). Large scale plasmid isolation was carried out from 100 mL cultures after 18 h of incubation according to the `QIAGEN Plasmid Midi and Maxi Protocol'.

3.15.2 Preparation of fungal genomic DNA for PCR approaches
Preparation was done according to Meyer et al (2002).1 mL of EPSmin17 was inoculated with a piece of mycelium and incubated overnight at 28 °C with shaking. Mycelia was harvested in a tabletop centrifuge (5 min, 12,000 rpm, RT) and washed once with 1 mL ddH_2O. Subsequently, the pellet was resuspended in 600 µL ddH_2O. Cell disruption was done by repeated (3 x) shock freezing in liquid nitrogen and following thawing at 75 °C for 5 min. DNA was purified by adding 500 µL phenol and homogenization for 2 min. Phases

were separated in a tabletop centrifuge (10 min, 12,000 rpm, 4 °C) ,the top phase was transferred to a new tube. After the addition of 400 µL chloroform, homogenization and centrifugation steps were repeated. Once again, the top phase was transferred to a new tube and two volume ethanol (abs.) and 1/25 volume 3 M Na-acetate were added. The DNA was precipitated for 1 h at -20 °C this steps followed a centrifugation of 15 min with 12,000 rpm at 4 °C. Subsequently, the pellet was washed once with 70% ethanol and afterwards air-dried. DNA was resuspended in 20 µL ddH_2O and 1 µL was used for PCR.

3.15.3 Isolation of genomic DNA from *S. rolfsii*

100 mL of respective medium were inoculated with mycelium of *S. rolfsii* (1/8 Petri Dish, grown for 5 days). Flasks were inoculated at 28 °C, 250 rpm on a magnetic stirrer. After 48 h of cultivation, mycelium was harvested by filtration through a piece of gauze. The Mycelia was washed twice with hot water (85 °C) for removing scleroglucan.
Mycelium was shock frozen in liquid nitrogen (which can be stored for several weeks at -80 °C, see Table A and for sclerotia see Table B). And 500 mg of mycelium was transferred to a prefrozen Teflon cup and 700 µL DNA extraction buffer (10 mM Tris/HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS) were added. Disruption of mycelia was performed with a dismembrator (Braun Biotech) for 2 min. After disruption, the mixture was thawed, transferred to a 2 mL tube and DNA extraction buffer was added to final volume of 1.8 mL. 10 µL of RNAse (10 µg/µL) were added and the tube was incubated for 10 min at 37 °C. After addition of 10 µL Proteinase K (20 µg/µL), a second incubation was carried out for 30 min at 50 °C. Subsequently, the mixture was divided in two equal parts, each mixed with 900 µL of phenol. The two resulting phases were homogenized by shaking for 2 min and afterwards centrifuged (10 min, 12,000 rpm, RT) in a tabletop centrifuge. The top phase was carefully transferred to a new 2 mL tube and again 900 µL of phenol were added. Homogenization and centrifugation were repeated. The top phase was again transferred to a new tube and 900 µL of chloroform/isoamylalcohol (24:1) were added. Homogenization and centrifugation were repeated once again. And both top phases were united and an equal amount of isopropanol was added. The two phases were not mixed for the first 5 min; subsequently they were mixed by inverting the tube carefully. The precipitation of DNA was carried out for 30 min (-20 °C) and then the sample was centrifuged (5 min, 8,000 rpm, 4 °C) in a tabletop centrifuge. The DNA-pellet was rinsed once with 70% ethanol, centrifuged (2 min, 8,000 rpm, 4 °C) and then air-dried. The pellet was re-dissolved in 50 µL ddH_2O and incubated for 10 min at 65 °C.

3.15.4 DNA isolation from *Hordeum vulgare*

One grain of barley was controlled crushed, until meal is gained. The flour was transferred to a prechilled (liquid N2) Teflon beaker and 750 µL of extraction buffer were added (0.02% of mercaptoethanol was added directly before usage). The mix was processed for 2 minutes at a dismembrator. The frozen solution was melted under RT and addition of 10 µL of protein kinase A.
Incubation was performed in a reaction tube for 30 min at 60 °C, then Chloroform/Isoamylalcohol (24:1) was added and solution was shaken thoroughly until a milky emulsion appeared. Next centrifugation was for 10 min at RT at 12,000 rpm. The aqueous phase was transferred to a new reaction tube and 0.6 Vol of Isopropanol was added. DNA precipitated at -20 °C for 30 minutes. Centrifugation followed at 4 °C for 15 min at 12,000 rpm. The pellet was washed with ice-cold 70% ethanol containing 10 mM ammonium acetate. Centrifugation, 15 min at 12,000 rpm and 4 °C. Washing step with 70% of ethanol followed. The pellet was redissolved in 50–100 µL of water.

Extraction buffer: 2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0;
0.2% β-mercaptoethanol.

3.15.5 Isolation of DNA fragments from agarose gel

DNA extraction from the agarose gel followed the `QIAquick Gel Extraction Kit Protocol´.

3.15.6 Purification of DNA

DNA fragments were purified according to the protocol of the `QIAquick PCR purification Kit´.

3.15.7 Restriction

Restriction analyses were carried out according to Sambrook et al. (2002). In general, 20 µL reaction mixtures were used, containing 1 µL of each restriction enzyme (10 U/µL), 2 µL 10 x restriction buffer and 200 ng of DNA. The restriction mixture was incubated at 37 °C for 1 hour.

3.15.8 Ligation

To ensure successful ligation, reaction mixtures were prepared, containing different ratios of insert DNA:vector DNA in a 16 µL volume of DNA in H_2O. Linearized vector was used to estimate the rate of self ligation. The DNA was melted at 70 °C for 45 sec, cooled down, first for 5 min to room temperature, then for 5 min at 4 °C on ice before 2 µL ligation buffer and 1 µL of T 4 DNA Ligase (5 U/µL) were added. Incubation was performed overnight at 14 °C. Dephosphorylation of vector DNA was performed using phosphatase at 37 °C for 30 min.

3.15.9 Isolation of RNA from *S. rolfsii* using CsCl-pad

Isolation of total RNA was performed according to Chirgwin et al. (1979). 100 mL of EPSmin17 or EPSmax13 were inoculated with mycelium of *S. rolfsii* (1/8 Petri Dish, grown for 5 days). The flasks were incubated at 28 °C, 250 rpm on a magnetic stirrer. After 48 h of cultivation, mycelium was harvested by filtration through a piece of gauze (Degussa) followed by washing with sterile aqua dest. Approximately 1 g was transferred to a prechilled (fluid N_2) Teflon cup, filled with two steal bulls, the mycelium was disintegrated for 2.5 min with dismembrator (Braun Biotech) and cooled again in liquid N_2. 5 mL of extraction buffer were provided to an autoclaved beaker (50 mL). The pulverised mycelium was resuspended and transferred to a SS34 centrifuge tube, sealed with Parafilm and agitated. 250 µL of 10% Na-Laurylsarcosine was added and the tube was turned gently. Centrifugation at 9,000 rpm, 10 min, RT followed. 5 mL of the CsCl-pad (3.5 mL 5–7 M CsCl + 0.01 M EDTA) were provided in an Ultracentrifuge tube and 1.5 mL of the supernatant was added (keep UZ tube angular for adding the supernatant gently at the boundary). The rotor AH650 was used and centrifugation at 32,000 rpm for 19 h, RT was performed. The supernatant was removed, except to approximately 1 cm above bottom level of the Ultra centrifuge tube. The bottom of the ultracentrifugation tube (containing the RNA pellet) was removed with a hot scalpel. The trimmed bottom was turned off and last supernatant was discarded. The pellet was dissolved in 300 µL TE-SDS buffer and 1/10 Vol of 8 M LiCl containing 2.2 Vol ice cold EtOH (96%) was added. A centrifugation step of 15 min, at 4 °C followed. The pellet was washed in 500 µL ice cold EtOH 80% and after a last centrifugation (15 min, at 4 °C) dissolved in 100 µL of DEPC-H_2O.

3.15.10 Gel electrophoreses

In order to separate DNA fragments according to their size, a horizontal gel electrophoresis was performed. Depending on the number of samples, either a midi gel (18x 10x 0.5 cm) or a mini gel (6x 10x 0.5 cm) containing 0.7–1.2% agarose in 0.5 TAE buffer was prepared (depending on the size of fragments). The samples were mixed with loading buffer. GeneRuler™ or λ-DNA digested with *Hind*III was separated along with the samples. The gels were run at 120 V for 45 minutes to 1 hour. The bands became visible after staining the gel in ethidium-bromide solution (0.4 mg/mL H_2O) for about 20 min by exposing it to UV light (254 nm). The results were documented with INTAS video documentation-system, Göttingen. The DNA concentration was determined by visual estimation compared to the GeneRuler™ after electrophoresis and staining with EtBr. For RNA analysis, 18 x 23 x 0.5 cm horizontal formaldehyde gels were used.
On a heated stir plate, using a stir bar, 2.25 g of agarose was combined with 128 mL of dH_2O in 300 mL Erlenmeyer flask and stirred until the agarose was in solution. (All the following steps were performed under the fume hood.) The mixture was cooled down to ~60 °C, and then 15 mL 10 x MOPS and 7.4 mL 37% formaldehyde were added. The gel was run in 1 x MOPS, just covering the gel, for 2–3 h at 120V. RNA samples were applied in RNA sample buffer which contained ethidium bromide, and the results were documented with INTAS video documentation-system, Göttingen.

3.15.11 Northern blot analysis

Northern analysis was performed according to Sambrook et al (1989). After gel electrophoresis; RNA was transferred to positively charged nylon membrane. RNA was blotted overnight and afterwards UV-cross linked. Hybridisations with radio labelled probes were carried out in Rapid Hyb buffer according to the manufacture's instructions (Amersham). Membranes were put in X-ray cassettes with intensifying screens and incubated at -70 °C.

3.15.12 Southern blot analysis

Southern analysis was performed according to Meyer et al. (2002). The genomic DNA was restricted and separated by gel electrophoresis in a 0.7% agarose gel. After staining and photographing the gel, it was shaking in 0.25 N HCl until the marker colour (bromophenol-blue) turns into yellow (~10 min) in order to fragment large DNA-molecules. Subsequently, the gel was rinsed with water and denaturation buffer (1.5 M NaCl, 0.5 M NaOH) was applied for 30 min to turn double strand DNA into single strand DNA. Afterwards neutralization buffer (1.5 M NaCl, 0.5 M Tris/HCl) was applied for 30 min. The DNA was transferred from the gel onto a positively charged nylon membrane by applying transfer solution (1.5 M NaCl, 0.15 M Na-citrate) using capillary force overnight. UV-cross linking followed. Hybridisations with radio labelled probes were carried out

in Rapid Hyb buffer according to the manufacture's instructions (GE Healthcare). Membranes were placed in X-ray cassettes with intensifying screens and incubated at -70 °C.

Radioactive labelling of DNA
PCR products were labelled by random primer labelling using [α-^{32}P] dATP (Megaprime labelling kit, GE Healthcare).

3.16 PCR

The polymerase chain reaction (PCR) was used to introduce appropriate restriction sites to constructed *oxoX and oxdc* expression cassettes. Moreover, PCR was also used to identify recombinant clones of interest. PCR products were purified using a "QIAquick PCR purification kit" and after purification they were directly ligated into pGEM-T or pJET1.2/blunt vector when it was necessary. All primers were synthesized by Metabion (Martinsried, Germany).

A general Biometra PCR reaction scheme

Component	**µL**
H_2O	9.8
4x reaction buffer	5
dNTP's (1mM total)	2
template 1 ng/µL (plasmid DNA) 100 ng/µL (genomic DNA)	1
forward primer (10-15 pmol)	1
reverse primer (10-15 pmol)	1
Taq polymerase (4 U/µL)	0.2

In the case of degenerated primers always the double concentration (20–30 pmol) was used

A standard Biometra PCR – program

Denaturation	94 °C	3'	
1st Primer annealing	60 °C	30''	
1st Elongation	72 °C	20''	
Denaturation	94 °C	15''	} 25x
Primer annealing	60 °C	15''	
Elongation	72 °C	30''	
Post-elongation	72 °C	2'	
Post-treatment	4 °C	5'	

For each PCR reaction, the program slightly differed; details are given in following table.

PCR programs differing from the usual

Primer used	1st annealing, 2–25 th annealing	Elongation
Hyg 1 Hyg 2	60 °C 30'' 62 °C 20''	72 °C, 30''
Bar 1 Bar 2	56 °C 30'' 60 °C 20''	72 °C, 30''
BI BT HI BT	60 °C 30'' 62 °C 30''	72 °C, 25''
Hyg 1 Hyg 2	60 °C 30'' 62 °C 15''	72 °C, 25''
GlyoxWob1 GlyoxWob2	50 °C 35" 55 °C 35"	72 °C, 25"
IST6 NS7	55 °C 35" 58 °C 35"	72 °C,30"
S5 5'5' S5 3'5'	55 °C 35" 58 °C 35"	72 °C, 45"
Spin1 Spin 2	55 °C 35" 55 °C 35"	72 °C, 45"
S9 3'5' S9 5'5'	50 °C 35" 54 °C 35"	72 °C, 45"

List of PCR primers used. Underlined sequences in primers Oxox fw2 (assembly)/AB2oxox and AB2oxdc/Oxdc 2fw (assembly) are homologous.

Primer name	Oligonucleotide sequence 5'-3'
BI BT	AAAGGATCCTCTGCGCACGTAAAG
HI BT	CGGAAGCTTGGCCAGCAGDAYAC
Glyoxwob fw	AATGTSAWSGAGTAYSAGGCC
Glyoxwob rev	ACATCCTTCCAGSTSAGGG
AB2 oxox	TTACTCTAAAAACCTTCGCAATCGATGGCGATAAGCTT
Oxox fw2 (assembly)	AGGTTTTTAGAGTAAGCAAGGTACCATGGGTTACTCTA
Hph rev BamHI	CGGGATCCCGCCTGTATCGAGTGGTGATT
AB2 oxdc	ATGGATGGATCCTTAATCAATCGATGGCGATAAGCTT
Oxdx fw2 (assembly)	TTAAGGATCCATCCTAAATTAAGGATCCATCCATCGC
Oxdc2rev BamHI	GGATCCAATACCTAGGTAGGAAATCATATCCGGCCG
gpdAB up BamHI	CGGGATCCCGACAGACGTCGCGGTGAGTT
Hyg 1	TCAGCTTCGATGDAYGAGGG
Hyg 2	TGTATTGACCGATTCCTTGCG
Oxdc 1 velutipes	ATTAAGGATCCATCCATCGCATTTCCGATG
Oxdc 2 velutipes	AATACCDAYGTAGGAAATCATATCCGGCCG
Bar1	GGTACGAACACGCTGGGC
Bar2	CCGGCCTCCACCCGAAGAG
OxoX barley down	GCAAGTCGACTGGGGCTCATGGAAGTTAAG
OxoX barley fw	GCAAGGTACCATGGGTTACTCTAAAAACCDAY
Oxdcrev BamHI	CGGGGATCCCGTGGGGCTCATGGAAGTTAAG
ITS 6	CCAGATTTCAAATTTGAGCT
NS 7	GAGGCAATAACAGGTCTG TGATC
3'Exon2	CGTAGCCAGTGTCCATGTC
3'Exon3	GGCAATCAGCAGGATGTC
3'oah-AN	TCTTGCTTACTACTGCCTGC
5'Exon1a	CAATGAGTTGCTCGTGTG TCC
5'Exon 1b	TGTACGACGGTCTGTCCG
5'Exon1c	GGCATGTACATGGTATGTT
5'Exon2	GACATGGACACTGGCTACG
5'oah-AN	AGGTTGATTGCTCGCTCG C
S5 5'	GACAGCCGATGATCCAGA
S5 3'	CACCGGTATTGAAGACCT
Spin1	AAATGTGAAGGAGTATGAGGC
Spin 2	GTCAGCTCAAATTCATCGCG
S9 3'	TCGTGCTTGCCGGAGAGAGGTA
S9 5'	GCTCGAGGATGCCGATCA

3.17 Construction of transformation plasmids for *S. rolfsii*

3.17.1 Cloning of oxalate oxidase from *Hordeum vulgare*

The mRNA for the oxalate oxidase gene from *Hordeum vulgare* (Accession Nr. Y14203) was extracted from 48 h old seedlings grown on wet cotton in a Petri Dish at 28 °C. Oxalate oxidase is known to be highly expressed in the coleorhizae of the seedling and not in the coleoptiles (Dumas et al. 1995). Due to that reason, only the area around the coleorhizae and a small part of the roots was used for RNA isolation. RNA was harvested by Promega™ pure yield kit. The primers for reverse transcriptase reaction were used according to Zhou et al. (1998). The reverse transcriptase reaction was set as follows. 25 mM dNTP mix (each), Array Script RT Buffer 10*, RNAse inhibition and 200 U/µL AMV reverse transcriptase (Amersham), were dispersed in DEPC water, together with 5 µL of RNA. Primer annealing was performed for 5 min at RT, than 1 h at 42 °C, and elongation was performed for 15 min at 70 °C.

The primers oxoX barley up and oxoX barley down (1 µL each) were added to 2 mL dNTP Takara mix in 2 µL Takara 10* buffer. 0.2 µL of Takara-Polymerase were added and 11.8 µL of HPLC purified water were added. As template 2 µL of cDNA for *oxoX* from *hordeum vulgare* was used. The PCR was performed by following scheme. The first denaturation step was performed for 30'' at 94 °C; the fist annealing temperature was set to 44 °C for 1 min. Elongation of 1 minute at 72 °C follows. Next denaturation was set to 30'' at 94 °C with annealing at 50 °C for 1 min and an elongation step of 1 min at 72 °C. Next step was a cycle of denaturation for 30'' at 94 °C with annealing for 1' at 56 °C and elongation for 1' at 72 °C, repeated for 38 times. Last step was an elongation phase for 5 minutes at 72 °C, with following cooling to 4 °C. The sequence was cloned into pjet1.2 vector and sequenced for verification.

3.17.2 Cloning of oxalate decarboxylase from *Flammulina velutipes*

The mRNA for the oxalate decarboxylase gene from *F. velutipes* (AF200683) was extracted from 4 days old culture, grown on EPSmax13 medium provided with cellophane, at 28 °C. Te RNA was extracted by conventional Phenol extraction. The primers for reverse transcriptase reaction were 1S55´ and 1S53´. The reverse transcriptase reaction was set as follows. 25 mM dNTP mix (each), Array Script RT Buffer 10*, RNAse inhibition and 200 U/µL AMV reverse transcriptase (Amersham), were dispersed in DEPC water, together with 5 µL of RNA. Primer annealing was performed for 5 min at RT, than 1 h at 42 °C, and elongation was performed for 15 min at 70 °C. The primers 1S55´ and 1S53´ (1 µL each) were added to 2 mL dNTP Takara mix in 2 µL Takara 10x buffer. 0.2 µL of Takara-Polymerase were added and filled up with 118 µL of HPLC purified water. As template 2 µL of cDNA for *oxdc* from *F. velutipes* was used. The PCR was performed by following scheme. The first denaturation step was performed for 30'' at 94 °C; the fist annealing temperature was set to 44 °C for 1 min. Elongation of 1 minute at 72 °C follows. Next denaturation was set to 30'' at 94 °C with annealing at 50 °C for 1 min and an elongation step of 2 min at 72 °C. Next step was a cycle of denaturation for 30'' at 94 °C with annealing for 1' at 56 °C and elongation for 2' at 72 °C, repeated for 38 times. Last step was an elongation phase for 5 minutes at 72 °C, with following cooling to 4 °C. The sequence was cloned into pjet1.2 vector and sequenced for verification.

3.17.3 Assembly PCR

The amplification of *oxoX* and *oxdc* constructs with appropriate restriction sites was achieved by an assembly PCR. First, *oxoX/oxdc* gene and *gpd* promoter region were separately amplified with suitable restriction sites. For *gpdA* promoter, AB2 BamHI and *gpd*Assrev, and for ORF of *oxoX*, *oxoX*xup and *oxoX*revBamHI primers were used. The 3' end of *gpdA* was homologous to the 5' end of *oxoX*. After 1st PCR, each product was purified using a `QIAquick PCR purification Kit´ and was used in subsequent amplification. In the 2nd step, the amplicons from the 1st PCR act as template DNA. The homologous ends which were introduced during the 1st PCR can hybridize and initiate new elongation steps. Together with two external primers (AB1 and oxoXrev BamHI) *oxoX* gene with appropriate restriction sites was obtained. The strategy of this method is shown in following figure.

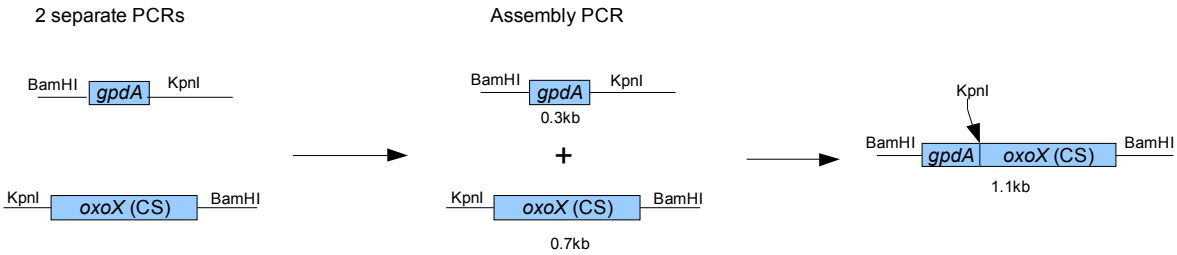

Figure of the strategy for the assembly PCR. In 1st round of PCR, oxoX/oxdc coding sequence and gpdA promoter region were separately amplified and in the 2nd round of PCR, both amplicons act as template DNAs and the amplification gives *gpd*:*oxoX* coding sequence and oxdc coding sequence with proper restriction sites for further introduction into cloning vector and fusion with terminator sequence.

3.17.4 Cloning of *oxoX*/*oxdc* expression cassettes

In order to establish *oxoX /oxdc* expression vectors several cloning steps were carried out. The overall cloning strategy is given in Figure B. At each cloning step, recombinant clones were selected via ampicillin, and confirmation by colony PCR, mini-prep plasmid DNA preparation and restriction analysis. Finally each clone was purified using QIAGEN plasmid Midi kits.

First the beta tubulin terminator (tubterm) from the plasmid pAN-NC3 was isolated with the primer pair HI-BT/BI-BT. At step 2, the pUC18 vector was linearized with HindIII/BamHI and dephosphorylated. The introduced restriction sites allowed direct ligation of the purified tubterm into the linearized pUC18 vector, resulting in the plasmid called pUCTUB. At step 3, the *hph* gene was isolated from the plasmid pBGgHg by use of the restriction enzymes XmnI and EcoRV and inserted into the pUCTUB plasmid cut by SmaI, resulting in plasmid pUCTUB hph. The *gpd* promoter was isolated from pBGgHg with the primer pair AB1/Ab2BamHI. The generated *oxoX* or *oxdc* respectively were fused to this promoter via assembly PCR. This was the fourth step that resulted in the assembly of the *gpdA* promoter and the *oxoX*/*oxdc* coding sequence by PCR with the primer pairs AB1/OxoXrev BamHI and AB1/OxDc BamHI respectively. *OxoX/oxdc* gene was introduced into pUCTUB hph vector by restriction with *Bam*HI yielding plasmid pUCOX or pUCDC respectively. A schematic overview of the cloning strategy is given in Figure B.

3.18 Fluorescence microscopy

Fluorescence was observed with a Zeiss Axioskop (Zeiss, Jena) fluorescence microscope equipped with a 48 79 09 filter set (Zeiss) for fluorescence detection (excitation wavelength 450 to 490 nm, emission wavelength 520 nm).

3.19 Microarray analysis

Tailor-made microarrays (44K multiplex chip, Agilent) were designed by imaGenes (Berlin, Germany) using an in-house developed method for empirical selection of best performing probes for each gene (Pre Selection Strategy). Briefly, up to ten probes were designed for each of the 454 and SSH sequences as well as for the *oxox* gene (60 bp long oligomers). The 244K Agilent test array was hybridized with pooled Cy3-labeled cRNAs gained form EPSmax13 and EPSmin17 cultures (see above) and (in average) two of the best performing oligos were selected for each sequence.

For comparative expression profiling, total RNA was isolated from *S. rolfsii*, cultured for 37 h in EPSmax13 and EPSmin17 media as described above. RNA quality control, synthesis of Cy3-labeled cRNA including cRNA purification and cRNA quality control, microarray hybridization, scanning and data extraction (Agilent´s feature extraction software) were performed by imaGenes GmbH. Expression data were analyzed by imaGenes GmbH using an in-house developed data analysis pipeline. After quantile normalization, genes were defined as differentially expressed if their expression levels varied at least 2 fold in EPSmax13 samples compared to EPSmin17 samples and if the difference was statistically significant (Student's t-test, *P*-value cut-off of 0.05).

4 Results

4.1 Establishment of a suitable transformation system for *Sclerotium rolfsii*

4.1.1 Selection system

Finding a safe and suitable selection system is the main prerequisite for a successful transformation. However, a transformation method that makes *S. rolfsii* accessible for genetic manipulations, which is a prerequisite for strain improvement, has not been developed so far. Several antibiotics were thus tested for growth inhibition of *S. rolfsii*. Antibiotics used were:

Antibiotic	Mechanism	Reference
hygromycin B	aminocyclitol antibiotic, inhibits protein synthesis, in prokaryotes and eukaryotes	Pettinger 1953
geneticin	G418, aminoglycoside, blocks protein biosynthesis in prokaryotes and eukaryotes	Davies and Jimenez 1980
benomyl	binds to microtubuli and inhibits cell division	Davidse and Flach 1978
phleomycin	glycopeptide antibiotic of the bleomycine family	Kuwahara and Sugiura 1988

Concentrations of 10, 50, 100 and 150 µg/mL of the different antibiotics were tested on six different media: Czapeck-Dox, Cove, Medium B, CYM, EPSmin17 and MGY+ media. Cultivations were carried out for 3 days at 30 °C on solid media. Cultivations without the addition of the respective antibiotic were performed for each medium as control.

An inhibiting effect was only seen at concentrations of 100 µg/mL in the case of G418 and hygromycin in Cove medium. There was marginal growth inhibition with phleomycin (Fig. 6).

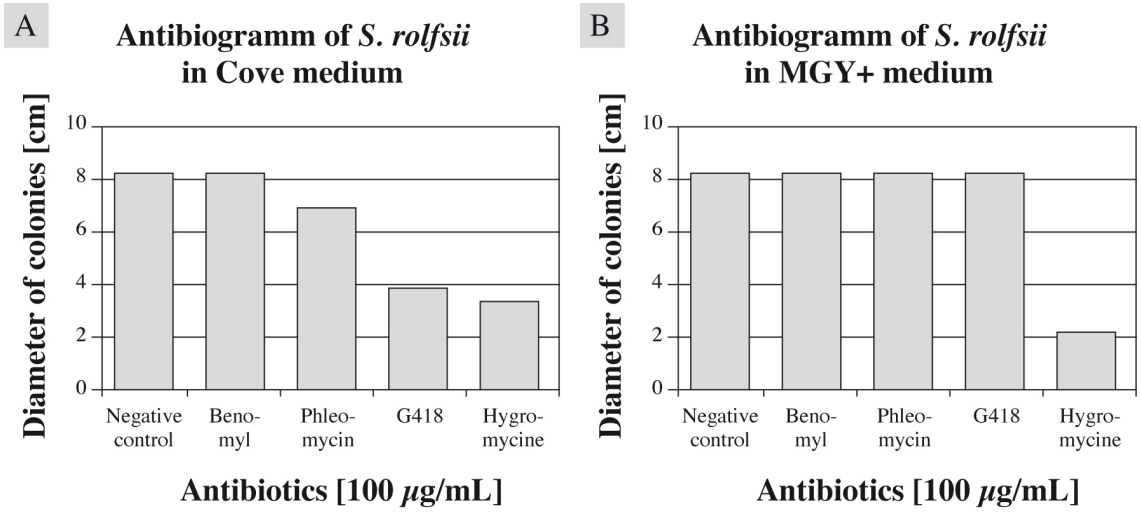

Figure 5: Antibiogram of *S. rolfsii* grown on A) Cove medium and B) MGY+ medium, supplemented with the antibiotics; benomyl, phleomycin, geneticin (G148) and hygromycin (final concentration of 100 µg/mL). The diameters of *S. rolfsii* colonies were determined after 3 days of cultivation at 30 °C on solid media.

Lower final concentrations did not result in growth inhibition of *S. rolfsii*. Hygromycin used in MGY+ medium was the only antibiotic found to be active in this medium (Fig. 6).
A further increase of concentration did not result in improved growth inhibition. Therefore, a final concentration of 100 µg/mL hygromycin was chosen for the transformation approaches. MGY+ medium, which showed the best growth inhibition with hygromycin, was used as selection medium in all transformation experiments with *S. rolfsii*.

4.1.2 *Agrobacterium tumefaciens* mediated transformation

In an attempt to facilitate the genetic transformation of *S. rolfsii*, two different transformation approaches, *Agrobacterium tumefaciens*-mediated transformation (AMT) and PEG-mediated transformation of protoplasts (PMT), were tested.
The literature on protoplast formation of *S. rolfsii* provides little information (Deshpande 1987; Kelkar 1990; Farina et al. 2004), while no information was available for the transformation by *A. tumefaciens*. These two different methods were thus adapted to *S. rolfsii* and compared with respect to handling and efficiency. Different strains of *A. tumefaciens* were used for AMT, (LB1100 and AGL1). Strain LB1100 is equipped with an unarmed helper plasmid containing the virulence genes for the infection of the host. The second plasmid, present in LB1100 is the binary vector pUR5750 carrying the *hph* gene of *E. coli* (conferring hygromycin resistance) under control of the *gpd* promoter and the *trpC* terminator from *Aspergillus nidulans*. Strain AGL1 was improved by introducing *vir* genes from *A. tumefaciens* strain A281 into the existent Ti-plasmid (pTiBO542). *A. tumefaciens* strains containing this modified Ti plasmid are regarded as highly virulent and cause fast growing tumours in plants (Hood et al. 1986; Ogawa and Mii 2001). AGL1 harbours the binary vector pBGgHg (Fig. A). This plasmid contains the *E. coli* hygromycin resistance gene *hph* driven by the *gpd* promoter of *Agaricus bisporus* and the CaMV 35S terminator of cauliflower mosaic virus (Chen et al. 2000).
For *A. giganteus*, a ratio of about 10^6 fungal conidia to $1.2*10^8$ cells of *A. tumefaciens* is necessary to obtain efficient transformation results (Meyer et al. 2003b). However, as *S. rolfsii* does not exhibit the most favourable constituents as conidia or sporangiospores, whole mycelium had to be transformed via AMT, making the procedure more difficult and laborious.
Several variables such as co-cultivation time, co-cultivation temperature and bacteria to fungus ratio were tested using both *A. tumefaciens* strains (Table 3). The *A. tumefaciens* concentrations used ranged from $6*10^6$ CFU/mL to $5*10^9$ CFU/mL.

Co-cultivation of mycelium grown in liquid medium as described for other fungi and plants (Huang et al. 2007) did not lead successful transformation with *S. rolfsii*. Whilst *S. rolfsii* grows on the surface of the liquid culture *A. tumefaciens* sedimented to the bottom of the flasks. Thus no transformants were obtained by this procedure (data not shown).

As submerse cultivated *S. rolfsii* suspensions proved to be inappropriate for AMT, solid mycelium was used for all transformation approaches. Thus, 1 cm^2 of *S. rolfsii* mycelium (grown 5 days at 30 °C) was placed in the centre of an agar plate (covered with cellophane membrane), and co-cultivated with an overlay of 300 µL of *A. tumefaciens* cell suspension. Prolonged co-cultivation periods (48 h to 72 h) revealed more transformants; however, further prolongations resulted in significantly reduced transformation efficiencies. The increased events obtained by co-cultivation of 72 h, are in a small amount, approximately 3 colonies more are found on every plate, which is equal to around 5% increase of transformation efficiency.

Table 3: Various conditions for transformation of *S. rolfsii* by AMT. Influence of different co-cultivation conditions using two different *A. tumefaciens* strains (LB1100 and AGL1). Hygromycin-resistance was determined after transfer of putative transformants on new selection plates containing 100 µg/mL hygromycin B. Co-cultivation as well as growth on selection plates were performed at 24 °C.

Strain	Ratio cells/cm^2	Co-cultivation [h]	Hygromycin resistance*	No. of putative transformants/[cm^2]
LB1100	6x 10^6 /1	36	weak	0
	5x 10^9 /1	36	weak	0
LB1100	6x 10^6 /1	48	weak	0
	5x 10^9 /1	48	weak	1
LB1100	6x 10^6 /1	72	weak	0
	5x 10^9 /1	72	weak	1
LB1100	6x 10^6 /1	96	weak	0
	5x 10^9 /1	96	weak	0
AGL1	6x 10^6 /1	36	weak	1
	5x 10^9 /1	36	weak	3
AGL1	6x 10^6 /1	48	weak/clear	3
	5x 10^9 /1	48	clear	2
AGL1	6x 10^6 /1	72	clear/weak	7
	5x 10^9 /1	72	clear	9
AGL1	6x 10^6 /1	96	weak	5
	5x 10^9 /1	96	weak	4

→ *Hygromycin resistance was determined by growth on new selection medium after 2 weeks of selection.

Transformants were checked on the molecular level for the transferred *hph* cassette by PCR with *hyg1*/*hyg2* primers and ITS6/NS7 primers as control for fungal DNA (Fig. 8).

4.1.3 PEG mediated transformation of protoplasts

Although protoplast formation in *S. rolfsii* has previously been reported, the described protocols significantly differed with respect to protoplast yields and regeneration rates among different *S. rolfsii* strains (Deshpande et al. 1987; Kelkar et al. 1990). In this work, the generation of protoplast for *S. rolfsii* was according to a modified protocol from Farina et al. (2004) including the hitherto described media. As regards lytic enzymes, three different products were tested - Novozyme 234, Glucanex and Vinoflow FCE. For all protoplastations, 45 h old mycelium pregrown in EPSmin 17 medium was used, which produced the highest yield of protoplasts.

The influence of lytic enzyme purity on protoplastation yield seemed to be negligible. Mixes of beta-glucanases and pectinases (Vinoflow FCE and Glucanex) resulted in the same amount of protoplasts compared to pure lytic enzyme (Novozyme 234). Therefore, cost efficient lytic enzyme mixes were used for further experiments. The ratio of biomass to lytic enzyme is the main factor for protoplast yield with *S. rolfsii*. Different ratios were tested and a strong increase of protoplast yield was observed using a ratio of 20:1 instead of 10:1 (w/w) lytic enzyme mix to bio dry mass (data not shown). A further increase in the amount of lytic enzyme did not improve protoplast yield. The optimal treatment time for the lytic enzymes was 90 minutes. Extended enzyme treatment resulted in a decrease in protoplast yield (Fig. 6). Optimized protoplast yields ranged from $1*10^6$ to $2*10^7$ per gram bio dry mass (BDM). This value is far below the $1*10^9$ protoplast per gram BDM reported by Farina et al (2004). Parallel protoplastation was set up to procure enough protoplast per µg plasmid DNA needed for transformation (Meyer et al. 2003b).

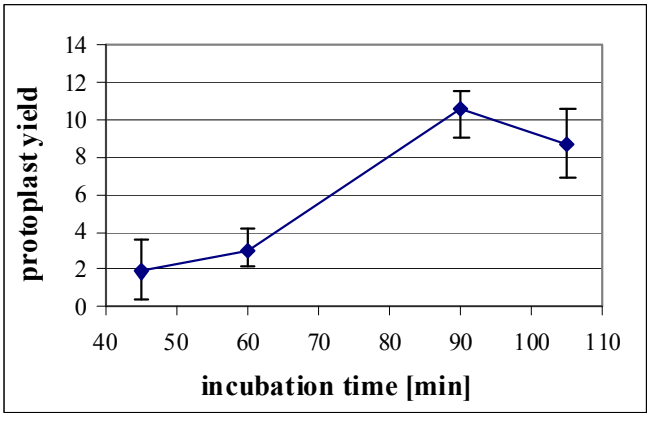

Figure 6: Kinetic of protoplast formation in *S. rolfsii*. Lytic conditions: 45 h old mycelium harvested from EPSmin17, 20:1 (w/w) biomass dry weight:enzyme ratio, lytic enzymes mix Vinoflow FCE, 28 °C, 70 rev min^{-1}. Bar markers show standard deviation from mean values of three independent experiments. The protoplast yield is calculated on protoplasts$*10^6*g^{-1}$ bio dry mass of mycelium.

Another critical factor is displayed by the concentration of hygromycin used in the top agar given to the protoplasts 18 h after transformation to raise selection pressure. Concentrations higher than 100 µg/mL resulted in nearly no regenerated protoplasts (Fig. 7).

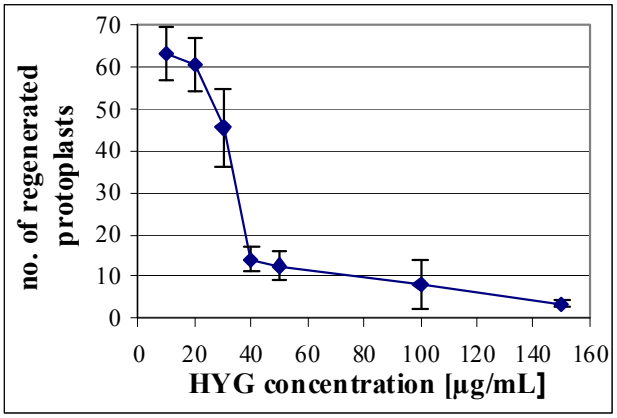

Figure 7: Influence of hygromycin B (HYG) concentration (final concentration per mL of medium, concentrated in poured soft agar, given to protoplasts 18 h after transformation). Regeneration ratio of protoplasts is displayed by the Y-axis (average number of protoplast was $2.5*10^8$).

A transformation rate of 1–5 transformants/µg DNA/10^6 protoplasts was determined for PMT. It was impossible to calculate a transformation for AMT rate due to the fact that putative transformants grew into each other during growth on selection plates. Therefore, only the transformation events per experimental approach and plate were used as degree of transformation efficiency. Both AMT and PMT were successfully established for *S. rolfsii,* which is the first report of transformation for this basidiomycete. No expression of GFP by fluorescent microscopy could be observed for the transformations performed using plasmid pBGgHg (carrying the EGFP gene). Homologous intron sequences within the gene are reported to be necessary for successful EGFP expression in basidiomycetes (Ma et al. 2001; Burns et al. 2005; Kües 2005; Rekangalt et al. 2007).

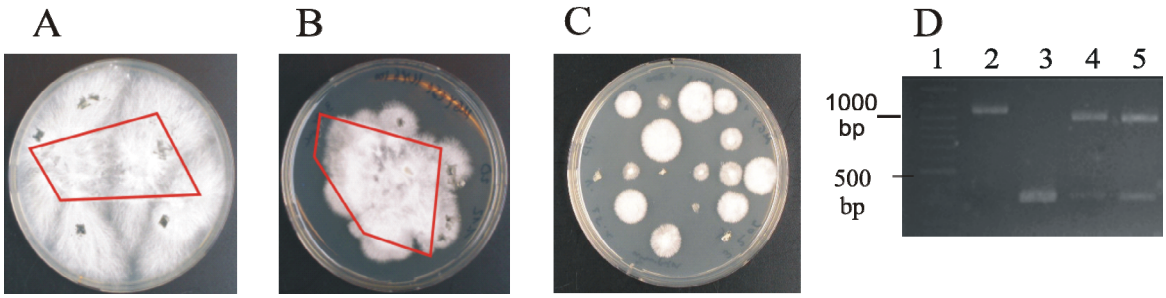

Figure 8: Growth of putative *S. rolfsii* transformants on selection plates containing 100 µg/mL hygromycin B.
A) Putative transformants of *S. rolfsii* obtained by AMT. B) Corresponding negative control for the AMT. Red lines in A and B indicate the excised piece of cellophane from the co-cultivation plate transferred to the selection plate. C) Transferred putative *S. rolfsii* transformants gained by PMT on selection medium. D) Successful transformation was confirmed on the molecular level by PCR. The hygromycin resistance gene of the expression cassettes was amplified; the internal transcribed sequence (ITS) of *S. rolfsii* was amplified as the control. Lane 1 GeneRuler™, lane 2 positive control for fungal DNA, lane 3 positive control for *hyg* gene, lane 4 positive confirmation of transformation via PMT by PCR, lane 5, positive confirmation of transformation via AMT by PCR.

In addition, the multinuclear state of some mycelia and protoplasts hampers the selection of transformants and renders the selection process tedious (Deed and Seviour 1989; Farina et al. 2004). Thus, the nuclear status of *S. rolfsii* protoplasts was analysed by DAPI staining of double stranded DNA. Protoplasts carried about 2–8 nuclei in approximately 80% and 0–1 nuclei in around 20% of protoplast were analysed (Fig. 9). This multinuclear status may explain the relatively low transformation rates observed for both methods used. Transformation of multinuclear compartments often results in transformation of only one nucleus, therefore minimizing transformation efficiency (Maheshwari 2005), and prolonging the selection of the transformants, until solely transformed mycelia can be obtained.

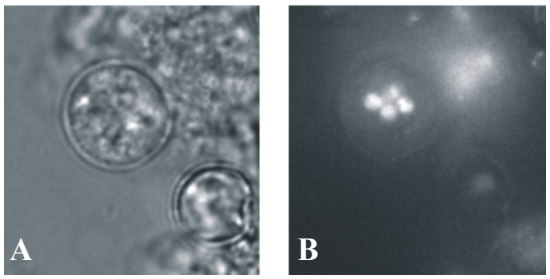

Figure 9: Microscopically investigation of the nuclear status in *S. rolfsii*. A) Transmission B) fluorescent microscopy, the protoplast were stained with DAPI for DNA labelling. Oil microscopy, magnification 1000x. Protoplasts were obtained by the improved protocol for protoplastation.

4.1.4 Development of suitable transformation vectors

Two cloning vectors for heterologous gene expression in *S. rolfsii* were constructed. Both vectors are based on the backbone from standard vector pUC18. The *hph* cassette from pBGgHg (driven by *gpdII* promoter from *A. bisporus* and terminated by the CaMV 35S terminator) was inserted as the selection marker. Two genes known to play a role in oxalate catabolism were chosen for heterologous expression in *S. rolfsii*, namely oxalate oxidase (*oxoX*) and oxalate decarboxylase (*oxdc*). It should be possible to identify transformants carrying either one of the genes by the decreased oxalate levels in the fermentation broth. For expression of *oxoX* gene from *Hordeum vulgare*, cDNA of oxalate oxidase was synthesized by 2 d old germlings of *H. vulgare*. The coding sequence of *oxoX* was fused with the *gpdII* promoter from *A. bisporus* and the *tub* terminator of *Neurospora crassa*, resulting in the plasmid named pUCOX with a size of 5.5 kb. For expression of oxalate decarboxylase, cDNA of oxalate decarboxylase was successfully synthesized by RNA extracted from 6 day old cultures of *Flammulina velutipes*. The coding sequence from *oxdc* gene of *F. velutipes* was fused with the promoter and terminator as mentioned above, and was also introduced, pUC18, resulting in the 7.3 kb sized plasmid called pUCDC. For detailed construction and cloning strategy see (3.17 and Fig. B).

S. rolfsii was transformed with pUCOX and pUCDC, using the PMT method. 10 µg of plasmid DNA was used for each transformation approach. Putative transformants were transferred to new MGY+ selection media (100 µg/mL hygromycin) twice. After two weeks of selection, 250 mL flasks, containing 50 mL of EPSmin17 or EPSmax13 liquid medium, respectively, were inoculated with 1 cm^2 of mycelium obtained from putative transformants. Samples were taken every 24 h and oxalate level was measured via HPLC in both media. More than 85 putative transformants were tested which yielded the same results. All putative transformants tested resulted in non reduced oxalate levels. Oxalate was also predominant (~2 gL^{-1} after 96 h) for all cultivations tested.

To verify whether instable integration of the expression cassette or an ineffective expression of the heterologous genes caused the effect of unaffected oxalate production, the DNA of 25 putative transformants was extracted and screened via PCR for the predominance of the respective expression cassette. It was not possible to confirm the expression constructs via PCR which indicates that no stable transformation was obtained by either pUCOX or pUCDC transformation plasmid. Instable integration might have been a result non stringent selection pressure caused by the use of hygromycin concentrations of 100 µg/mL. In addition, the further increase of hygromycin concentration to 150 µg/µL on primary transformants did not result in stable integration of the heterologous constructs. Furthermore, an increasing resistance of the *S. rolfsii* wildtype after longer cultivations on hygromycin containing medium was observed. This increasing resistance was observed by incubating the wild type for two weeks on medium containing hygromycin. The transfer to new selection plates resulted in a nearly normal growth behaviour for the non-transformed *S. rolfsii*.

The adaption of *S. rolfsii* to hygromycin hampers selection of transformants and heightens the risk of obtaining false positive transformants. PCR checks for *hph* and *oxoX* or *oxdc* respectively, were negative for all putative transformants after a selection period of two weeks. Transformations were performed five times with an average of 32 putative transformants for each heterologous gene, all resulting in false positive transformants and non influenced oxalate metabolism.

4.2 Development of different cultivation media for *S. rolfsii*

Media with scleroglucan inducing and non-inducing properties had to be developed for the examination of scleroglucan and oxalate biosynthesis in *S. rolfsii*. Different cultivation conditions can in general influence the transcriptional status of any microorganism. The

analysis of RNA populations obtained from cultivation of *S. rolfsii* in inducing/non-inducing media could thus lead to the identification of genes involved in the biosynthesis of scleroglucan or oxalate, respectively.

Most literature reports deal with the identification of cultivation media resulting in enhanced scleroglucan production (Wang and McNeil 1994; Wang and McNeil 1996; Farina et al. 1998; Schilling 2000; Survase et al. 2006; 2007b). The main influence was reported to be the C-source. Previous experiments have identified sucrose and glucose to be the most efficient scleroglucan inducing C-sources, whereas, fructose reduces scleroglucan yield. The focus on media development was thus to identify scleroglucan non-inducing conditions to obtain the lowest scleroglucan yields possible.

According to Farina et al (1998), a medium composition was used as basis medium and different carbon and nitrogen sources were tested. Scleroglucan production was qualitatively estimated by visual inspection of the medium viscosity (Table 4.).

Table 4: Estimation of growth of *S. rolfsii* and viscosity of cultivation medium dependent on different media compositions after 72 h of cultivation. *S. rolfsii* was cultivated in 100 mL medium on a magnetic stirrer (250 rpm) or shaker at 28 °C for three days. Abbreviations: - no growth/non viscosity + slight growth/slight viscosity, ++ good growth/good viscosity, +++ very good growth/high viscosity, (+) indicates different results in the biological duplicates Glc: glucose; Fru: fructose, Suc: sucrose.

Medium	C-source (mM)	N-source (mM)	Growth shaker/magnetic stirrer	Viscosity shaker/magnetic stirrer	pH-value shaker/magnetic stirrer
EPSmin1	55 Glc	56 $(NH_4)_2SO_4$	++/+++	+/+	2.65/2.59
EPSmin2	55 Glc	87 NH_4Cl	++/+++	++/++	2.71/2.47
EPSmin3	55 Fru	56 $(NH_4)_2SO_4$	++/+++	-/-	2.35/2.51
EPSmin4	55 Fru	87 NH_4Cl	++/+++	-/-	2.57/2.62
EPSmin5	55 Fru	174 NH_4Cl	++/+++	-/-	2.39/2.43
EPSmin6	55 Fru	285 NH_4Cl	-/+	-/-	2.42/2.50
EPSmin7	27 Fru	285 NH_4Cl	+(+)/+++	-/-	2.77/2.80
EPSmin8	138 Fru	285 NH_4Cl	+++/++	-/-	2.77/2.82
EPSmin9	27 Fru	26 $NaNO_3$	++/+++	-/-	2.68/2.69
EPSmin10	166 Fru	17 NH_4Cl	++/++	-/-	2.70/2.65
EPSmin11	166 Fru	35 NH_4Cl	++/++	-/-	2.72/2.74
EPSmin12	222 Fru	17 NH_4Cl	+++/++	-/-	2.43/2.39
EPSmin13	222 Fru	35 NH_4Cl	++/++	-/-	2.45/2.38
EPSmin13	166 Fru	17 NH_4Cl	++/++	-/-	2.52/2.54
EPSmin14	166 Fru	17 NH_4Cl	+++/++	-/-	2.60/2.58
EPSmin15	166 Fru	35 NH_4Cl	++/+	-/-	2.51/2.47
EPSmin16	222 Fru	17 NH_4Cl	+++/+	-/-	2.77/2.82
EPSmin17	**222 Fru**	**35 NH_4Cl**	**+++/+++**	**-/-**	**2.4/2.5**
MOPT	438 Suc	26 $NaNO_3$	++/+++	+/+	2.52/2.61
EPSmax1	27 Glc	187 NH_4Cl	++/++	-/-	2.56/3.13
EPSmax2	27 Glc	26 $NaNO_3$	++/+++	-/+	2.61/2.68
EPSmax3	55 Glc	26 $NaNO_3$	+++/++	++/++	2.49/2.55
EPSmax4	111 Glc	26 $NaNO_3$	+++/+++	++/++	2.42/2.47
EPSmax5	277 Glc	26 $NaNO_3$	+++/+++(+)	(+)++/+++	2.63/2.53
EPSmax6	166 Glc	17 $NaNO_3$	++/+++	++/++	2.24/2.43

Medium	C-source (mM)	N-source (mM)	Growth shaker/magnetic stirrer	Viscosity	pH-value
EPSmax7	166 Glc	35 $NaNO_3$	+++/+++	++/+++	2.27/2.27
EPSmax8	222 Glc	17 $NaNO_3$	+++/+++	+++/++(+)	2.29/2.3
EPSmax9	222 Glc	35 $NaNO_3$	+++/+++(+)	+(+)/++	2.29/2.32
EPSmax10	166 Glc	17 $NaNO_3$	+++/+++	+(+)/++	2.85/2.74
EPSmax11	166 Glc	35 $NaNO_3$	+++/+++	++(+)/+++	2.67/2.34
EPSmax12	222 Glc	17 $NaNO_3$	+++/+++	++(+)/++	2.43/2.21
EPSmax13	**222 Glc**	**35 $NaNO_3$**	**+++/+++**	**+++/+++**	**2.1/1.9**

All media containing glucose resulted in good growth and also usually in increased viscosity of the medium, which is an indication for scleroglucan production. Concentrations equal or higher than 27 mM Glc resulted in high scleroglucan production in all media tested.

The influence of the N-source was seen in most media by the enhanced scleroglucan production with $NaNO_3$, whereas NH_4Cl ensured good growth conditions, but nearly no scleroglucan production. Highest viscosity was observed in EPSmax13 medium (222 mM Glc, 35 mM $NaNO_3$). Thus, EPSmax13 was chosen as the medium for scleroglucan producing conditions. EPSmin17 (222 mM Fru, 35 mM NH_4Cl) was chosen as medium for scleroglucan non-producing conditions. Both media yielded nearly the same biomass and showed comparable growth behaviour (Fig. 10), which was important for performing a comparative transcriptomic analysis. As mentioned before, the addition of L-threonine or NH_4Cl in molarities of 10^{-2} mM or 10^{-3} mM, respectively, was reported to inhibit oxalate production (Bateman and Beer 1965; Kritzman et al. 1976; Survase et al. 2006). Interestingly, oxalate production occurs at approximately the same level in both media. This demonstrates that oxalate synthesis does not seem to be dependent on different C- or N-sources in *S. rolfsii*. An inhibiting effect of high amounts of NH_4Cl for oxalate production was not observed in all media tested (data not shown). Both the use of NH_4Cl in high molarities and the addition of L-threonine did not result in a lowered oxalate level which is in sharp contrast to previous reports (Kritzman et al. 1977; Wang and McNeil 1996; Farina et al. 1998; Survase et al. 2006).

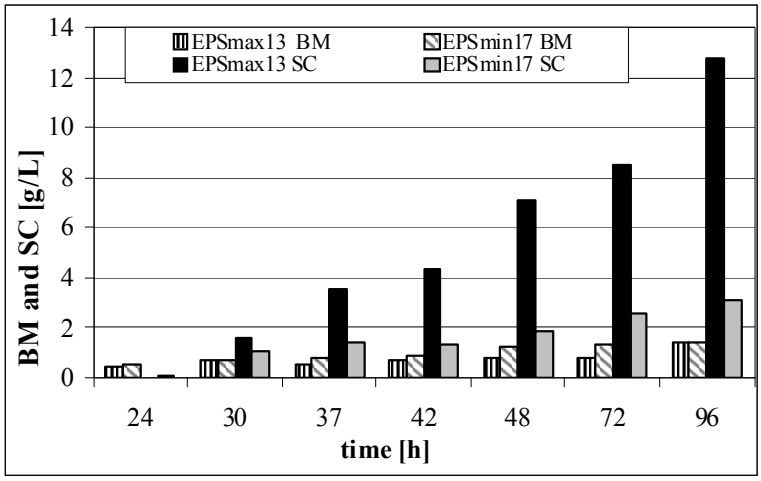

Figure 10: Biomass and scleroglucan production of *S. rolfsii* in the improved media EPSmax13 for induced scleroglucan production and EPSmin17 for non-induced scleroglucan production, respectively. Cultivation was performed over the time span up to 96 h. All cultures were grown in 250 mL Erlenmeyer flasks on a magnetic stirrer, 250 rpm at 28 °C. BM represents biomass and SC scleroglucan.

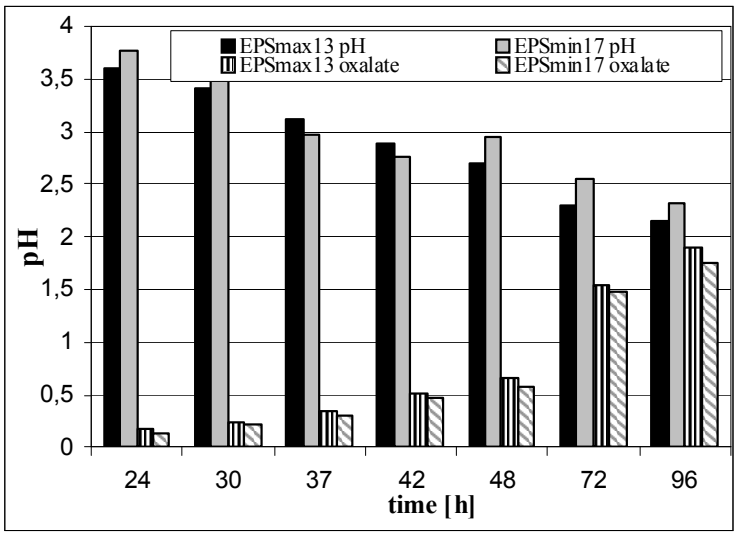

Figure 11: Formation of oxalate and corresponding pH in the fermentation broth of the cultivations in the improved media EPSmax13 and EPSmin17 for *S. rolfsii*. Cultivation was performed over the time span up to 96 h. All cultures were grown in 250 mL Erlenmeyer flasks on a magnetic stirrer, 250 rpm at 28 °C.

The media were named according to their scleroglucan production behaviour: EPSmax (for maximal ExoPolySaccharid production) and EPSmin (for minimal ExoPolySaccharid production) respectively.

4.3 Identification of genes involved in oxalate biosynthesis

It was generally assumed that two approaches for metabolic engineering are conceivable one aiming at reduction of oxalate biosynthesis, the other one aiming at enhanced oxalate

degradation. Therefore, the aim of this approach was to identify and isolate *S. rolfsii* genes putatively involved in oxalate biosynthesis. A literature survey of microbial oxalate metabolism was conducted to obtain a basis for the selection of interesting candidate genes. A schematic overview of all identified genes by this survey is given in Figure 13. Genes, their functions, as well as attempts to isolate them from *S. rolfsii* by PCR will be explained in the following section.

4.3.1 Oxaloacetate hydrolase (*oah*, EC 3.7.1.1)

Oxalic acid in most wood-rotting fungi is formed by oxaloacetate hydrolase (no. 1 in Fig. 13) (Akamatsu et al. 1991; 1992), or glyoxylate oxidase (Akamatsu and Shimada 1993; 1994). The extracellularly secreted enzyme glyoxal oxidase (no. 2 in Fig. 13) was identified in *Phanerochaete chrysosporium* (Kersten and Kirk 1987) and *Coriolus verisicolor* (Akamatsu and Shimada 1994). Using glyoxylate oxidase, an efficient sequence of oxidations from glycolaldehyd over glyoxal to glyoxylate was performed, the latter being subsequently oxidised to oxalate (Hammel et al. 1994). Oxaloacetate hydrolase is also present in ascomycetes such as *Botrytis fuckeliana* or *A. niger* (Pedersen et al. 2000; Joosten et al. 2008). Acetate and oxalate are built by the reaction of oxaloacetate and H_2O, catalyzed by oxaloacetate hydrolase. Oxaloacetate itself is delivered by the TCA. Due to the lack of sequence information for oxaloacetate hydrolase (*oah*) from basidiomycetes, primers were designed based on alignments of *oah* genes of *Botrytis cinerea* (Q6PNM8) and *A. niger* (DQ426539). Several amplicons were obtained from *S. rolfsii* using an annealing temperature of 47.5 °C; one with the primer pair 5'exon1a/3'exon3 with the size of 700 bp, two with the primer pair 5'exon1b/3'exon3 in the size of 700 and 400 bp and one with the primer pair 5'exon2/3'exon3 with the size of 250 bp. However, subcloning of these fragments as well as sequencing delivered no sequence similarity to an oxaloacetate hydrolase (data not shown).

4.3.2 Glyoxylate oxidase (EC 1.2.3.5)

No sequence information is available in public databases for glyoxylate oxidase (no. 2 in Fig. 13), the only sequence information given is for a glycolate oxidase (EC 1.1.3.15), an enzyme that catalyzes the reaction from glycolate to glyoxylate (no. 12 in Fig. 13). Glycolate oxidase (as glyoxylate oxidase) is a peroxisomal enzyme catalyzing the oxidation of α-hydroxy acids by using the cofactor flavin mononucleotide (FMN). The reaction is divided into two parts. In the first half reaction, the hydroxyl group of the substrate is oxidized and FMN is reduced. In the second half-reaction, reduced FMN is reoxidized by oxygen and hydrogen peroxide is

produced (Lindqvist and Branden 1985; Lindqvist and Branden 1989). This enzyme is widely distributed and has mainly been studied in mammals and plants. Some reports describe the over oxidation from glycolate over glyoxylate to oxalate by glycolate oxidase (but without sequence information) (Davies and Asker 1983). Due to these findings and former reports on an identified glyoxylate dehydrogenase (Balmforth and Thomson 1984) and a putative glycolate oxidase (Schilling 2000) in *S. rolfsii*, both without sequence data, the best described glycolate oxidases, derived from *Spinacia oleracea* (P05414), was used as a sequence template for primer design (Lindqvist and Branden 1980; 1985, 1989; Cederlund et al. 1988; Lindqvist et al. 1991).

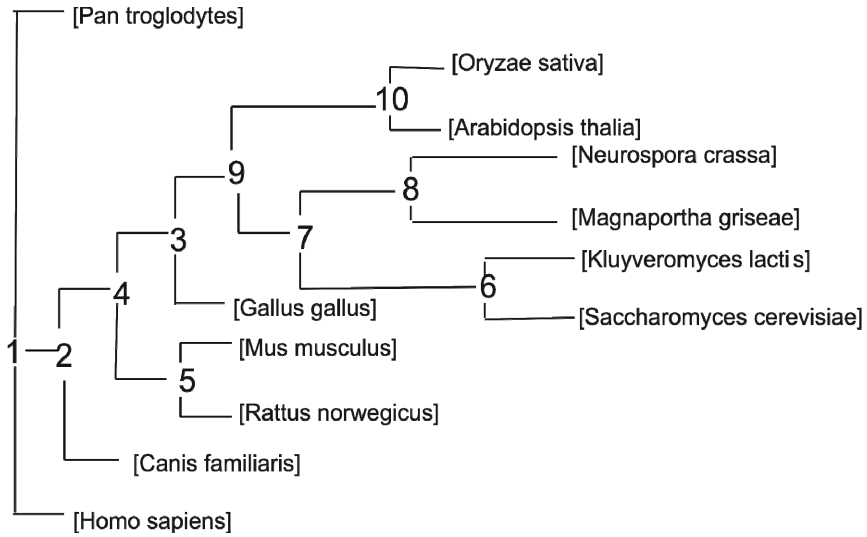

Figure 12: Phylogenetic analysis of different (S)-2-hydroxy-acid oxidases, selected by HomoloGene: (http://www.ncbi.nlm.nih.gov/sites/entrez?db=homologene**).** Sequences of branch 6 and 8 were used for the design of degenerated primers to isolate glycolate oxidase in *S. rolfsii*.

Specific primers for the spinach glycolate oxidase (Spin1/2) were designed and spinach DNA was used as a template for a PCR control. In a second PCR approach, degenerated primers GlyOxWob1/GlyOxWob2, developed on the basis of a phylogenetic tree (Fig. 12) were used for genomic *S. rolfsii* DNA. Surprisingly, PCR with spinach DNA yielded no amplicons. The fragment obtained by PCR with *S. rolfsii* DNA was subcloned and sequenced, however, tblastx analysis showed nucleotide series with no similarity to any glycolate oxidase known.

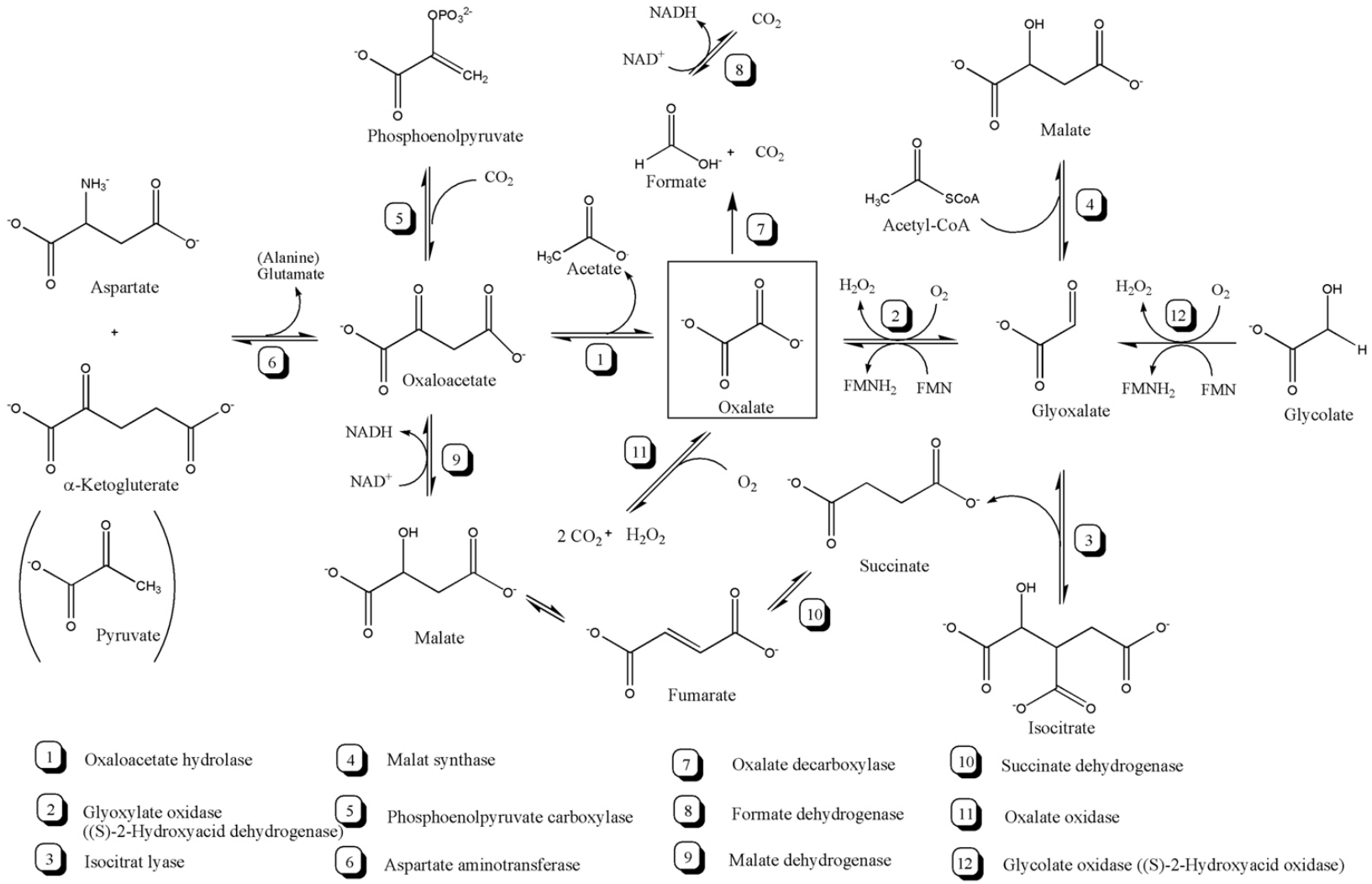

Figure 13: Schematic overview of enzymes known to be involved in oxalate metabolism by microbial, plants and mammals, summarized by a literature survey.

4.3.3 Oxalate oxidase (*oxoX*, EC 1.2.3.4)

Oxalate oxidase is described to be involved in H_2O_2 production for lignin degradation by the dikaryotic white rot fungus *Ceriopsis subvermispora* (Escutia et al. 2005). Oxalate oxidase decomposes oxalate directly to CO_2 and H_2O_2 (no. 11 in Fig. 13) and is reported to be found mainly in plants (Lane et al. 1993; Dumas et al. 1995; Requena and Bornemann 1999; Patnaik and Khurana 2001).

The sequences of two isoforms identified in *C. subvermispora* revealed that they are both bicupins which unexpectedly share greatest similarity to microbial bicupin oxalate decarboxylases rather than monocupin plant oxalate oxidases (Escutia et al. 2005). The *oxoX* of *C. subvermispora* identified which seem to be involved in the pathway leading to H_2O_2, is histochemically located in membrane bound vesicles (Aguilar et al. 1999). This finding is consistent with its proposed role in extracellular H_2O_2 production.

Since enzyme forms in *C. subvermispora* and other oxalate oxidases known from various organisms differed in a high degree, an alignment of the *oxoX* from *Hordeum vulgare* (CAA74595) and *C. subvermispora* (CAD91553) was used. The *H. vulgare oxoX* gene has been extensively investigated and was therefore chosen for primer design. Primers (Bar1/2) specific for *H. vulgare* sequence were tested in barley, yielding the expected amplicon with a size of 275 bp–677 bp (Y14203.1). PCR with *S. rolfsii* DNA resulted in an amplicon of approximately 850 bp (Fig. 14).

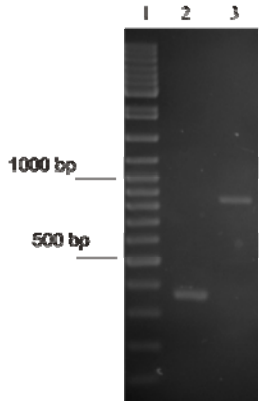

Figure 14: PCR results for the oxalate oxidase gene with primer designed by *Hordeum vulgare* oxalate oxidase. Lane 1 GenerulerTM, lane 2 PCR product with Primer Bar1/Bar2 from *Hordeum vulgare* DNA, lane 3 amplicon with primers Bar1/Bar2 and *S. rolfsii* DNA as template.

The amplified PCR product was subcloned into pGEMT and sequenced. Blast analysis and ClustalW alignment (Thompson et al. 1994) showed 55% similarity to *H. vulgare oxoX*.

Brown rot fungi produce large amounts of oxalic acid, whereas white rot fungi do not accumulate the acid to such a great extent, but rather decompose it to formate and carbon dioxide (Shimada et al. 1997).

4.3.4 Oxalate decarboxylase (*oxdc*, EC 4.1.1.2)

Oxalate decarboxylase degrades oxalate to formate and CO_2 by utilization of NAD (no. 7 in Fig. 13). Oxalate decarboxylase was identified in bacteria (Tanner and Bornemann 2000) and fungi (Mehta and Datta 1991; Kesarwani et al. 2000). Oxalate decarboxylase is present in the brown rot fungi *Postia placenta* (Micales 1995) and *Flammulina velutipes* (Azam et al. 2002). The enzyme is located on the fungal surface or in the hyphal sheath (Micales 1997). Oxalate decarboxylase (*oxdc*) is a member of the cupin family of proteins which is defined by a characteristic β-sandwich domain having one six-stranded β-sheet and one five-stranded β-sheet. The *F. velutipes oxdc* gene (AF200683) was used as a template for the design of specific primers. The resulting primers annealed to a region highly conserved in oxalate decarboxylases, as was confirmed by a protein alignment with all available Brenda sequences (http://www.brenda-enzymes.info/). For *F. velutipes*, the primers yielded the expected amplicon with a size of approximately 1,022 bp. PCR in *S. rolfsii*, resulted in an amplicon of about 900 bp (Fig. 15).

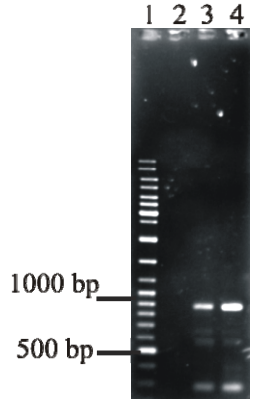

Figure 15: PCR screen for oxalate decarboxylase of *S. rolfsii*. Line 1, Generuler^{TM}, line 2, putative *oxdc* amplicon (800 bp) obtained by the primers 11S55` and 2S53` designed for oxalate decarboxylase of *F. velutipes*.

The PCR fragment was subcloned into pGEMT and sequenced. Surprisingly, Blast analysis showed similarity to NAD dependent formate dehydrogenase of 94% (AB307682), but no similarity to oxalate decarboxylase.

4.3.5 Formate Dehydrogenase (*fdh*, EC 1.2.1.2)

Formate dehydrogenase (FDH) is described to be the enzyme in oxalate metabolism, removing formate produced by oxalate decarboxylase (no. 8 in Fig. 13) from fungal metabolism. The resulting predominance of formate dehydrogenase may be an indication of active oxalate degradation in *S. rolfsii*. A wide diversity of formate dehydrogenase types is found in bacteria where they are involved in respiration; see Sawers (1994) for review - and possibly in the maintenance of a reducing environment (Haynes et al. 1995). Formate produced by oxalate decarboxylase is converted to carbon dioxide by FDH, yielding NADH. Primers were designed using a sequence of the *fdh* gene from *Neurospora crassa* (L13964.1). The primers (4-S9 3'/3-S9 5) should result in an amplicon of 1,364 bp (from nt 1,252–2,616). The obtained PCR product was approximately 1000 bp in size**.** Different amplicons were found in the case of the tested *N. crassa* DNA. A DNA fragment in the size of approximately 1,000 bp was obtained with tests using *S. rolfsii*. The fragment was cloned into pGEMT and subsequently sequenced. Blast analysis of the amplicon sequence showed no similarity to any formate dehydrogenase (data not shown).

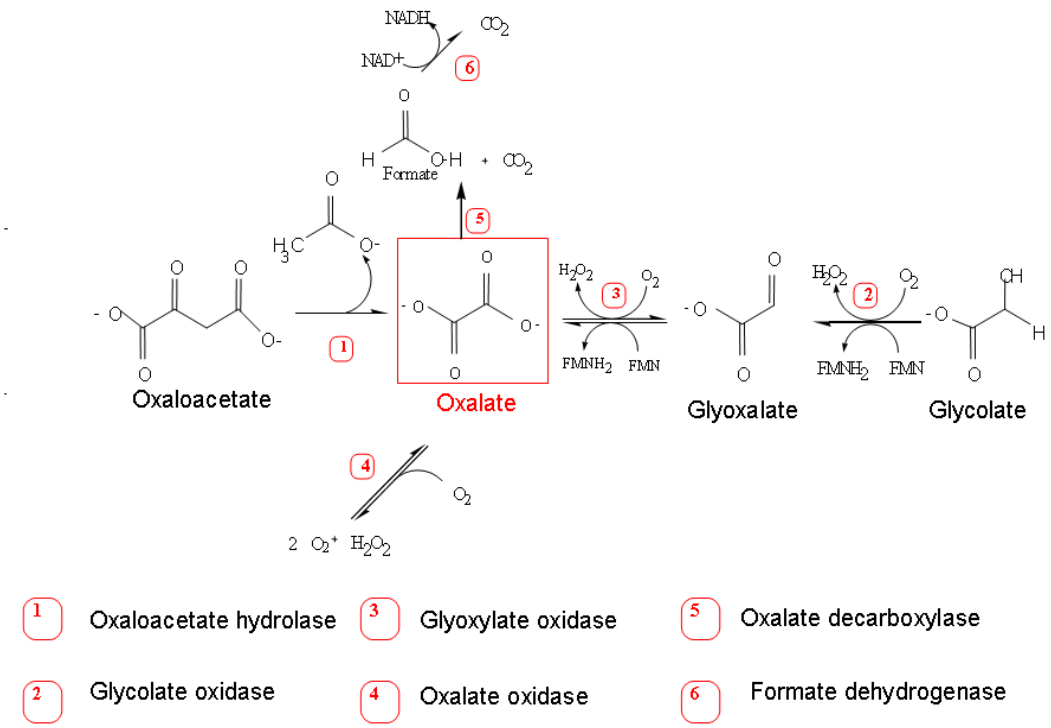

Figure 16: Summary of microbial oxalate metabolic genes whose putative presence was tested in the genome of *S. rolfsii* via PCR. Using this approach, the presence of two enzymes (formate dehydrogenase and oxalate oxidase could be verified.

In summary, PCR amplicons of approximately the corresponding size of putative oxaloacetate hydrolase, oxalate decarboxylase, oxalate oxidase, formate dehydrogenase and

glycolate oxidase genes were obtained using different primers with *S. rolfsii* DNA as template (Fig. 16). However, Sequence data revealed that of the obtained amplicons, only a formate dehydrogenase and oxalate oxidase could be identified in *S. rolfsii* by sequence analysis. Exact identification was not possible for the remaining sequences due to insufficient similarity.

4.4 The Suppression Subtractive Hybridisation (SSH) approach

In order to unravel the biosynthesis and regulation of scleroglucan production, a transcriptomic approach was applied. Suppression Subtractive Hybridisation (SSH) aimed at identifying transcripts which are up-regulated under scleroglucan-producing conditions. Growth media EPSmax13 and EPSmin17 were used for high and low scleroglucan-producing condition, respectively. SSH is a powerful technique for the generation of subtracted cDNA libraries. It is based primarily on a recently described technique called suppression PCR and combines normalization and subtraction in a single procedure (Diatchenko et al. 1996). The normalization step equalizes the abundance of cDNAs within the target population and the subtraction step excludes the common sequences between the target and driver populations. In a model system, the SSH technique enriched rare sequences over 1,000- fold in one round of subtractive hybridisation. The SSH technique has proven to be applicable to many molecular genetic and positional cloning studies for the identification of disease, developmental, tissue-specific, or other differentially expressed genes (Sternberg and Gepstein 2007; Boeuf et al. 2008) .

Suppression Subtractive Hybridisation

The PCR-Select^{TM} cDNA Subtraction Kit from BD Biosciences was selected for the SSH approach. mRNA from *S. rolfsii* grown in EPSmin17 medium was used as `driver´ and EPSmax13 medium as `tester´, respectively. Both RNA samples were extracted after 37 h of cultivation because a clear difference in scleroglucan production was observed for this time point (Fig. 10). Using this technique, approximately 400 differentially expressed genes could be isolated and were cloned into pUC18. In order to validate the SSH results, 180 of 400 clones derived were used for reverse Northern analysis (Fig. 17). DNA of clones was spotted via slot blot technique on nylon membranes and then hybridised with cDNAs derived from EPSmax13 and EPSmin17 media, respectively.

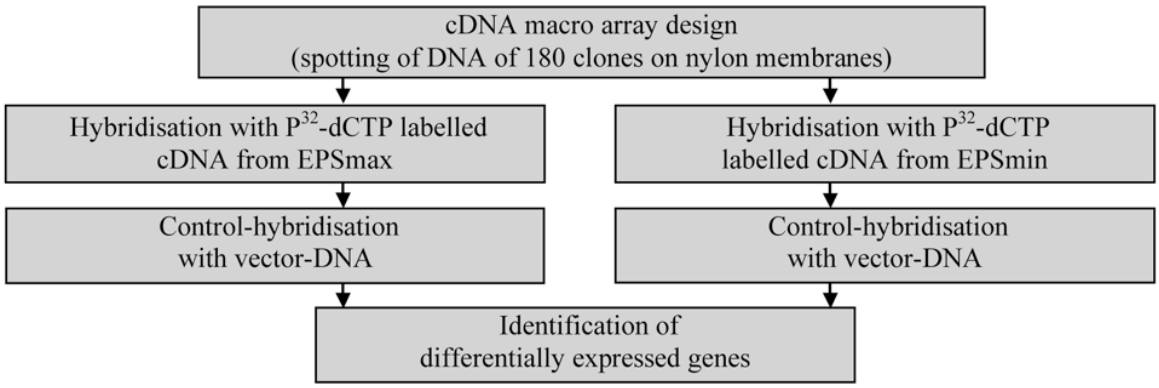

Figure 17: Flow chart of the reverse Northern analysis of cDNAs selected via Suppression Subtractive Hybridisation (SSH).

An example of the hybridisation with different cDNAs is given in Figure 18. To validate different expression profiles, the membranes were hybridised with vector DNA which ideally should result in equal hybridisation signals for all clones.

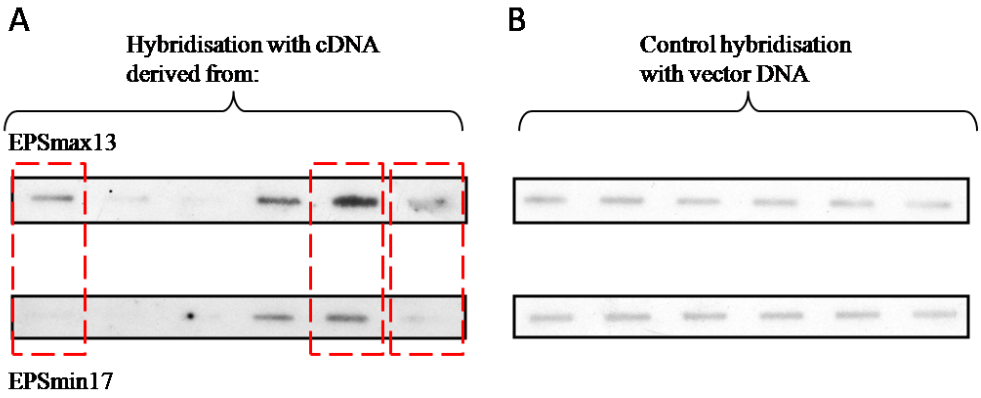

Figure 18: Example for reverse Northern blotting to identify differentially expressed genes during scleroglucan production in *S. rolfsii*. A) Spotted clone DNA hybridised with P^{32}-dCTP labelled cDNA from EPSmax13 (upper line) and with P^{32}-dCTP labelled cDNA from EPSmin17 (lower line) after 37 h of cultivation. Differentially expressed genes are marked by a frame. B) Control hybridisation with pUC18 vector DNA for normalisation.

49 of 180 tested putative ESTs could be identified as indeed being differentially expressed during scleroglucan biosynthesis. The 49 differentially expressed EST's were sequenced by T7 forward and reverse primers which anneal in the vector pUC18 backbone. The resulting sequences of forward and reverse reactions were assembled (SeqManTM^{II} DNASTAR, Madison, USA), analysed via tblastx (Altschul et al. 1990) and assigned to their predicted functional activity within different biochemical pathways (Table 5). The isolated DNA sequences had an average length of 300 bp (Excel sheet A). With only two cDNAs in duplicate, redundancy was low. No self ligated vectors were obtained.

Table 5: Categorisation of tblastx results and length of the differentially expressed sequence tags identified by SSH performed with mRNA from *S. rolfsii*. Signal intensity of the reverse Northern blotting after normalisation with vector DNA was set to Xmax for EPSmax13 and Ymin for EPSmin17 media, respectively.

TargetID	Predicted function according to tblastx hit	Length (bp)	Xmax	Ymin
C-metabolism				
4 VI-8	Formate dehydrogenase	186	1.46	0.00
27 V-36	Formate dehydrogenase *	486	0.32	0.00
33 XI-28	Pyruvate decarboxylase *	318	1.55	0.90
E4.	Pyruvate decarboxylase	242	0.87	0.00
G2	Pyruvate decarboxylase	236	2.99	1.57
G8	Pyruvate decarboxylase	242	0.86	0.00
12 VII-3	Glucan phosphorylase	421	2.10	1.36
3 VI-7	Isocitrate dehydrogenase *	546	0.83	0.00
C2	Glycogen phosphorylase	470	0.38	0.00
B5	UTP-glucose-1-phosphate uridylyltransferase	429	1.24	0.87
B8	Trehalose phosphorylase	178	0.97	0.00
9 VI-19	Oxoglutarate dehydrogenase *	325	0.79	0.19
7 VI-14	Glycogen phosphorylase	421	2.35	0.00
D1	Glucosamine-6-phosphate isomerase	441	0.30	0.13
E3	Beta-fructofuranosidase/invertase	419	2.78	1.90
D2	Phosphopyruvate hydratase	183	1.72	0.54
F3	Mannitol-1-phosphate dehydrogenase	357	1.30	0.20
F4	UDP-glucose 4-epimerase	531	2.40	1.72
Lipid metabolism				
29 X-12	Acetyl-CoA hydrolase/transferase	911	0.49	0.34
34 XI-34	Oleate 12-hydroxylase gene	337	0.32	0.00
8 VI-18	Acyl-CoA-Deyhdrogenase *	877	2.30.	0.31
Transport				
13 VII-5	Endoplasmic reticulum-derived transport	451	0.94	0.57
A3	Copper transporter	481	0.98	0.00
Amino acid metabolism				
22 VIII-45	Acetylornithine Aminotransferase	146	0.52	0.00
6 VI-12	Acetylornithine Aminotransferase	140	0.99	0.25
21 VIII-38	Aminotransferase *	589	0.57	0.37
A2	Aspartate aminotransferase	476	0.87	0.56
B7	Aspartate amino-transferase	538	0.77	0.12
Respiration and oxidative stress				
D4	Manganese superoxidismutase	229	1.56	0.12
C6	2 OG-Fe(II) oxygenase superfamily	575	0.53	0.36
Others				
D3	Superfamily of calcium sensors and calcium signal modulators	351	0.54	0.32
18 VII-50	ATP synthase vacuolar proton pump	358	0.74	0.43
20 VIII-21	GAL4-like DNA-binding domain	340	0.39	0.27
31 X-30	Plasma membrane H^+ transporting ATPase	348	0.37	0.00
B4	Intradiol dioxygenase	570	0.37	0.00
Hypothetical				
A4	hypothetical protein UM02463.1	352	0.50	0.00
14 VII-6	XP_001828655.1 CC1G_10527	345	0.56	0.00
24 IV-17	XP_001873967.1	470	1.34	0.00
28 X-11	XP_001875220.1	624	1.66	0.00
5 VI-11	XP_001873416.1	392	0.54	0.00
B2	XP_001830146.1 CC1G_09306	540	0.67	0.00

TargetID	Predicted function according to tblastx hit	Length (bp)	Xmax	Ymin
15 VII-9	Hypothetical transcription factor	352	0.91	0.64
No hit below 10^{-5}				
B6	No hit	590	2.34	0.00
A8	No hit	193	1.67	0.00
A7	No hit	193	1.89	0.24
1 VI-4	No hit	207	1.20	0.00
11 VII-2	No hit	241	0.24	0.00
G7	No hit	537	1.90	0.00
F8	No hit (putative cytochrome b)	367	1.27	0.00

→ * marks the candidate genes for further validation of SSH results

The metabolic function of glycogen phosphorylase and beta-fructofuranosidase (syn. invertase) are involved in glycogen and sucrose degeneration, respectively. Other genes belonging to the category of carbohydrate metabolism, up-regulated in the EPSmax13 medium are the trehalose phosphorylase (EC 2.4.1.64) and mannitol-1-phosphatase (EC 3.1.3.22). The latter enzyme belongs to hydrolases and the first to the family of glycosyltransferases, both ending up in Pi, and single sugar or sugar alcohol units. Furthermore, genes as isocitrate dehydrogenase, 2 oxoglutarate deyhdrogenase and phosphopyruvate hydratase, belonging to TCA and glycolysis, respectively, were identified.

Six out of the 49 differentially expressed genes were selected as candidate genes for further validation of the SSH results. RNA was harvested at time points between 30 to 85 h of *S. rolfsii* cultivation in EPSmax13 and EPSmin17 medium respectively, and the expression profile was determined via Northern blotting. Candidate genes were selected according to their categorisation with putative roles in biosynthesis of scleroglucan or oxalate: formate dehydrogenase (presumably involved in oxalate metabolism), pyruvate decarboxylase (marker enzyme for oxygen limitation), aminotransferase, glycogen phosphorylase (stress induced), isocitrate dehydrogenase (key enzyme of TCA) and acyl-CoA-deyhdrogenase (first step of fatty acid oxidation). The expression profile of three of these genes is given in Figure 19. The different expression pattern between growth in EPSmax13 and EPSmin17 media could clearly be seen in the case of formate dehydrogenase (A) and pyruvate decarboxylase (B). The expression level was below the detection limit for aminotransferase and the other three candidate genes**.**

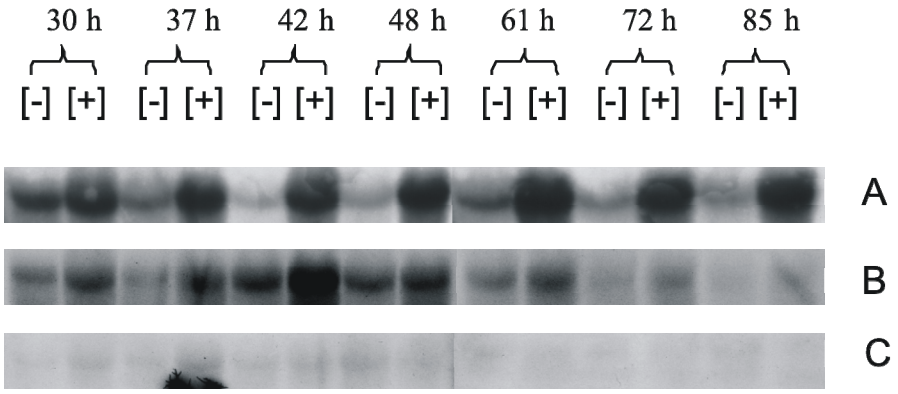

Figure 19: Gene expression profiles by Northern blot analysis of three different candidate genes from *S. rolfsii*. A) Formate dehydrogenase (TargetID 4 VI-8 in SSH). B) Pyruvate decarboxylase (TargetID 3 II-18 in SSH). C) Aminotransferase.(TargetID 6 VI-12 in SSH) Hybridisation (2.5 h at 70 °C) was performed with mass balanced RNA extracted at different time points from the growth curve of *S. rolfsii* (30–85 h) cultivated on scleroglucan inducing medium EPSmax13 = [+] and scleroglucan non-inducing medium EPSmin17 = [-].

The expression patterns of formate dehydrogenase and pyruvate decarboxylase confirm the signal intensities obtained by SSH; both enzymes are overexpressed in EPSmax13 medium. Formate dehydrogenase displays nearly constitutively strong expression over the whole time course in EPSmax13 medium, whereas pyruvate decarboxylase seems to be overexpressed mainly between 30 to 48 h of cultivation. Furthermore, the expression profiles of the candidate genes confirm the time point of 37 h chosen for comparative transcription analysis of *S. rolfsii*. A putative involvement of the candidate genes in metabolism of *S. rolfsii* will be discussed in the next section.

4.5 Sequencing of the EST library of *S. rolfsii*

EST library sequencing proved to be a powerful tool to analyse the metabolic status and the transcriptional machinery induced by selected conditions. In order to circumvent sequencing of the whole genome, this technique was applied as it is a fast and convenient method to obtain insights into the genetics of any organism. In addition, highly repetitive sequences which are predominant in basidiomycete genomes do not impede with the assembly of contiguous sequences. To realize effective transcriptomic analysis of the transcriptional status of *S. rolfsii*, a cDNA library was synthesised for the purpose of pyrosequencing. The sequence data obtained were used for the annotation of genes involved in metabolism as well as for microarray based approaches to obtain further insight into the biosynthesis of oxalate and scleroglucan in *S. rolfsii*. A cDNA library obtained from *S. rolfsii* was sequenced by 454 Life Sciences™ (Branford, USA) with triplicate runs. For sequencing, RNA was extracted from 37 h old cultures of *S. rolfsii* grown in EPSmin17 and EPSmax13 medium, respectively. Both RNA populations were pooled using a 1:1 ratio to guarantee equal predominance of

different RNA populations and a complete cDNA library was synthesized (Agowa, Berlin). Putative constitutively expressed genes, such as glycerol phosphate dehydrogenase and glucoamylase were used for normalization. The cDNA library was synthesised with the Clonetch SMART System (Clonetch, Japan) modified by AGOWA (Agowa, Berlin).

The cDNA library was sequenced by the ultrafast pyrosequencing method. Three runs of shotgun sequencing were performed followed by 454 Life Sciences™ Newbler assembling technique, resulting in 21,937 contiguous sequences (Excel sheet C), composed of 3.68 mill. bases. From out of these 21,973 contigs, 437 large contigs, with an average of 654 bases were obtained; the remaining contigs were 100 to 200 bp in length. Table 6 summarises general sequencing information.

Table 6: Sequencing of the EST library. Characteristics and statistics of sequenced EST library of *S. rolfsii* by 454 Life Sciences™ and assembly approach.

Number of GS FLX runs	3
Number of reads	356,098
Number assembled	343,410
Number partial	54
Largest contig size	1,256 bases
Average large contig size	654 bases
Number of bases in large contigs	286,124 bases
Number of large contigs	437
Number of contigs	21,937
Number of bases	3,681,160 bases

The 21,937 contigs obtained were blasted via the Sequence Analysis and Management System (SAMS, http://www.cebitec.uni-bielefeld.de/groups/brf/software/sams_info/). This automatique annotating tool originally was developed for quality supervision of genome sequencing projects (Meyer et al. 2003a). In addition to the quality assessment of shotgun nucleotide sequence data, SAMS is well suited for the analysis of individual sequence fragments. In this thesis, the system was used to annotate short EST contigs. Similar to the annotation of predicted chromosomal coding regions, individual short sequences were analyzed and functionally annotated in SAMS using an automated approach. A function prediction was computed for this purpose by interpreting the combined results of standard bioinformatics tools such as BLAST. For the functional annotation of short comparative transcriptomic contigs, the analysis pipeline was applied with four different BLAST tools: BLAST2x vs. the NCBI NR protein database (E-value cut-off of 10^{-5}), BLAST2x vs. the KOG protein database (E-value cut-off of 10^{-5}), BLAST2n vs. the NCBI NT nucleotide database (E-value cut-off of 10^{-5}) and BLAST2n vs. a selected database of fungal EST sequences (http://www.tigr.org/TIGRFAMs/, http://www.jgi.doe.gov/genome-projects, and

http://www.basidiomycetes.org/geno.htm, E-value as well as in the other blasting tools was set up to an cut-off of 10^{-5}). The EuKaryotic Orthologous Groups database (KOG) is the eukaryote-specific version of the Clusters of Orthologous Groups (COG). To obtain insight into biological processes operating in both different media conditions, sequences were annotated on a contig basis according to KOG categories, thus assigning predicted functions to coding sequences (http://www.ncbi.nlm.nih.gov/COG/). A total of 6,951 contigs (~31%) were assigned to one or more KOG functional categories. The remaining sequences were excluded by the chosen cut off E value of 10^{-5}. KOG categorisation of the analysed data set is shown in Figure 21. Among all functional KOG categories, 'Energy production and conversion (C)' and 'Carbohydrate transport and metabolism (G)' are of particular interest as carbohydrates are metabolised into further components during the production of scleroglucan and oxalate.

Table 7: *S. rolfsii*, selected Clusters of Eukaryotic Orthologous Groups of proteins (KOGs) identified in the analysed ESTs with putative relation to oxalate and scleroglucan biosynthesis.

KOG category/ KOG no.	Predicted function	TargetID
Energy production and conversion (C)		
KOG1260	Isocitrate Lyase	contig18241, contig16780, contig18241
KOG2617	Citrate synthase	contig10242, contig17695, contig16122, contig15326, contig16122, contig10242, contig16122, contig16122,contig10242
KOG0538	Glycolate Oxidase	contig21032, contig15511, contig17818
KOG0069	Glyoxylate/hydroxypyruvate reductase	contig21037, contig04723, contig16914, contig11312, contig04947, contig16572, 27_V
KOG1494	NAD-dependent malate dehydrogenase	contig21582, contig18066, contig16174, contig19518
KOG0756	Mitochondrial tricarboxylate/ dicarboxylate carrier proteins	contig17238, contig18806, contig13789, contig05048
Carbohydrate transport and metabolism (G)		
KOG4153	Fructose 1,6-bisphosphate aldolase	contig14386, contig00532, contig01198, contig13214, contig01200
KOG2099	Glycogen phosphorylase	contig15192, contig00741, 12_VII3_contig,, 7_VI14
KOG0471	α-amylase	contig21141
KOG1220	Phosphoglucomutase	contig13244,contig15646,D6
KOG2431	1,2-α-mannosidase	contig16537,contig18908
Posttranslational modification, protein turnover, chaperones (O)		
KOG2292	Oligosaccharyltransferase, STT3 subunit	contig21080,contig15093, contig20921, contig11560

In sum, 413 clusters were identified by screening the *S. rolfsii* EST library against the KOG database (Excel sheet B). The SSH approach as well as the successful development of scleroglucan inducing and non-inducing media was confirmed by recovery of targetID's

within the KOG specific clusters, as formerly obtained by SSH. Such as were glycogen phosphorylase (12_VII3 and 7_VI14) and phosphoglucomutase (D6). However, sequence of targetID 27_V (SSH approach) classified to glyoxylate/hydroxypyruvate reductase (KOG0069) by blast analysis using the KOG database, was identified as a formate dehydrogenase by tblastx analysis. Interestingly, a single cluster containing targetIDs for glycolate oxidase (KOG0538), which corresponds to the literature survey conducted to unravel oxalate biosynthesis in *S. rolfsii.*

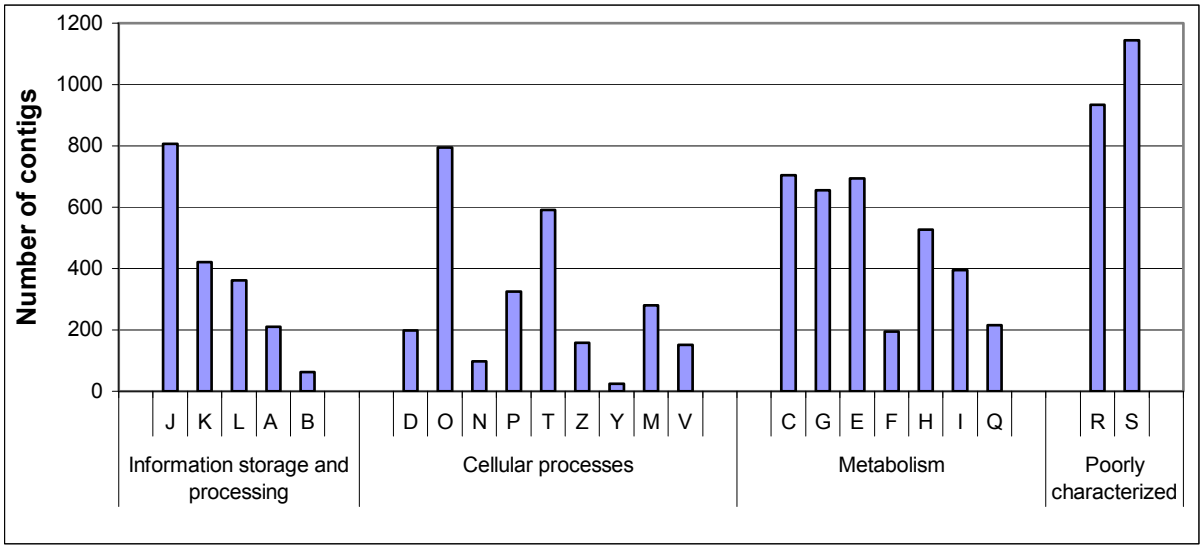

Figure 20: Categorisation of *S. rolfsii* contigs according to Eukaryotic Clusters of Orthologous Groups of proteins (KOGs). Categories are abbreviated as follows: J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination and repair; A, RNA processing and modification; B, chromatin structure and dynamics; D, cell cycle control, cell division, chromosome partitioning; O, posttranslational modification, protein turnover, chaperones; N, cell motility; P, inorganic ion transport and metabolism; T, signal transduction mechanisms; Z, Cytoskeleton; Y, Nuclear structure, M, cell wall/membrane/envelope biogenesis; V, defence mechanisms; C, energy production and conversion; G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown.

Distributions of the KOG classification for an overview of fungal metabolism, performed in the chosen conditions are given in Figure 20. Metabolism and posttranslational modification appears to be main active categories.

4.6 Genome expression profiling using microarray hybridisation

The sequence data obtained from the EST library sequencing provided an opportunity for further transcriptomic approaches based on microarray analysis. This, in contrast to the SSH approach; can only be performed with prior knowledge of underlying sequence information, which are now available.

Three Agilent Multiplex 44K Arrays were designed for transcriptomic analysis of *S. rolfsii*. For all 21,937 contigs assembled by 454 Life Sciences™ and all other sequences obtained from the SSH approach, suitable oligonucleotides, 60 bp long were designed (RZPD, Berlin). To optimize specific and reliable hybridisation of mRNA, the most suitable oligonucleotides were evaluated by a testing hybridisation run, and the two most adequate were chosen for hybridisation experiments (File A).

The DNA chips were hybridised with *S. rolfsii* mRNA, obtained from four different cultivation conditions (Fig. 21). Hybridisations were performed in triplicate using mRNA isolated from three independent cultures (biological triplicate). The time point of 37 h was used for comparison of early scleroglucan (++) induced and non-induced (-) conditions, and 61 h were used for the high scleroglucan production (+++) in glucose based medium and adequate scleroglucan production in EPSmin17 medium (+). Furthermore, the time enhanced comparisons in the same medium (vertical comparison) may result in the identification of housekeeping genes.

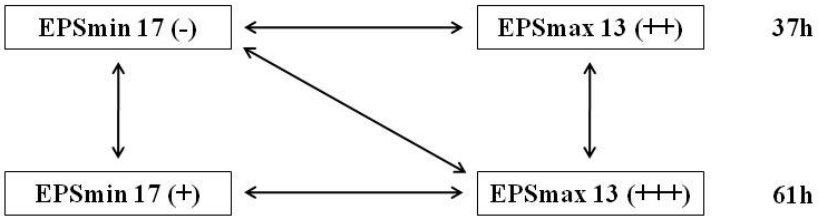

Figure 21: Scheme of the comparison of microarray data of *S. rolfsii*, concerning the different media conditions and time points of RNA extraction. (+) indicates scleroglucan production (-) indicates no or only low scleroglucan production. Horizontal comparisons were among the 37 h of cultivation conditions, in the upper line and in the 61 h of cultivation conditions in the lower line, respectively. The vertical comparisons describe the comparison of the prolonged cultivations up to 61 h in the different media.

A horizontal comparison of the EPSmin17 and EPSmax13 medium, once for the cultivation of 37 h and for the cultivation of 61 h was performed to unravel the genes differentially expressed in scleroglucan inducing and non-inducing cultivation conditions. Vertical comparison was performed by the composition of conditions of 37 h and 61 h of cultivation within the same media. The last comparison was performed by a crosswise comparison of the cultures cultivated in EPSmin17 for 37 h and the cultures cultivated in EPSmax13 medium for 61 h. To simplify the handling of microarray data, the different cultivation conditions are renamed by following scheme; EPSmin17/37, EPSmax13/37 for cultivation in the different designed media for 37 h and EPSmin13/61, EPSmax13/61 for the cultivations in the different developed media for the time of 61 h respectively.

RNA extracted from one of the three cultivations in EPSmin17/37 had to be excluded as their data significantly differed from the other two RNA samples. Therefore, only a biological duplicate was used in the case of EPSmin17/37.

Exclusion by t-test and p-value cut-off identified a total of 5,736 contigs to be significantly up-or down-regulated under the different conditions. Not for all of these 5,736 'genes' annotation of KOG's categorisation was available. Circumventing the restriction of automatique annotation based on highly conserved protein domains, manually curation via tblastx analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied to all differentially expressed contigs revealed by the microarray approach. Indexing of annotated `genes´ was performed by, following mapping on Munich information centre for protein sequences (MIPS, http://mips.gsf.de/projects/funcat) functionally grouped categorisation (FunCat) based scheme. Via tblastx analysis, 2,120 contigs could be grouped into FunCat categories.

Following the assumption that scleroglucan production differs from 37 h on within the varied media, expression profile data from EPSmin17/37 and EPSmax13/37 was regarded to provide maximum information on differentially expressed genes involved in scleroglucan synthesis during early scleroglucan producing period (Fig. 10). Applying this comparison, strong up-regulation of carbon metabolism, lipid metabolism and cellular transport was observed (Fig. 22).

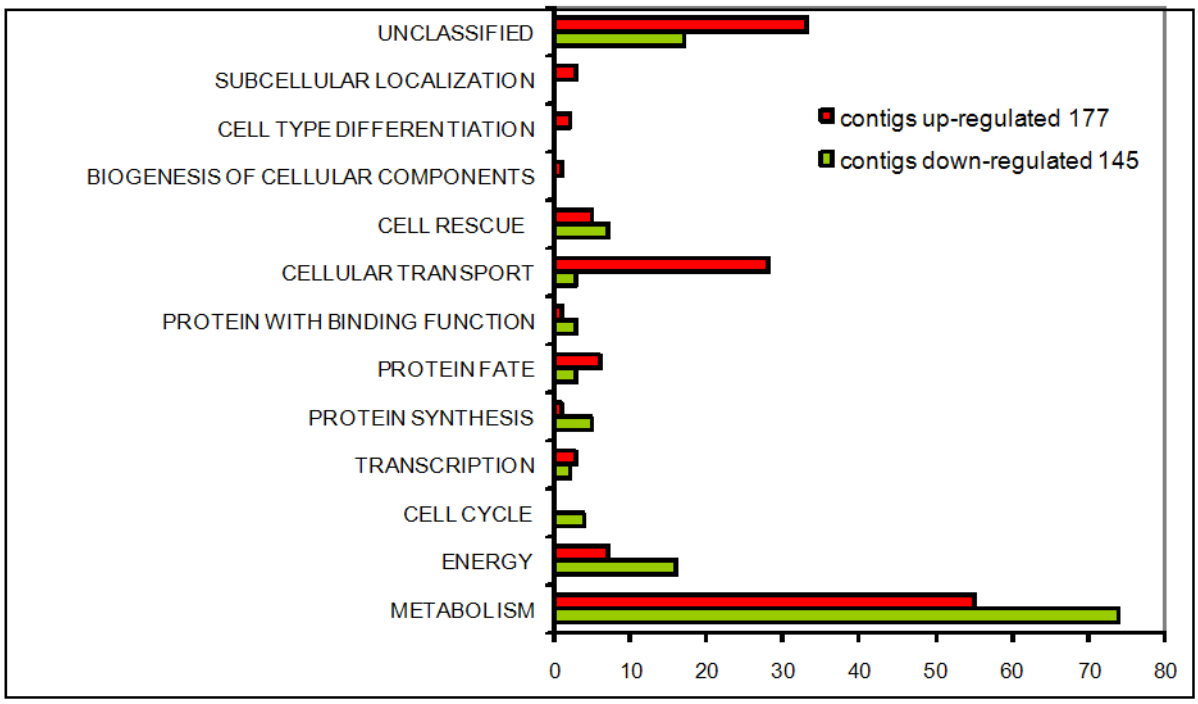

Figure 22: Horizontal comparison of microarray data of *S. rolfsii*, concerning EPSmin17 and EPSmax13 medium after 37 h cultivation. From 723 contigs delivered by the microarray analysis 322 meet the cut off ($<10^{-5}$) by tblastx analyses. These 322 contigs were classified by the FunCat categories to sort them into fungal metabolism. The x-axis presents the number of contigs differentially expressed in the EPSmax13/37 medium.

Table 8 shows the top ten of the most up-regulated classified contigs. The main affected contigs were analysed for formate dehydrogenase, squalene epoxidase, alternative oxidase, cytochrome P450, and stress response related proteins. Several membrane transporter coding genes, mainly mitochondrial and phosphate carriers were significantly up-regulated. In contrast, laccases and further secondary-metabolism involved enzymes as aryl alcohol dehydrogenase and various cytochrome P450s were significantly down-regulated.

Table 8: Top ten of the up-regulated genes from *S. rolfsii* delivering positive tblastx results in the horizontal comparison for 37 h of cultivation. Fold change and p-value of the selected contigs is given as well as their classification to MIPS and FunCats. A fold change of e.g. 2 means twofold up-regulation in EPSmax13/37.

EPSmin17/37 compared to EPSmax13/37				
TargetID	**Fold change**	**p-value**	**tblastx**	**FunCat category**
contig04723	2.95	0.002	Formate dehydrogenase	02.13 respiration
contig00863	2.95	0.010	GABA/polyamine transporter	20.01.11 amine/polyamine transport
contig15675	2.56	0.044	Heat shock protein	32.01.05 heat shock response
contig10652	2.31	0.025	stress response related ATPase	32.01 stress response
contig01446	2.26	0.021	Pectate lyase	01.05 C-compound and carbohydrate metabolism
contig04863	2.25	0.043	Squalene epoxidase	01.06 lipid, fatty acid and isoprenoid metabolism
contig04867	1.90	0.027	Glycogen debranching enzyme	01.05 C-compound and carbohydrate metabolism
contig14837	1.87	0.007	C-5 sterol desaturase	01.06 lipid, fatty acid and isoprenoid metabolism
contig21758	1.58	0.010	Phosphate transport protein	20.01.01.07.07 phosphate transport
contig20926	1.54	0.016	Glucan (1→3)-β-glucosidase	01.25.01 extracellular polysaccharide degradation

The second comparison is thought to examine the conditions within the high scleroglucan induction in EPSmax13/61 cultivation, and the comparison to the ambient scleroglucan production in EPSmin17/61. This horizontal comparison revealed a much higher number of contigs (3,070) to be differentially expressed than by the comparison based on 37 h of cultivation. Via tblastx analysis 2,043 contigs were rejected by missing the E-value cut off. Arrangement oft resulting FunCat categorisation is given in Figure 23. When compared to Figure 22, it becomes obvious that proteins involved in the category ‘protein fate’ become more important in the later cultivation times.

Within this comparison, again, formate dehydrogenase is observed to be highly up-regulated in combination with phosphate transport and various cytochrome P450s. Furthermore, laccase, alternative oxidase, nitrite reductase, sulfite oxidase, phosphoglycerate mutase, β-glucan synthesis-associated proteins and UDP-glucose epimerase were identified to be significantly up-regulated. The top ten of the mainly up- regulated genes are summarized in Table 9.

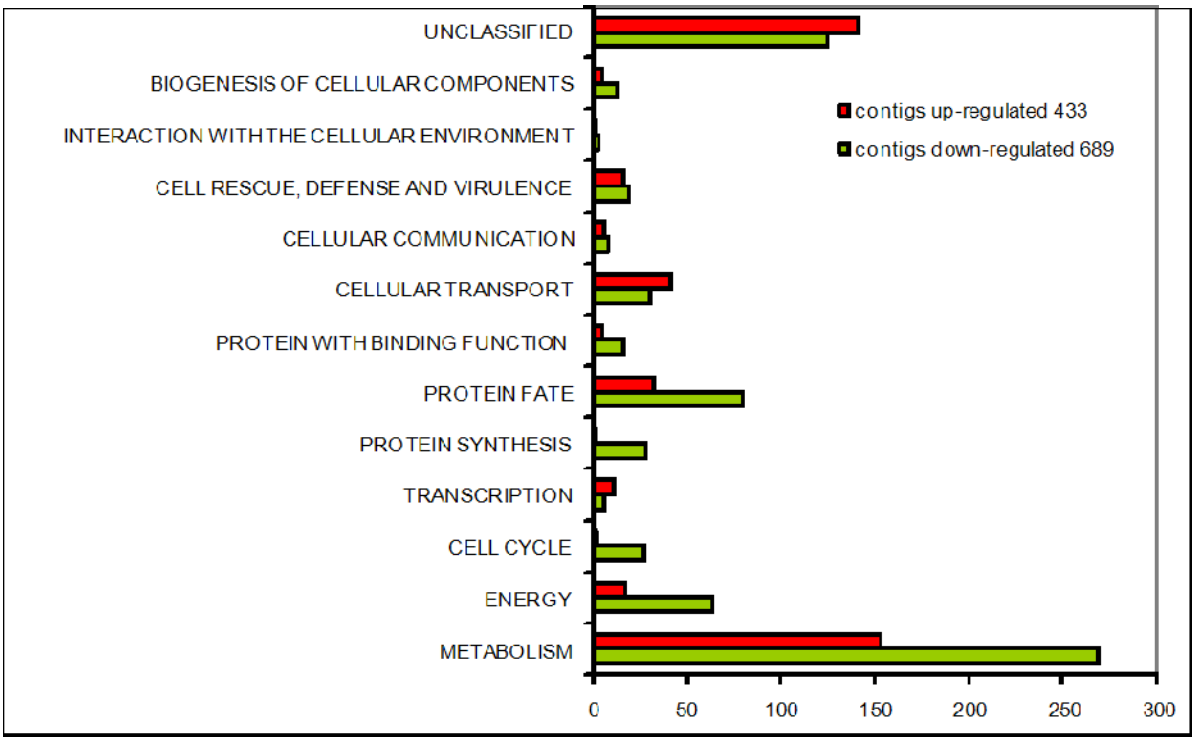

Figure 23: Statistics of the microarray data of *S. rolfsii*, by the horizontal comparison of EPSmin17 and EPSmax13 medium at 61 h of cultivation. From 3,070 contigs delivered by the microarray analysis 1,122 meet the cut off ($<10^{-5}$) by tblastx analyses. These 1,122 contigs were classified by the FunCat categories to sort them into fungal metabolism. The x-axis presents the number of contigs differentially expressed in the EPSmax13/61 experiment.

Table 9: Top ten of the up-regulated genes of *S. rolfsii* delivering positive tblastx results in the horizontal comparison for 61 h of cultivation. A fold-change of e.g. 2 corresponds to twofold up-regulation in the"EPSmax13/61" experiment.

EPSmin17/61 compared to EPSmax13/61				
TargetID	**Fold change**	**p-value**	**tblastx**	**FunCat category**
contig11978	5.00	0.025	Cytochrome P450	02.13 respiration
contig16572	3.10	0.006	Formate dehydrogenase	01.05 C-compound and carbohydrate metabolism
contig14489	2.75	0.017	Laccase	01.20.50 catabolism of secondary metabolites
contig20165	2.48	0.026	Glutamine aminotransferase	01.01.01 amino acid biosynthesis
contig02834	2.45	0.005	Glucan (1→3)-β-glucosidase	01.05.01 C-compound and carbohydrate utilization
contig12301	2.43	0.041	Phosphate transport protein	20.01.01.07.07 phosphate transport
contig16537	2.23	0.000	Class I alpha-mannosidase	01.05.03 polysaccharide metabolism
contig15675	2.12	0.016	Heat shock protein	32.01.05 Heat shock response
contig11349	1.98	0.000	Alpha mannosidase	01.05.01 C-compound and carbohydrate utilization
contig11137	1.97	0.003	Pyruvate dehydrogenase	01.05.01 C-compound and carbohydrate utilization

Next, the vertical comparison i.e. EPSmin17/37 and EPSmin17/61 and EPSmax13/37 and EPSmax13/61, respectively, was done.

With respect to the EPSmin17 media, 691 differentially expressed contigs were observed, 410 out of which, however, delivered no tblastx hits. The different classification by FunCat categorisation of the remaining 281 hits is depicted in Figure 24. Again, the metabolism is

the most prevalent differentially regulated category followed by the category, involved in protein fate. The top ten up-regulated contigs are depicted in table 10.

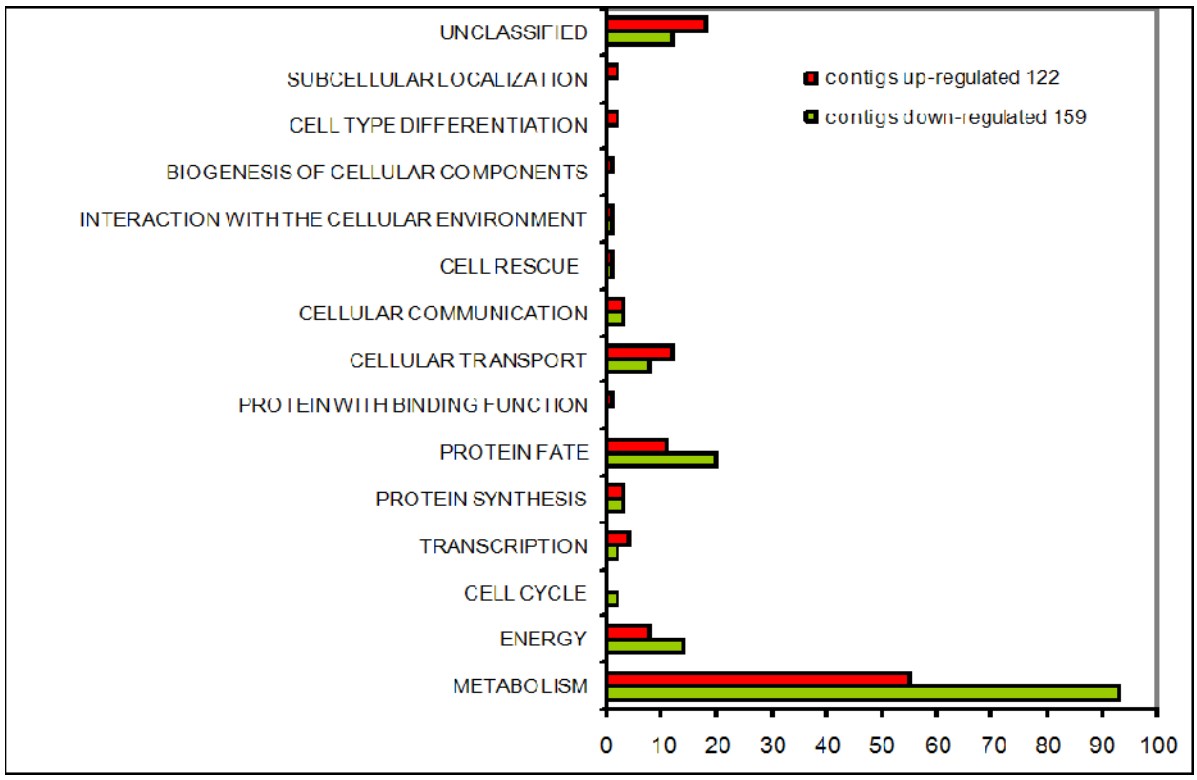

Figure 24: Classification of the microarray data of *S. rolfsii* by the vertical comparison of EPSmin17/37 and EPSmin17/61. From out of 691 contigs delivered by the microarray analysis 282 meet the cut off ($<10^{-5}$) by tblastx analysis. These 281 contigs were classified by the FunCat categories to sort them into fungal metabolism. The x-axis presents the number of contigs differentially expressed in the EPSmax17/61 experiment.

Table 10: Top ten of the up-regulated genes of *S. rolfsii* delivering positive tblastx results in the vertical comparison of the cultivation in EPSmin medium from 37 to 61 h. A fold change of e.g. 2 indicates that the gene is twofold up-regulated in the"EPSmin17/61" experiment.

EPSmin17/37 compared to EPSmin17/61				
TargetID	**Fold change**	**p-value**	**tblastx**	**FunCat category**
contig12979	3.59	0.017	Amino acid permease	01.01.07 amino acid transport
contig08049	3.41	0.023	Cytochrome P450	20.01.15 electron transport
contig19159	3.28	0.004	Glycosyl transferase family 2	01.05 C-compound and carbohydrate metabolism
contig02949	2.81	0.023	Aryl alcohol dehydrogenase	01.20 secondary metabolism
contig05838	2.68	0.039	Oxidoreductase	02.13 respiration
contig09744	2.57	0.001	Serine esterase	30.05 signal transduction
contig01466	2.34	0.006	NADH-dependent flavin oxidoreductase	metabolism of vitamins, cofactors, and prosthetic groups
contig05257	1.96	0.009	Delta 4-sphingolipid fatty acid desaturase	01.06 lipid, fatty acid and isoprenoid metabolism
contig16534	1.71	0.002	Oligosaccharyltransferase	01.05 C-compound and carbohydrate metabolism
contig09547	1.75	0.022	3-carboxymuconate cyclase	01.05 C-compound and carbohydrate metabolism

The comparison within the EPSmax medium from cultivation of 37 h to 61 h, delivered 2,123 contigs to be differentially expressed. 861 there from were classified to FunCat categories by

their tblastx result. Arrangement of the FunCat classification is depicted in Figure 25 and the top ten up-regulated contigs are given in Table 11.

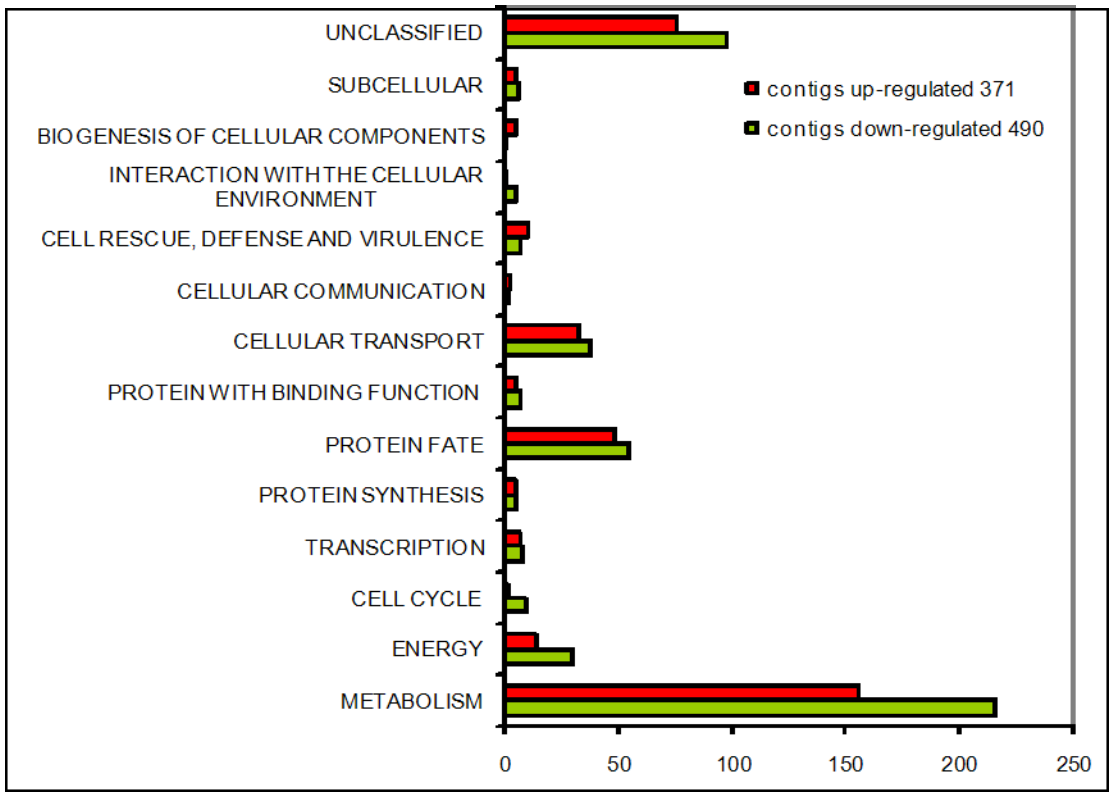

Figure 25: Arrangement of the microarray data by the vertical comparison of EPSmax13 medium at 37 h of cultivation compared to cultivation for 61 h. From 2,123 contigs delivered by the microarray analysis, 861 meet the cut off (<10^{-5}) by tblastx analyses. These 861 contigs were classified into FunCat categories. The x-axis presents the number of contigs differentially expressed in the EPSmax13/61 experiment.

Reconfirmed, to be highly up-regulated, was the FDH as well as aryl alcohol dehydrogenase, dienlacton hydrolase and cytochrome P450 involved in secondary metabolism. Down-regulated, were (1→6)-β-glucan synthase, acetyl-CoA synthetase, oxaloacetate hydrolase isocitrate lyase and mitochondrial carrier proteins (Fig. 25 and Table 11).

Table 11: Top ten of the up-regulated genes of *S. rolfsii* delivering positive tblastx results in the vertical comparison of the cultivation in EPSmax13 medium from 37 to 61 h. A fold-change of e.g. 2 represents twofold up-regulation in the"EPSmax13/61" experiment.

EPSmax13/37 compared to EPSmax13/61				
TargetID	**Fold change**	**p-value**	**tblastx**	**FunCat category**
contig09204	4.80	0.026	Formate dehydrogenase	02.16 Fermentation
contig21569	4.20	0.017	Aryl alcohol dehydrogenase	01.20 secondary metabolism
contig02150	3.45	<0.001	Dienlacton hydrolase	01.20 secondary metabolism
contig20165	3.12	0.018	Glutamine amidotransferase	01.01 amino acid metabolism
contig11978	2.76	0.036	Cytochrome P450	01.20 secondary metabolism
contig05497	2.66	<0.001	N-acetyl glucosamine-phosphotransferase	14.07.02 modification with sugar residues
contig10970	2.54	0.001	Mitochondrial carrier protein	20.09.04 mitochondrial transport
contig16524	2.32	0.002	Acid phosphatase	01.04 phosphate metabolism
contig16920	2.20	0.001	Oligosaccharyl transferase	01.05 C-compound and carbohydrate metabolism
contig06503	2.16	0.012	Glutamate decarboxylase	01.01 amino acid metabolism

Arrangement and top ten up-regulated contigs of the last crosswise comparison between EPSmin17/37 and EPSmax13/61 is summarized in Table 12 and Figure 26, in order to get a closer insight into the pathways mainly affected by different growth conditions.

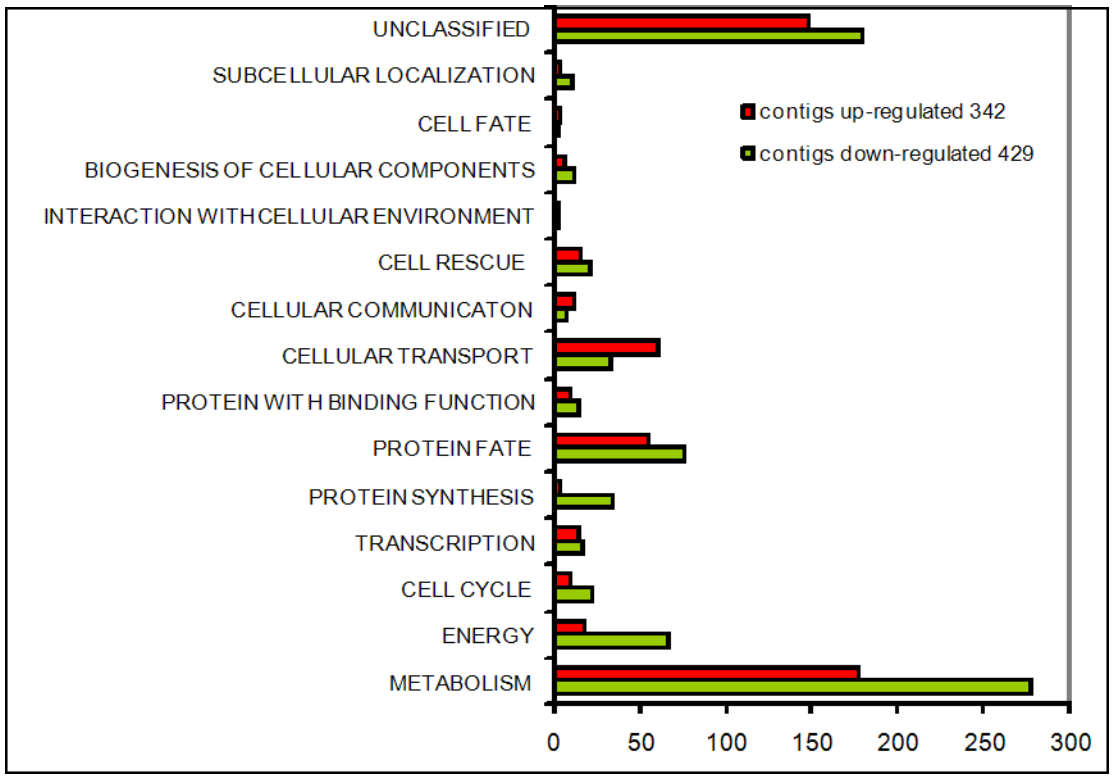

Figure 26: Arrangement of the microarray data of *S. rolfsii* by the crosswise comparison of EPSmin17 medium at 37 h of cultivation compared to cultivation in EPSmax13 for 61 h. From 2,123 contigs delivered by the microarray analysis 771 meet the cut off ($<10^{-5}$) by tblastx analyses. These 771 contigs were classified by the FunCat categories to sort them into fungal metabolism. The x-axis presents the number of contigs differentially expressed in the EPSmax13/61 medium.

The comparison of the EPSmin17 to EPSmax13 conditions revealed up-regulated formate dehydrogenase, GABA/polyamine transporter and stress response related ATPases. Further on, cytochrome P450, sulfite and nitrite reductase and in addition, many genes involved in squalene and fatty acid metabolism as well as the mannitol-1-phosphate dehydrogenase and alternative oxidase were significantly up-regulated.

Remarkably, the comparison of EPSmin17/37 vs. EPSmax13/61 appeared to uncover very interesting indications for metabolic pathway genes involved in both scleroglucan and oxalate syntheses. A model of the profiled fungal metabolism is schematised in Figure 28, which will be discussed later.

Table 12: Top ten of the up-regulated genes of *S. rolfsii* delivering positive tblastx hits in the crosswise comparison of the cultivation in EPSmax13 for 61 h and EPSmin17 medium for 37 h. A fold-change of e.g. 2 represents twofold up-regulation in the"EPSmax13/61" experiment.

EPSmin17/37 compared to EPSmax13/61				
TargetID	**Fold change**	**p-value**	**tblastx**	**FunCat category**
contig05271	3.02	0.026	Phosphate transport protein	20.09.04 mitochondrial transport
contig01249	3.09	0.047	C5-sterol desaturase	01.06 lipid, fatty acid and isoprenoid metabolism
contig20823	3.02	0.026	Aryl alcohol dehydrogenase	01.20 secondary metabolism
contig20001	4.10	0.009	2 OG-Fe(II) oxygenase superfamily	01.20 secondary metabolism
contig12166	3.80	0.002	Cytochrome P450 monooxygenase	32.07 detoxification
contig17496	3.70	0.005	2 OG-Fe(II) oxygenase superfamily	01.20 secondary metabolism
contig21758	3.60	0.027	Mitochondrial carrier protein	20.09.04 mitochondrial transport
contig00863	3.30	0.005	GABA/polyamine transporter	20.01.11 amine / polyamine transport
contig12326	3.30	0.018	2-alkenal reductase	01.20 secondary metabolism
contig02150	3.30	0.001	Dienelactone hydrolase	01.20 secondary metabolism

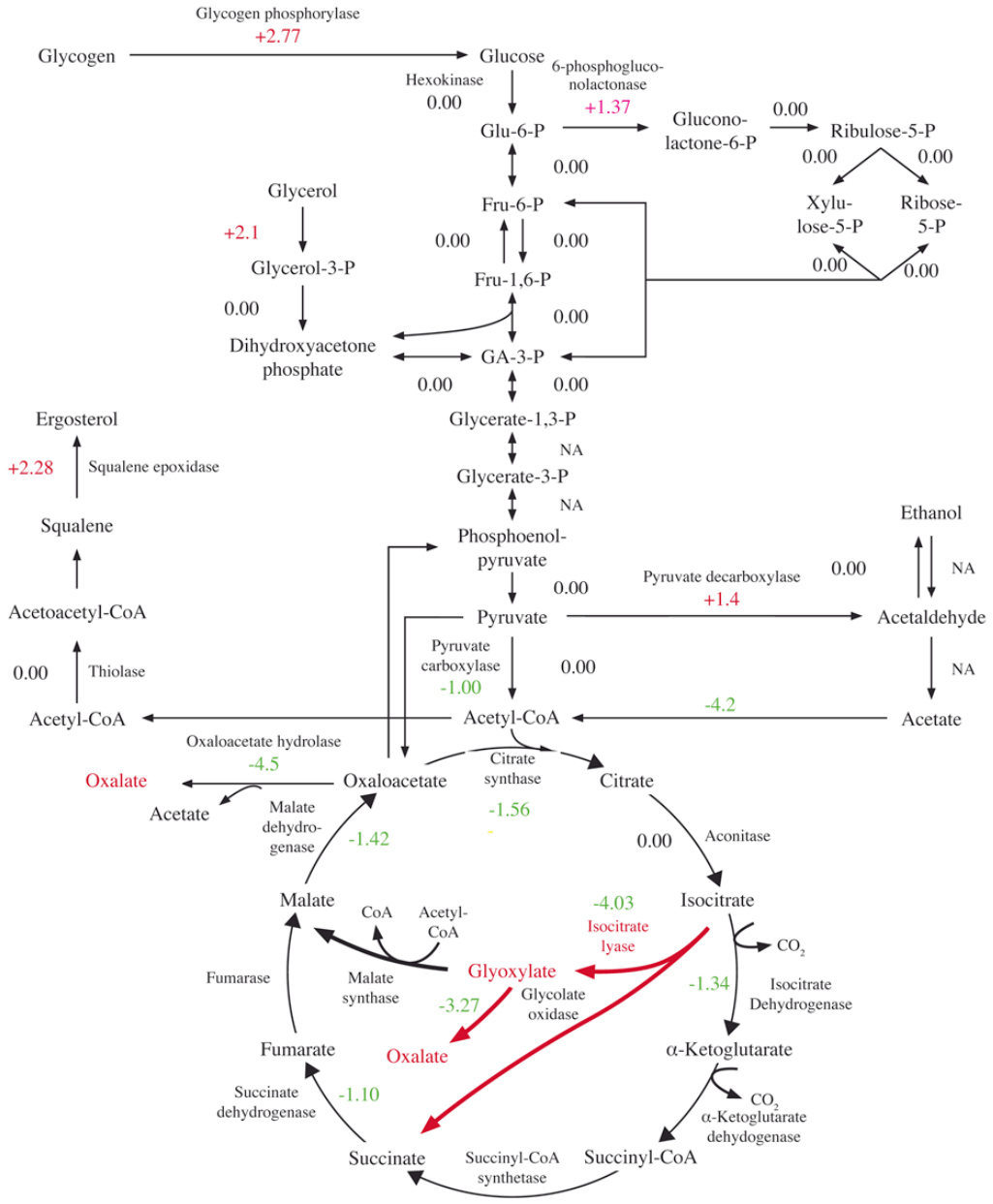

Figure 27: Overview of central metabolic pathways unravelled in *S. rolfsii* by comparison of cultivation conditions from EPSmin17/37 to EPSmax13/61. Only the key intermediates are indicated. Double headed arrows denote reversible reactions. The genes encoding the reactions are identified by gene names and the corresponding regulation (p-value <0.05) is highlighted in green for down-regulation and in red for up-regulation in EPSmax13/61 experiment. (0.00) represents unaffected expression of the genes. NA indicates non-identified genes by sequence analysis of the EST library. The targetIDs for the named contigs are given in Table C.

From the horizontal and vertical comparison of the various microarray data, potentially interesting and important contigs were selected and their temporal expression monitored (Table 13 and Table 14). Table 13 summarizes selected contigs whose expression seems to be time-dependent, i.e. their up-/down-regulation under EPSmax13 conditions is dependent on cultivation time. For example, a strong up-regulation of (1→3)-β-glucosidases seems to be more important at 37 h compared to 61 h, whereas UDP-glucose-epimerase up-regulation seems to be more enhanced during later time in growth.

However, for few genes a constant level of up-/down-regulation was observed, suggesting, that their influence on scleroglucan production is rather growth independent.

Table 13: Selected *S. rolfsii* ESTs commonly up- or down-regulated in EPSmax13 medium compared to EPSmin17 medium. The temporal expression is monitored for an insight in fungal metabolism influenced by cultivation in the improved media for scleroglucan inducing and non-inducing properties and different cultivation times. A fold-change of e.g. 2 indicates that the gene is twofold up-regulated in the"EPSmax13"experiment.

TargetID	Genes	Fold change 37 h	Fold change 61 h
contig07276	Laccase **Δ**	-2.25	-2.65
contig04723	Formate dehydrogenase **Δ**	2.95	2.74
contig07074	Nitrite reductase **Δ**	1.53	1.75
contig14534	Mannitol-1-phosphate dehydrogenase **Δ**	1.59	1.35
contig15675	Heat shock protein **Δ**	2.56	2.18
contig02136	Alternative oxidase **Δ**	1.44	1.65
contig10652	Stress response related ATPase **Δ**	2.31	2.02
contig04863	Squalene epoxidase *	2.25	1.46
contig05030	Major Facilitator Superfamily*	1.05	0.00
contig10387	Ammonium transporter *	1.16	0.00
contig02066	Aminophospholipid-transporting ATPase*	2.36	1.13
contig00863	GABA/polyamine transporter*	2.55	1.47
contig11092	C-4 methyl sterol oxidase*	1.62	1.19
contig02834	Glucan 1,3-beta-glucosidase*	2.96	1.47
contig19827	Oxidoreductase **O**	-1.09	1.49
contig16036	Gluconolactonase **O**	-1.14	-2.82
contig08327	Glucoamylase **O**	-1.07	-2.57
contig11978	Cytochrome P450 **O**	2.01	5.02
contig19066	UDP-glucose epimerase **O**	1.01	1.76
contig09610	Glycosyltransferase family 39 **O**	0.00	1.32
contig04275	Glutamate decarboxylase **O**	0.00	2.39
contig05766	Aryl-alcohol dehydrogenase **O**	0.00	2.70
contig03348	Cytochrome P450 monooxygenase **O**	0.00	2.34
contig07970	Major Facilitator Superfamily **O**	0.00	1.61
contig 05747	Sugar (and other) transporter **O**	0.00	1.05
contig15257	Glucose dehydrogenase **O**	0.00	1.40

→ * indicates to be important in the early growth phase
→ O indicates to be important in the late growth phase
→ Δ indicates to be growth independent

4.7 Data mining for putative target genes

As clearly indicated by Table 14 and Figure 28, all genes involved in TCA and GLOX cycle, are significantly down-regulated at 61 h conditions in EPSmax13 conditions, compared to EPSmin17. In contrast, expression of TCA and GLOX cycle contigs seems to be similar in EPSmax13 and EPSmin17 media at 37 h, i.e. during early growth conditions (Table C). Theses data suggest that high production of scleroglucan is accompanied by reduced activity of TCA and GLOX cycles.

Table 14: Differentially expressed genes of the TCA in *S. rolfsii*, with their corresponding fold changes. The comparisons of prolonged cultivation within scleroglucan inducing and non-inducing conditions are given, as well as the fold changes in the vertical comparison concerning cultivation for 61 h. A fold-change of e.g. -2 indicates that the gene is twofold down-regulated in the"EPSmax13/61"experiments.

TargetID	Fold Changes in the comparisons			Gene
	EPSmin13/61 vs. EPSmax13/61	EPSmax13/37 vs. EPSmax13/61	EPSmin17/37 vs. EPSmax13/61	
contig15326	0.00	-1.15	-1.56	Citrate synthase
contig00773	-4.04	-2.50	-3.27	Glycolate oxidase
contig01017	0.00	-1.10	0.00	Isocitrate dehydrogenase
contig14763	-3.94	-4.49	-4.54	Isocitrate Lyase
contig05630	-3.91	-4.46	-4.53	Oxaloacetate hydrolase
contig21272	0.00	-1.18	-1.00	Pyruvate carboxylase
E4.seq	0.00	0.00	1.40	Pyruvate decarboxylase
contig11137	1.97	0.00	1.14	Pyruvate dehydrogenase kinase
contig19544	-1.69	-1.46	-1.83	Pyruvate dehydrogenase kinase
contig21237	-1.10	0.00	-1.10	Succinate dehydrogenase
contig18806	-2.93	-3.34	-3.53	Succinate/fumarate transporter

Regarding scleroglucan synthesis and general polysaccharide metabolism in *S. rolfsii*, a large amount of genes putatively involved in polysaccharide metabolism were identified by sequencing of the EST library (Excel sheet C).

The postulated general scheme of genes involved in polysaccharide synthesis (Fig. 1) was supported by the identification of hexokinase, phosphoglucomutase, UTP-glucose-uridylyl-transferase and (1→3)-β-glucan synthase in the *S. rolfsii* EST library, mostly not affected on transcriptional level under the different growth conditions (Excel sheet C).

Of main interest, appear the oligosaccharyltransferase, the *eln3* coded glycosyltransferase and the high amount of different glycosyltransferases. Also very interesting are a β-glucan synthase and an amylo-(1→6)-hydrolase, the human glycogen branching enzyme, which may be involved in the branching of scleroglucan. Solely, two short contigs (contig03355, contig16869) were addressed to (1→6)-β-glucan synthases (*dag3* gene) of *Agaricus bisporus*. Both were down-regulated in EPSmax13/61 compared to EPSmax13/37 and one (contig03355), down-regulated in EPSmax13/61 compared to EPSmin17/37. Whereas, their expression levels were unaffected in the remaining comparisons.

Table 15: Differentially expressed genes putatively involved in scleroglucan biosynthesis of *S. rolfsii*, with corresponding fold changes. The comparisons of prolonged cultivation within scleroglucan inducing and non-inducing properties are given, as well as the fold changes in the vertical comparison concerning cultivation for 61 h. A fold-change of e.g. 2 indicates that the gene is twofold up-regulated in both, the EPSmax13/61 and the EPSmax13/37 experiment.

TargetID	Fold changes in the comparisons				Genes
	Min37 vs. Max37	Min61 vs. Max61	Max37 vs. Max61	Min37 vs. Max61	
contig13812	0.00	1.25	0.00	0.00	(1→3)-β-glucan synthase component.
contig12094	0.00	1.57	0.00	1.91	(1→4)-α-glucan branching enzyme
contig17329	0.00	0.00	0.00	1.01	(1→6)-α-mannosyltransferase
contig04502	1.46	1.23	0.00	2.13	Amylo-α-1,6-glucosidase.
contig03355	0.00	0.00	-1.17	-1.80	(1→6)-β-glucan synthase
contig16869	0.00	0.00	-1.18	0.00	(1→6)-β-glucan synthase
contig12971	0.00	0.00	1.83	1.41	(1→3)-β-exoglucanase
contig04211	0.00	0.00	0.00	1.12	(1→3)-β-glucan synthesis-associated protein
contig09598	0.00	1.80	0.00	0.00	(1→3)-β-glucan synthesis-associated protein
contig10575	0.00	1.89	0.00	2.54	Glycosyltransferase (*eln3* gene)
contig20926	1.54	2.39	1.35	2.92	Glucan (1→3)-β-glucosidase
contig04589	-2.33	-3.13	0.00	-3.37	Glucoamylase G2
contig18908	0.00	1.41	1.77	2.19	Glycosyl hydrolase family 47
contig16330	0.00	1.29	0.00	0.00	Glycosyl hydrolase family 92
contig04867	1.90	1.46	0.00	2.44	Glycogen debranching enzyme
contig12735	0.00	1.53	1.16	1.97	Glycogen debranching enzyme
C2.seq	1.49	0.00	0.00	2.78	Glycogen phosphorylase-like protein
contig04531	0.00	1.42	1.89	2.33	Glycoside hydrolase family 47 protein
contig01604	1.54	2.01	0.00	2.41	Glycosyl hydrolase family protein
contig09616	0.00	0.00	0.00	1.26	Glycosyl hydrolases family 31
contig12238	0.00	0.00	1.15	1.16	Glycosyltransferase
contig08856	0.00	0.00	0.00	1.33	Glycosyltransferase family 32
contig09610	0.00	0.00	1.13	1.32	Glycosyltransferase family 39
contig14534	1.59	1.35	0.00	1.59	Mannitol-1-phosphate dehydrogenase
contig20286	0.00	0.00	1.53	1.91	Oligosaccharidyl-lipid flippase partial
contig16522	0.00	1.18	1.88	2.03	Oligosaccharyl transferase β subunit
contig20921	0.00	1.55	2.10	2.39	Oligosaccharyl transferase subunit (Stt3)
contig16534	0.00	1.15	1.67	2.24	Oligosaccharyltransferase, α subunit
contig19066	1.01	1.76	1.03	2.10	UDP-glucose epimerase

5 Discussion

The subject of this thesis was the identification of key genes involved in the biosynthesis of scleroglucan and oxalate in the basidiomycete *Sclerotium rolfsii*, and to predict promising target genes for genetic engineering of industrial scleroglucan production. The basis for genetic modification, however, is the availability of molecular biological tools to manipulate *S. rolfsii*. Therefore, a transformation system including suitable transformation plasmids had to be established. Two approaches aiming at the elucidation of scleroglucan and the oxalate pathway were followed. The first approach based on a literature and database survey for genes putatively involved in the respective biosynthesis pathways, as well as on the identification of candidate genes in *S. rolfsii*. The second holistic approach based on transcriptomic analysis and was used to reconstruct a model for scleroglucan and oxalate biosynthesis in *S. rolfsii*.

5.1 Scleroglucan production inducing and non-inducing media

In order to enhance the research of genes involved in scleroglucan production on transcriptome level, the focus was based on media designed enabling scleroglucan inducing and non-inducing properties.

The concentration of the C-source used revealed that the main influence on scleroglucan production is the raising of viscosity from concentrations above 55 mM, as is it is the case for glucose. Osmosity exhibited a significant influence on scleroglucan production, leading to stimulation of exopolymer production. The exopolymer secreted represents the osmotic buffer (Tilser et al. 1996; Farina et al. 1998). Interestingly, the MOPT medium, described as optimal polysaccharid medium for *S. rolfsii* by Farina et al (1998), revealed non-optimal scleroglucan production, indicative of different behaviours of strains used in the discriminatory experiments.

The influence of N-source appears to be secondary compared to C-source concentration as seen by medium viscosity in low concentrated glucose media with exchange of $NaNO_3$ and NH_4Cl (Table 3). Rising glucose concentrations and balanced C/N-ratio heighten the scleroglucan yield as reported for *S. rolfsii* and other microorganisms (Hamilton et al. 1979; Farina et al. 1998; Bautista-Gallego et al. 2008). The use of fructose and NH_4Cl successfully inhibits scleroglucan production in nearly all media compositions tested, except for EPSmin2 where a high concentration of NH_4Cl was used. *S. glucanicum,* in contrast, appears to enhance scleroglucan production through the use of NH_4Cl as the inorganic N-source (Taurhesia and McNeil 1994). It seems certain that when YE is added to all the various media, it represents an additional non defined N-source, whereas a clear distinction of N-source is not given.

The leading effect on polysaccharide production is ascribed to the carbon and nitrogen source, with glucose and sucrose known to result in high exopolysaccharide yields (Kim et al. 2006; Xu et al. 2006; Shih et al. 2008). It was shown that fructose prevents scleroglucan production in submerse culture by inhibiting the scleroglucan synthesizing enzymes in *S. rolfsii* (Survase et al. 2006). Also, $NaNO_3$ rather than NH_4Cl yield better glucan levels in fungi (Farina et al. 1998; Survase et al. 2006) as well as in other microbial systems (Sutherland 1989; Cerning 1990; Kimmel et al. 1998). In contrast to other fungi, in the case of *S. rolfsii* it is known that depletion of nitrogen stimulates glucan formation (Rau 2004). Other components such as phosphate, salts and citric acid were provided in the same amounts and dosage forms and are reported to have no influence on glucan formation to a great extend. Zn^{2+} was supplied in the form of none defined YE in both media, as scleroglucan production is known to be dependent on Zn^{2+} availability in the cultivation media (Pilz 1991).

The influence of lowered oxygen levels on scleroglucan synthesis as reported by Schilling et al. (2000) was ignored since this is not practical in shaking flask fermentations. The improved media resulted in similar growth conditions of *S. rolfsii* and nearly the same biomass production (Fig. 10). Moreover, scleroglucan production differed greatly in the developed scleroglucan inducing and non-inducing media. The oxalate level remained constant in both media (around 1.5 g L^{-1} after 72 h), even after adding L-threonine or NH_4Cl in high molarities, which were reported to inhibit oxalate synthesis in *S. rolfsii* (Kritzman et al. 1976) and other fungi (Survase et al. 2007b).

The simultaneous production of oxalate and scleroglucan as reported in many cases (Wang and McNeil 1994; Survase et al. 2007b), is unconfirmed for *S. rolfsii*, as oxalate formation occurred in the same amount in scleroglucan inducing and non-inducing media. Furthermore, the reports of glucose-induced repression of ß-glucanases formation (Rau 2004) was only partially confirmed within the framework of this thesis, whereas there were found some up and some down-regulated beta glucanases on transcriptomic level in the glucose based cultivations (Excel Sheet D).

5.2 *S. rolfsii* is accessible by different transformation techniques

A prerequisite for a successful transformation is the establishment of an adequate selection system based on antibiotics active in the used media (Brenner and Sherris 1972; Butaye et al. 2000). A selection system was established by using hygromycin and MGY+ medium; other antibiotics tested were exposed to inactivity in the tested media as shown by the antibiograms (Fig. 6). The final working concentration of 100 µg/mL is within the lower limit of medium

range as for other fungi (Weidner et al. 1998; Meyer et al. 2003b; Arechiga-Carvajal and Ruiz-Herrera 2005), but appeared to be suitable by *S. rolfsii* sensitivity (Fig. 6).

Two different DNA-transfer methods were tested and compared in an attempt to facilitate the genetic transformation of *S. rolfsii*. A hygromycin resistance gene *hph* from *E. coli* was chosen to select positive transformants. The transfer of appropriate constructs was realized either by protoplast mediated transformation (PMT) or by *Agrobacterium tumefaciens* mediated transformation (AMT), both methods were established successfully. The transformation yield of 1–5 transformants per 10^6 protoplasts and µg DNA obtained by PMT was far below the yield reported for other fungi (Mullins et al. 2001; Koukaki et al. 2003; Meyer et al. 2003b), and may be caused by the multinucleous status of the protoplast, as described for other fungi (Hara et al. 2002). Young cultures (45 h in submerse culture), proved to be the most suitable for protoplastation, and the use cell wall degrading enzyme mixes of Vinoflow® FCE or Glucanex® in higher amounts (0.5 g /g BDM) resulted in the same protoplast yields as for Novozyme 234. The highest yield of protoplasts was obtained with a ratio of 500 mg Vinoflow FCE to 0.5 g BDM of *S. rolfsii*. Regenerated protoplasts transformed with *hph* could be selected by their ability to grow on an agar overlay containing 50 µg/mL hygromycin. Higher concentrations of hygromycin in the top agar resulted in non regenerating protoplasts, even with successful transformants.

Co-cultivation for AMT performed in submerse cultures proved to be inefficient; thus solid mycelium was used as described for other fungi. The best transformation results were obtained by use of 1 cm^2 *S. rolfsii* mycelium of a 3 d old preculture in EPSmin17 medium. Co-cultivation time and the fungus:bacterium ratio was found to alter the transformation efficiency. Although 28 °C is the optimal incubation temperature for *A. tumefaciens*, this temperature was not appropriate for T-DNA transfer. It has been proposed that the T-DNA transfer machinery is greatly affected by temperature. For example, the expression of some *vir* genes of *Agrobacterium* those are necessary for interaction between the bacterium and the recipient is impaired at temperatures above 26 °C, thus affecting the transformation efficiency (Fullner and Nester 1996; Baron et al. 2001). The optimal temperature resulting in the highest transformation rate was then found by co-cultivation at 24 °C.

There was a low correlation between transformation efficiency and duration of co-cultivation of fungal mycelium with *A. tumefaciens* cells. However, a significant correlation between the number of *A. tumefaciens* cells present during the co-cultivation period was found. The period of co-cultivation was set to 72 h which is about one day longer than described for the most fungi (Rho et al. 2001). This extension, however, resulted in a marginal increase in

transformation efficiency (+ 5%). The number of *A. tumefaciens* cells present during co-cultivation clearly demonstrate how the fungus:bacterium ratio influences DNA transfer events. Highest transformation events were obtained by *A. tumefaciens* concentration of $2*10^9$ cells mL^{-1} per cm^2 of fungal mycelium. Lower concentrations (10^6–$5*10^7$) yielded in lowered transformation efficiencies of around 20%.

The fact that the expression of the EGFP gene was unsuccessful (located on pBGgHg) could be attributed to the missing introns in the coding sequence. The need for introns within the gene or promoter region for effective heterologous expression in basidiomycetes has been described (Lugones et al. 1999; Burns et al. 2005; Kües 2005). Transformants were screened via PCR on molecular level for hygromycin resistance and 98% of putative transformants proved to be true transformants.

A disadvantage of the selection of AMT transformants is that putative transformants grow into each other and also overgrow non-transformed mycelia, which in turn makes identification and purification of putative transformants difficult and tedious.

It could also be shown that the *Agrobacterium* strain used plays an important role in transformation efficiency in *S. rolfsii* as has also been described for other plants and fungi (Gelvin and Habeck 1990; Chabaud et al. 2003; Grant et al. 2003). The hypervirulent strain AGL1 (Ti plasmid TiBo542) infected more fungal cells than LB1100.

Effective transformation of *S. rolfsii* depends greatly on functional promoters controlling and conferring complete hygromycin resistance. Whereas the *gpd*AB::*hph* construct resulted in a good transformation efficiency rates and stable hygromycin resistant clones, the *gpd*AN::*hph* construct was shown to be ineffective.

5.3 Heterologous expression in *S. rolfsii*

The heterologous expression of oxalate oxidase and oxalate decarboxylase was successfully described in plants and fungi. The PMT in *S. rolfsii* based on self-constructed transformation vectors carrying the heterologous genes of oxalate oxidase (*Hordeum vulgare*) and oxalate decarboxylase (*Flammulina velutipes*), resulted, however, in non-oxalate reduced transformants. After two weeks of selection, *S. rolfsii* appeared to have lost the complete transformation construct. Screens on a molecular level via PCR and Southern blot (data not shown) confirmed this assumption as none of the transformed genes could be detected. The loss of the transformation construct may originate from the ability of *S. rolfsii* wild type to adapt to hygromycin when incubated on hygromycin containing media for 2 weeks. Loss of selection pressure may enhance mitotic instability frequency, as has been reported for other

fungi, even with the use of AMT (Tooley 1992; Monfort et al. 2003; Nyilasi et al. 2005; Migheli 2008). However, this high frequency of mitotic instability cannot compare with rates obtained with other fungi (Weld et al. 2006), since the protoplast of *S. rolfsii* are multinuclear. Thus it is possible that the resultant colonies contained both transformed and untransformed nuclei and this may have contributed to the mitotic instability. A decrease in hygromycin resistance was also observed in zygomycetes (Nyilasi et al. 2008) and dermatophytes (Kaufman et al. 2004). Employment of restriction-enzyme-mediated integration (REMI) was reported to enhance a stable transformation rate in the ascomycete *Cochliobolus heterostrophus* (Lu et al. 1994).

However, the identification of many major facility superfamily (MFS) transporters in the *S. rolfsii* EST library (Excel sheet C) may indicate a high probability that *S. rolfsii* detoxifies or degrades poisonous substances. Fungicide resistance has been reported for *S. rolfsii* on polyoxin-D (Maria 2008) as well as for many plant pathogens against benomyl and different phenyl amides and benzimidazoles (Damicone 1997). Reduced sensitivity is thought to be a result of genetic mutations, reduced antibiotic uptake or detoxification by the resistant organism (Damicone 1997).

The need to optimise selection and transformation systems becomes obvious when examining the data presented. A stringent and non resistance evoking antibiotic has to be found for a safe and reliable transformation of *S. rolfsii*. The expression system may be further optimized when stably transformed to *S. rolfsii*. The need for intron sequences (as seen in EGFP) within the gene sequences for successful expression has to be evaluated in *S. rolfsii*.

5.4 Unravelling the scleroglucan biosynthesis in *S. rolfsii*

Fungal exopolysaccharide synthesis is still speculative as regards glucan synthesis branching and secretion of glucans. This work represents the initial report on genetic and transcriptional approaches aiming at a deeper insight into the scleroglucan biosynthesis of *S. rolfsii*.

Combined data from databases, literature surveys and transcriptome based approaches revealed a more distinct picture of *S. rolfsii*'*s* scleroglucan metabolism. Genes involved in the hypothetical biosynthesis of fungal exopolysaccharides and scleroglucan respectively, as reported elsewhere, (Fig. 28) were identified by the SSH-, microarray-approach and EST sequencing, to the step where UDP-glucose is available as scleroglucan precursor. From this step on, initiation, chain elongation and branching is catalysed by glucan synthase

sequentially adding UDP-glucose to the supposable membrane–anchored scleroglucan assembling protein.

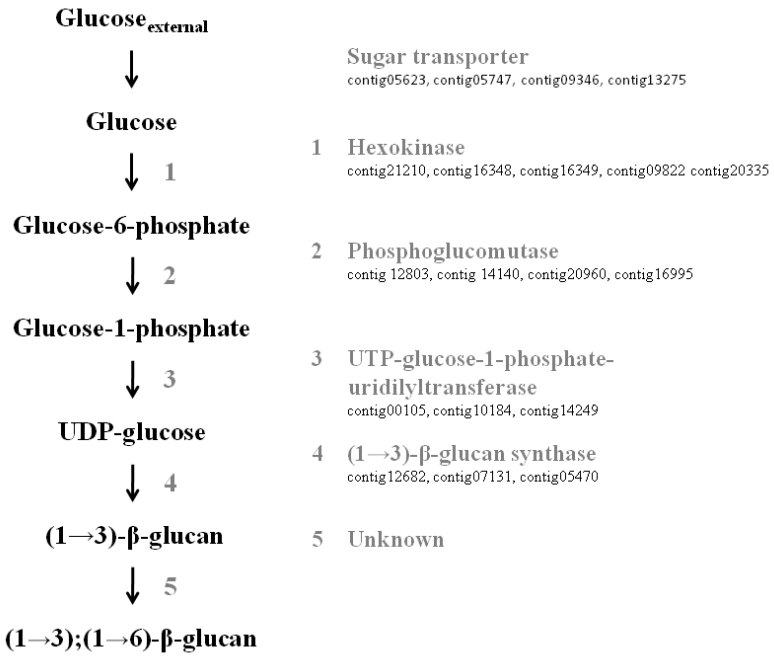

Figure 28: postulated scleroglucan synthesise as proved to be functional in *S. rolfsii.*

^{14}C incorporation experiments with following Smith degradation in *S. rolfsii* indicate that the backbone extension of scleroglucan is performed from the non-reducing end on (Batra et al. 1969). Using a parallel branch supporting mechanism, the incorporation of single side chain residues is recognized to appear simultaneously with the glucan backbone elongation (Schmid et al. 2006). The (1→3)-β-glucan synthase is known to accept only UDP-glucose as substrate (Orlean 1982). However, it was only slightly up-regulated in EPSmax13/61. Therefore, posttranslational regulation and activation via GTP directed by Rho1 subunit of the glucan synthase or by use of phospholipids is assumed for *S. rolfsii* glucan synthase as reported by other glucan synthesizing complexes (Mazur and Baginsky 1996; Beauvais et al. 2001; Douglas 2001). The significantly up-regulated phosphate transport in all cultivations in EPSmax13/61 during high scleroglucan production indicates the use of high amounts of phosphate during scleroglucan formation. It is putatively used for the phosphorylation of GTP thus inducing the glucan synthase.

Another enzyme complex putatively involved in scleroglucan synthesis is the oligosaccharyltransferase (OST) which is up-regulated under all scleroglucan producing conditions (Excel sheet D). The OST is described to catalyze the asparagine-linked glycosylation of proteins in the lumen of the endoplasmic reticulum in many organisms

(Karaoglu et al. 1995). This complex enzyme consists of several subunits (e.g. 9 in *S. cerevisiae*) and has been named as the gatekeeper of the secretory pathway (Kelleher and Gilmore 1994; Dempski and Imperiali 2002; Lennarz 2007). The different subunits interact with the mannosyl and glucosyltransferases which assemble the lipid-linked oligosaccharide donor (Kukuruzinska and Robbins 1987; Burda and Aebi 1998) and are shown to have different functions *in vivo* and *in vitro* (Spirig et al. 1997). In addition, the transport of complete glycans by the OST subunit STT3 has already been described (Castro et al. 2006). As regards the role of the glucosidase subunit of OST in the biosynthesis of the cell wall (1→6)-β-glucan, there are reports ranging from indirect involvement (Abeijon and Chen 1998) to a more direct involved function (Shahinian et al. 1998) in the biosynthesis in *S. cerevisiae*, whereas the direct mechanism is still unclear (Chavan et al. 2003). Therefore, it may be postulated that in combination with the further described genes of the common polysaccharide biosynthesis the OST of *S. rolfsii* may also be involved in scleroglucan synthesis in a manner yet unknown.

The highly up-regulated UDP-glucose epimerase interconvert's the present nucleotide sugars UDP-glucose and UDP-galactose, both known to be precursors of many polysaccharides (Boels et al. 2001). However, this is not true for scleroglucan biosynthesis. UDP-glucose-epimerase and OST are involved in the cell wall morphogenesis and in cell wall thickening for the protection against oxidative stress and high acidic conditions (Singh et al. 2007). Although the pH after 61 h is about 2.5 in both media, there is no upregulation in EPSmin17/61 medium but only in EPSmax13/61. Therefore, it may be presumed that both enzymes are indirectly involved in scleroglucan synthesis.

Furthermore, a large amount of different glycosyl hydrolases which were up-regulated in EPSmax13 medium were identified in the transcriptome of *S. rolfsii*. Several glycosyl hydrolases were shown to have high transglycosylation and transferase activity *in vitro* (Faijes and Planas 2007). Therefore, they may also have been involved in scleroglucan biosynthesis, putatively in the branching on every third unit. This presumption may be strengthened by the fact of a down-regulated (1→6)-β-glucan synthase in the enhanced glucose cultivations, indicating that the (1→6)-β-glucan synthase is not involved in the branching mechanism. In addition, the finding that gluconolactonase, a β-glucan synthase inhibitor (Lopez-Romero and Ruiz-Herrera 1978), is down-regulated under all high scleroglucan forming conditions and

up-regulated in EPSmin17/61 indicates that scleroglucan producing enzymes are inhibited in fructose-based media such as EPSmin17.

5.5 Identification of genes involved in oxalate biosynthesis

A literature survey revealed different enzymes of various microorganisms which are involved in the oxalate metabolism. Based on this literature survey, a PCR approach was developed to identify oxalate synthesis/degrading genes. Engineering of theses genes then was intended to employ an oxalate deficient strain for optimized downstream processing of scleroglucan.

The PCR approach gave hits for most targeted genes; however, after sequencing the PCR products only formate dehydrogenase and a putative oxalate oxidase could be confirmed. In this approach, finding of correct genes was limited by insufficient sequence information from the literature search. To compensate a missing homology between identified genes of other organisms highly degenerate primers had to be used for the PCR. On the other hand, the phylogenetic distance between *S. rolfsii* and the other organisms chosen was too far. These facts may have led to negative or false positive results.

Combining the results of the bibliome based PCR approach, EST pyrosequencing data, and microarray based data mining, genes for oxaloacetate hydrolase, formate dehydrogenase and glycolate oxidase were identified in *S. rolfsii* genome.

The blast analysis of the EST library exposed 5 contigs according to KOG category of glycolate oxidase (FMN-dependent) and three contigs for oxaloacetate hydrolase (Excel sheet C). Isocitrate lyase/PEP mutase enzyme superfamily represents the family to which the decrypted oxaloacetate hydrolase of *S. rolfsii* belongs to. The oxaloacetate hydrolase of the basidiomycete *Moniliophthora perniciosa* (Rio et al. 2008) contains active site serine, which is necessary for an oxaloacetate hydrolase activity as has been described by Joosters et al. (2008). The same serine site is present in the *S. rolfsii's* oxaloacetate hydrolase, which is 98% homologous to the actually published sequence of putative oxaloacetate hydrolase in *Moniliophthora perniciosa* (Rio et al. 2008).

Currently, two models describe the oxalate synthesis in *S. rolfsii*. In the first model, oxalate is produced via a NADH dependent glyoxylate dehydrogenase (Balmforth and Thomson 1984). The second model prefers a more direct conversion via FMN dependent glycolate oxidase (Schilling 2000). The use of NADH dependent glyoxylate dehydrogenase will depend on oxalyl-CoA transferase to provide oxalate (Fig. 29).

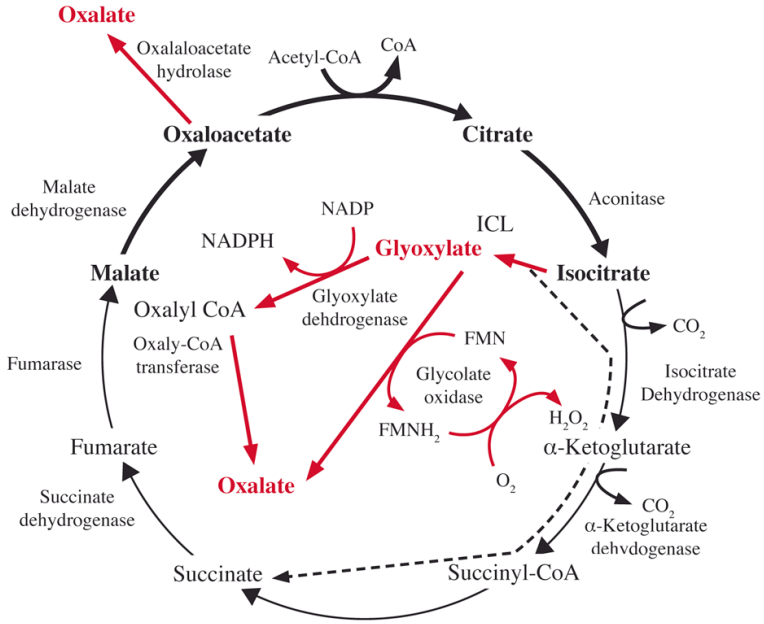

Figure 29: Postulated oxalate metabolism in *S. rolfsii*. Three different oxalate synthesising routes are described by use of the glyoxylate shunt, or alternatively, by C-C bond cleavage within oxaloacetate. ICL represents the isocitrate lyase.

However, sequence analysis of the *S. rolfsii* transcriptome did not reveal any data showing an oxalyl-CoA transferase. In addition, a sequence for glyoxylate dehydrogenase is not available in databases. The observed oxygen dependence of the oxalic acid synthesis in cultivations with *S. rolfsii* as, reported by Schilling et al. (2000), led to the conclusion that oxalic acid also might be produced via the enzyme glycolate oxidase. After enzyme-catalysed FMN reduction to $FMNH_2$, FMN is regenerated in the presence of molecular oxygen (Richardson and Tolbert 1961). Therefore the simultaneous presence of glycolate dehydrogenase, glycolate oxidase and oxaloacetate hydrolase may be possible in *S. rolfsii*.

A very interesting finding is that a cytochrome c dependent glyoxylate dehydrogenase in *Formitopsis palustris* has been reported by Tokimatsu et al. (1998). In addition to oxaloacetate hydrolase and glyoxylate oxidase, *F. palustris* also exhibits this third oxalate producing enzyme by which neither NAD nor NADP was utilized. Oxaloacetate hydrolase belongs to the enzyme class of hydrolases, whereas glyoxylate dehydrogenase and glycolate oxidase both belong to the oxidoreductases. The high amount of short sequence fragments in the *S. rolfsii* EST library, classified among the unknown oxidoreductases, may be a further hint at the hitherto putative glycolate dehydrogenase in *S. rolfsii*.

As there is only a limited offer of glucose in natural environment, there seems to be a lack in the economical balance in *S. rolfsii* caused by energy waste via the glyoxylate shunt for oxalate biosynthesis. Nevertheless, oxalate production may have a metabolic advantage for

S. rolfsii apart from its role in phytopathogenesis. This vantage point may be due to a link between energy production and oxalate biosynthesis. Oxalate biosynthesis may play in concert with energy yielding metabolic linkage of TCA and GLOX cycles. This highly ordered metabolic coupling system can serve as metabolic machinery for the oxalate biosynthesis accompanied by energy production for fungal growth. This scheme, as described in *F. palustris* by Munir et al. (2001b), might also be functional in *S. rolfsii* within early periods of cultivation in both media (Fig. 30). The highly sophisticated linkage of TCA and GLOX cycle by using glyoxylate and succinate as transfer shuttles, which replenish the neighboured biochemical pathway, results in a constant oxidation of acetyl-CoA to yield oxalate, which in turn accumulates in the cultivation medium. The use of acetate recycling routes (A and D in Fig. 30) also results in enhanced carbon net yield by additional charging of acetyl-CoA into the GLOX cycle.

Transport of succinate and glyoxylate from glyoxysomes to mitochondria, and vice versa, might be enabled in *S. rolfsii* by highly active mitochondrial transporters (Excel sheet C/D) as has been described in *F. palustris* (Sakai et al. 2006), to maintain the constitutive metabolic coordination of the TCA and GLOX for oxalate biosynthesis.

Taking into account the biochemical energy generated and consumed in the metabolic system (Fig. 30), overall oxidation of 2 mol acetyl-CoA yields 2 mol oxalate and results in the generation of a net amount of 4 NADHs and 2 ATPs (based on the assumption that no other metabolic pathway oxidizes acetyl-CoA). The TCA driving enzymes as isocitrate lyase and malate synthase were confirmed by Munir et al (2001a) not to be dependent on glucose repression as also has been reported for many other organisms (Westergaard et al. 2007; Usaite et al. 2008). Therefore, glucose will not cause a switch in fungal metabolism when used as the main carbon source.

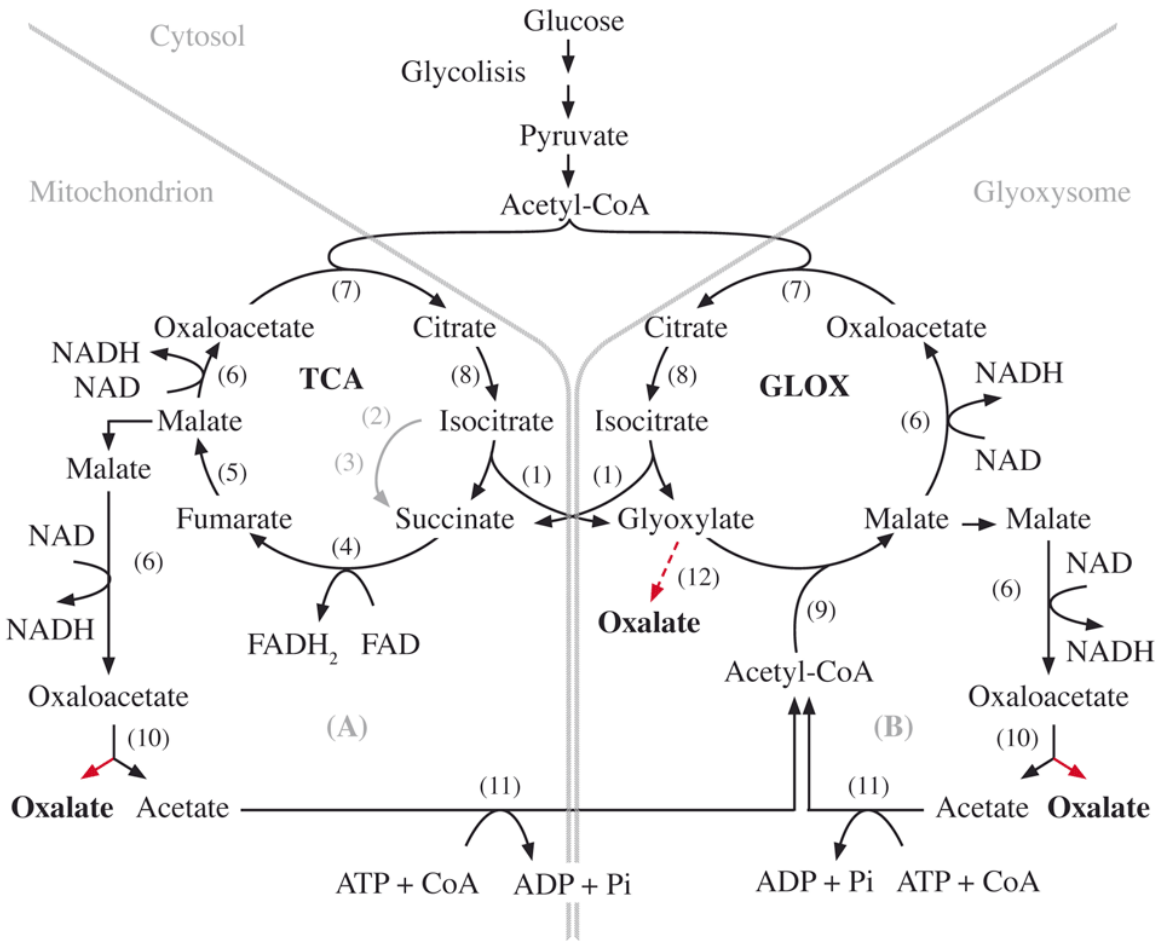

Figure 30: Metabolic system of TCA and GLOX according to Munir et al. (2001b), adapted to *S. rolfsii*. Coupling of the TCA and the GLOX cycles for NADH regeneration and energy extraction. (1) Isocitrate lyase (2) Isocitrate dehydrogenase (3) 2-oxoglutarate deyhdrogenase (4) Succinate dehydrogenase (5) Fumarase (6) Malate dehydrogenase (7) Citrate synthase (8) Aconitase (9) Malate synthase (10) Oxaloacetate hydrolase (11) Acetyl-CoA-synthase (12) Glycolate Oxidase. (A) and (B) represents the acetate recycling routes.

In addition, by the equal transcription within the first 37 h of cultivation in both media, no glucose repression of TCA linked enzymes on transcriptional level has been observed for *S. rolfsii* Therefore, the elaborate energy generating system as recommended by Munir et al. (2001b) will be functional in EPSmin17 and EPSmax13 conditions for ambient pH values and aerobic conditions. The rising content of oxalate decreases the pH in media and putatively in cytosol by longer cultivation in both media, therefore the TCA is repressed in the presence of organic acids. Isocitrate lyase, the key enzyme regulating the flux of carbohydrates from glyoxylate shunt to TCA or vice versa, mainly is regulated by pH and known to be inhibited by organic acids, especially by oxalate (Ozaki and Shiio 1968; Walshaw et al. 1997; Matsuzaki et al. 2008). The high amount of oxalate present in both designed media after 61 h (pH ~2.5); announce downregulation of glyoxylate shunt in prolonged cultivation. In fungal phytopathgenesis, the first attack of plant by oxalate will open the way to cellular components and further carbon source.

5.6 Energy production for fungal metabolism involved in scleroglucan production

Interpretation of microarray data caused the idea of a change in fungal metabolism during the scleroglucan production. Insights into the fungal metabolism during scleroglucan production may lead to further access to scleroglucan biosynthesis and may deliver putative new target genes for the metabolic engineering of scleroglucan synthesis. For this reason, the use of a time-grouped approach is performed to vary the changing conditions during batch fermentation of *S. rolfsii*.

5.6.2 Energy production during the early scleroglucan producing period

Energy supply for scleroglucan synthesis has to be separated into two parts. All TCA involved enzymes are identified to be active for the cultivation in EPSmin17/37 and EPSmax13/37 (Table A). The early scleroglucan biosynthesis process in submerse cultivations is characterized by sufficient oxygen supply, as has been described for other cultures (Schedel 1991). Therefore, cultivations up to 37 h can be regarded as aerobe, and the main energy production appears to be obtained by TCA and respiratory chains. The highly ordered metabolic coupling of both energy providing pathways, circumventing the carbon waste by decarboxylation steps of the TCA will be preferred. The use of GLOX bypass and thermodynamic based mining of energy sources warrants the high amount of oxalate produced. Following this metabolism scheme, glucose available is charged to scleroglucan biosynthesis and oxalate production, combined to a maximum yield of energy. The use of this high sophisticated energy utilizing system guarantees the plant pathogen fungus, virulent status via oxalate secretion by simultaneous energy benefit; providing advantages in natural environment against any kind of competitors.

Accumulation of secreted and partially linked scleroglucan to the hyphal sheath, increase viscosity of media and will reinforce the diffusion barrier of oxygen transfer, as has been reported for other fungi (Zaragoza et al. 2008). In addition, the high amount of secreted oxalate lowers pH resulting in acidic conditions (~pH 3.0) after cultivation periods of 48 h and longer (Fig. 11).

5.6.2 Hypoxic energy production during the late scleroglucan producing period

The cumulating effects of increased viscosity and lowered pH evoke a total change in the fungal metabolism, directing it towards anaerobe adaption. This anaerobic shift is indicated by the increased expression of enzymes known to be induced by hypoxic or anaerobic conditions. Pyruvate decarboxylase and formate dehydrogenase (FDH) are thus induced initiating a change in the fungal energy household.

Scleroglucan synthesis, therefore, is exposed to be uncoupled from the TCA within the late polysaccharide producing phase. All genes involved in the TCA and GLOX cycle respectively, are significantly down-regulated by organic acids and limited oxygen supply (Fig. 27).

Nevertheless, energy is essential for the polysaccharide synthesis process as is already known from other polysaccharide producers (Fu et al. 1995). Therefore, alternative energy producing strategies have to be performed during the hypoxic period in order to achieve enhanced scleroglucan production in *S. rolfsii.* The highly up-regulated FDH (found in at least two copies) in the EPSmax13/61 appears to play an important role in the hypoxic fungal metabolism and is closely linked to energy and scleroglucan production.

Hypoxia leads to strong FDH induction coupled with ATP synthesis, as has been described for plant mitochondria (Oliver 1981). FDH decomposes formate to CO_2 and acetate by retrieval of one NADH. NADH thus produced can be used for electron transfer on respiratory chain, generating ATP for the scleroglucan synthesis in *S. rolfsii*. The use of this TCA circumventing energy producing strategy may charge the glucose available, directly into scleroglucan synthesis. Such as uncoupling of product formation and TCA has been reported for *Penicillium chrysogenum,* where the enhanced production of beta lactame by the addition of formate was shown (Harris et al. 2007). The total or only partial uncoupling of TCA and enhanced product formation by use of formate as an energy source but not as a carbon substrate has been reported for metabolic engineered *S. cerevisiae* strains (Geertman et al. 2006). Additional supplemented formate replenished the cytosolic NADH for enhanced glycerol production. Formate oxidation is associated with high values of membrane potential and direct coupling to the three proton translocation sites in plant mitochondria (Hourton-Cabassa et al. 1998).

Formate may be produced by the direct decarboxylation of pyruvate via pyruvate formate lyase ending up in acetyl-CoA and formate (Kuwazaki et al. 2003; Hemschemeier et al. 2008). This system has been identified for many prokaryotes (Berks et al. 1995) and some

eukaryotes (Atteia et al. 2006). The pyruvate formate lyase could not be detected by sequences analysis in *S. rolfsii*. The high amount of short and non-annotated contigs (Fig. H) may result in a distinct loss of sequence data, and therefore non identification of pyruvate formate lyase within the EST library. An alternative source of formate in *S. rolfsii* might be realized by the non-enzymatic conversion of formate directly via H_2O_2 from glyoxylate, as has been described for many white rot fungi (Kuan and Tien 1993). However, this reaction is dependent on pH and the decomposition of glyoxylate will not proceed below a pH value of 5.5, other than at extremely high concentrations of glyoxylate and H_2O_2 (Halliwell and Butt 1974; Yokota et al. 1985). Stability of TCA and GLOX intermediates as glyoxylate, the prerequisite for this non enzymatic transformation has not been reported for *S. rolfsii*, and has been neglected as an alternative, due to the high turn over rate of formate dehydrogenase needed for scleroglucan biosynthesis in *S. rolfsii*.

In most eukaryotes FDH's appear to be cytosolic enzymes (Overkamp et al. 2002). The regulation of FDH is described to occur transcriptionally as it is induced by nitrate, hypoxia and environmental stress factors (Sakai et al. 1997; Hourton-Cabassa et al. 1998; Suzuki et al. 1998). Inhibition of FDH occurs by 2-oxoglutarate and oxaloacetate, NADH, ADP and ATP in high levels, indicating intracellular regulation on energy status (Watanabe et al. 2003; Sanchez et al. 2005). NH_4Cl as N-source exposed non-inducing effect on FDH in *Candida* (Sakai et al. 1997), emphasizing downregulation of FDH in NH_4Cl based EPSmin17 medium.

Unaffected scleroglucan yields along with simultaneously lowered oxalate production through fermentation under oxygen limited conditions has been reported by Schilling et al (2000), and emphasize the alternative energy producing route of *S. rolfsii* under hypoxic and $NaNO_3$ based conditions. Bacteria (Pirog et al. 2003), and rumal fungi (Wachenheim and Patterson 1992) also exposed oxygen independent polysaccharide production, highlighting hypoxic energy producing strategies uncoupled from TCA. The observation of up-regulated FDH in the EPSmin17/61 medium cultures, defined by low viscosity and, therefore, sufficient oxygen supply could be explained by existing pH of ~2.5 caused by oxalate secretion. FDH have been reported for the first time in fungi, including *Fusarium oxysporum* (Uchimura et al. 2002), *Ceriporiopsis subvermispora* (Watanabe et al. 2003; 2005; 2008) and now in *S. rolfsii*.

Furthermore, the genome of *S. rolfsii* has been revealed by sequence and pathway analysis to code for alternative oxidase and nitrate assimilating proteins. Nitrate- as well as nitrite-reductase has been identified. Therefore, *S. rolfsii* exhibits the property of a complete denitrification system; functionality, however, has to be proofed by suitable experiments.

Beyond primary metabolism, bypassing and overflow pathways such as GABA shunt and linkage of urea and TCA via argininosuccinate lyase have been identified. In addition, stress related and osmolysis relevant components such as mannitol and trehalose have been described to be vitally important under the examined cultivation conditions.

S. rolfsii genes have been shown to function in efflux or detoxification, secondary metabolite biosynthesis, GABA shunt, mannitol cycle and other accessory metabolic pathways. Identified genes include major facility superfamily transporters and ATP-binding cassette (ABC) transporters, various oxidoreductases and cytochrome P450s. Furthermore, *S. rolfsii* possesses an approximately complete set of lignin degrading enzymes such as laccases, pectinases, cellobiose dehydrogenase, manganese peroxidase, aryl alcohol dehydrogenase and a high variety of cytochrome P450s.

5.7 Sequencing and annotation

Here, the large amounts of short contigs impede gene prediction and complete coverage of the transcriptome. The high number of small contigs results from the short length of single reads obtained by the first generation 454 Life Science™ sequencing approach. Assembling of such short fragments without comparative sequences is challenging (Wommack et al. 2008), particularly when a transcriptomic approach is used and therefore, assembling of one single contig is almost unfeasible.

Around 12,000-14,000 genes can be expected to be present in the genome for *S. rolfsii,* as indicated by other comparable basidiomycete genomes (Kersten and Cullen 2007). The number of expected genes will further be decreased by EST sequencing.

Therefore, the 21973 contigs delivered by 454 assembling technology with an average length of 150-200 bp (Fig. C) indicate the coverage of given genes. However, as regards the total number of assembled bps (3,600,000), a complete coverage of the transcriptome may not have been accomplished. The high number of short contigs, combined to the high amount of non identifiable sequences, results in a loss of information. Therefore, the first generation of high throughput pyrosequencing has to be regarded critically in the case of *ab initio* sequencing projects and putatively high repetitive sequences, as typical for basidiomycetes (Goldberg et al. 2006). The new generations of pyrosequencing will enhance the length of single reads (up to 500 bp) and may solve the problems of assembling and annotation (Mashayekhi and Ronaghi 2007; Wommack et al. 2008).

Computational gene structure prediction for bacterial genomes may, in essence, be considered a solved problem, whereas gene structures are simple and a number of algorithms

have been developed that work well on prokaryotic sequences (Audic et al 1998; Delcher et al 1999; Besemer et al 2001; Suzek et al 2001). Currently, the problems in computational eukaryotic gene structure prediction have been well documented and are far from being solved (Guigo et al. 2000; Korf et al. 2001; Rogic et al. 2002; Zhang et al. 2003; 2004). Therefore, *ab initio* sequencing approaches are regarded as the main challenge in fungal genome annotation (Loftus 2003).

The comparison of the different blast analysis tools identified the BLAST2x versus the KOG database to be the best tool to provide a fast overview of present genes. However, clustering by protein domains is not specific to the single genes and has to be proven by manual curation. Data handling of all blast analysis tools was very time consuming and complex. Whilst tblastx analysis revealed the highest amount of sequence information, it is also the most expensive tool as regards computational-, human- and time-capacity. Manual curation of sequencing and microarray data takes far too long to complete the annotation and pathway analysis within an acceptable period of time (Baumgartner et al. 2007) Therefore; new computational curation methods have to be developed.

Furthermore, new aspects of the *S. rolfsii* metabolism were presented in this work which may lead to new applications of this fungus in industry, or even new targets for metabolic engineering with the focus on industrially interesting products.

Further studies may contribute to a more holistic understanding of fungal metabolism and growth under anaerobic conditions and stress response to acidic conditions such as caused by oxalate in *S. rolfsii*.

5.8 Outlook

The results in this thesis strongly indicate that scleroglucan synthesis in *S. rolfsii* occurs via the hypothetically assumed pathway, up to the (1→3)-β-glucan chain. The targeted deletion or silencing of subunits of the (1→3)-β-glucan synthase may lead to further insights into the branching mechanism and may reveal the exact role of the subunits in scleroglucan biosynthesis. In addition, the silencing of OST may provide insight into the putative involvement of this enzyme complex in the scleroglucan biosynthesis.

The metabolic engineering process as regards scleroglucan synthesis involves the overexpression of the identified enzymes, yielding the (1→3)-β-glucan precursors.

In this regard, the data obtained in this work provide a fair amount of sequence information on promising target genes in the biosynthesis pathway for scleroglucan by *S. rolfsii*. To obtain a deeper insight into scleroglucan synthesis, e. g. targeted mutagenesis of subunits putatively involved in scleroglucan production would be advisable for future work.

The identification of a formate based energy production in *S. rolfsii* (hitherto unknown) provides new targets as regards the engineering of scleroglucan biosynthesis. Increasing the net energy yield may also increase the scleroglucan yield. Furthermore, the use of formate in a mixed cultivation with glucose, which may result in enhanced scleroglucan production, has to be elucidated.

As postulated in this work, oxalate biosynthesis in *S. rolfsii* proceeds by the oxaloacetate hydrolase and glycolate (glyoxylate) oxidase enzyme system. This system is then further used by the fungus for energy production and regeneration of redox equivalents exhausted by scleroglucan production. Further in-depth analyses will have to be performed to elucidate the interaction of TCA with GLOX in more detail. Elucidation of the carbon flux towards diverse routes can be realized by creating knock out strains of oxaloacetate hydrolase and glycolate oxidase. The use of a metabolomic approach may also lead to a deeper insight into the distribution of glucose in-between the glucose consuming biosyntheses.

6 Summary

Scleroglucan is an exopolysaccharid produced by the basidiomycete *S. rolfsii* and consists of (1→3)-β-linked glucose with a (1→6)-β-glycosyl branch on every third unit. The scleroglucan biosynthesis as well as the pathway leading to formation of the unwanted by-product oxalate were analysed on sequence level with the aim to enable metabolic engineering in *S. rolfsii*. A transformation system for *S. rolfsii* was established in order to enable functional genetics. The *ab initio* pyrosequencing of an EST library combined with two transcriptomic approaches, namely suppression subtractive hybridisation and microarray technique, delivered initial insights into the genomics of *S. rolfsii* during scleroglucan production. Based on the data obtained, it can be postulated that scleroglucan synthesis is carried out by a five-step-biosynthesis. The nucleotide sugar supply for scleroglucan synthesis was confirmed by the identification of four protein coding genes, namely hexokinase, phosphoglucomutase, UTP-glucose-1-phosphate uridylyltransferase and the backbone elongating (1→3)-β-glucan synthase. The last enzyme (or enzymes), responsible for branching on every third unit, remains speculative.

The biosynthesis of oxalate could be further unravelled by the identification of glycolate oxidase and oxaloacetate hydrolase, both acting within the GLOX cycle. Putative target genes were identified by sequence analysis for oxaloacetate hydrolase, glycolate oxidase and isocitrate lyase. Theses genes represent promising target genes for metabolic engineering, in order to improve *S. rolfsii* as a production host for industrial scleroglucan synthesis.

The data obtained indicate that there is a novel energy saving metabolic system in *S. rolfsii* via coupling of TCA and GLOX cycle and alternatively by oxidation of formate. This lead to the suggestion that the aerobic fungus *S. rolfsii* possesses several mechanisms to adapt and survive anaerobic growth conditions caused by excessive scleroglucan production.

6 Zusammenfassung

Scleroglukan, ein Exopolysaccharid, bestehend aus (1→3)-β-verknüpfter Glukose mit einer (1→6)-β-glycosidischen Verzweigung an jeder dritten Einheit, wird von dem Basidiomyzeten *Sclerotium rolfsii* sekretiert. Sowohl die Biosynthese von Scleroglukan als auch der Syntheseweg des Hauptnebenprodukts Oxalat wurden auf Sequenzebene analysiert, um durch Metabolic Engineering die Scleroglukan Produktion optimieren zu können. Hierzu war es nötig ein Transformations-System für *S. rolfsii* zu etablieren.

Eine *ab initio* Pyrosequenzierung in Verbund mit zwei zusätzlichen Untersuchungen auf Transkriptomebene, namentlich subtraktive suppressive Hybridisierung und Microarray Analyse, gewähren erstmals Einblick in die Molekulargenetik und den Stoffwechsel von *S. rolfsii*. Für die Biosynthese von Scleroglukan konnte dadurch eine Abfolge der Genprodukte Hexokinase, Phosphoglukomutase, UTP-Glukose-1-Phosphat Uridylyl-transferase und (1→3)-β-Glukan Synthase postuliert werden, der letzte Schritt der (1→6)-β-Verzweigung bleibt weiterhin spekulativ.

Für die Oxalat Biosynthese wurden zwei Enzyme identifiziert welche direkt an der Oxalat Synthese über den GLOX Zyklus in *S. rolfsii* beteiligt sind, Glykolat Oxidase und Oxalacetat Hydrolase. Beide Enzyme wurden zum ersten Mal in *S. rolfsii* nachgewiesen. Die gewonnenen Sequenzdaten liefern somit Angriffspunkte für ein Metabolic Engineering des Oxalat Stoffwechsels über die Gene für Oxalacetat Hydrolase, Glykolat Oxidase oder auch der Isocitrat Lyase.

Die nötige Energie für die Scleroglukan Biosynthese wird durch eine Kopplung des TCA mit dem GLOX Zyklus geliefert oder alternativ, über die Oxidation von Formiate. Anaerobe Respiration erscheint ebenfalls möglich in *S. rolfsii*, was ein absolutes Novum für diesen bisher als aerob betrachteten Pilz darstellt und einen Hinweis für vielfältige Adaptionsmöglichkeiten von *S. rolfsii* verkörpert.

7 References

Abe, K., K. Gomi, F. Hasegawa and M. Machida (2006). "Impact of *Aspergillus oryzae* genomics on industrial production of metabolites." Mycopathologia **162**(3): 143-53.

Abeijon, C. and L. Y. Chen (1998). "The role of glucosidase I (Cwh41p) in the biosynthesis of cell wall beta-1,6-glucan is indirect." Mol Biol Cell **9**(10): 2729-38.

Adrio, J. L. and A. L. Demain (2006). "Genetic improvement of processes yielding microbial products." FEMS Microbiol Rev **30**(2): 187-214.

Aguilar, C., U. Urzua, C. Koenig and R. Vicuna (1999). "Oxalate oxidase from *Ceriporiopsis subvermispora*: biochemical and cytochemical studies." Arch Biochem Biophys **366**(2): 275-82.

Akamatsu, Y., A. Ohta, M. Takahashi and M. Shimada (1991). "Enzymatic formation of oxalic acid from oxaloacetate with cell free extracts of the brown-rot fungus *Tyromyces palustris* in relation to the biodegradation of cellulose." Mokuzai Gakkaishi **25**: 375-377.

Akamatsu, Y. and M. Shimada (1993). "A novel glyoxylate oxidase of the brown rot fungus *Tyromyces palustris*." Proc FEMS Symp. Lisbon. Portugal: 149-150.

Akamatsu, Y. and M. Shimada (1994). "Partial purification and characterization of glyoxylate oxidase from the brown rot basidiomycete, *Tyromyces palustris*. ." Phytochemistry **37**: 649-653.

Akamatsu, Y., M. Takahashi and M. Shimada (1992). "Cell-free extraction of oxaloacetase from white-rot fungi, including *Coriolus versicolor*." Wood research **79**: 1-6.

Akashi, H., S. Matsumoto and K. Taira (2005). "Gene discovery by ribozyme and siRNA libraries." Nat Rev Mol Cell Biol **6**(5): 413-22.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). "Basic local alignment search tool." J Mol Biol **215**(3): 403-10.

Anantharam, V., M. J. Allison and P. C. Maloney (1989). "Oxalate:formate exchange. The basis for energy coupling in Oxalobacter." J Biol Chem **264**(13): 7244-50.

Andersen, M. R., M. L. Nielsen and J. Nielsen (2008). "Metabolic model integration of the bibliome, genome, metabolome and reactome of *Aspergillus niger*." Mol Syst Biol **4**: 178.

Arechiga-Carvajal, E. T. and J. Ruiz-Herrera (2005). "The RIM101/pacC homologue from the basidiomycete *Ustilago maydis* is functional in multiple pH-sensitive phenomena." Eukaryot Cell **4**(6): 999-1008.

Askolin, S., T. Nakari-Setala and M. Tenkanen (2001). "Overproduction, purification, and characterization of the *Trichoderma reesei* hydrophobin HFBI." Appl Microbiol Biotechnol **57**(1-2): 124-30.

Atteia, A., R. van Lis, G. Gelius-Dietrich, A. Adrait, J. Garin, J. Joyard, N. Rolland and W. Martin (2006). "Pyruvate formate-lyase and a novel route of eukaryotic ATP synthesis in *Chlamydomonas mitochondria*." J Biol Chem **281**(15): 9909-18.

Azam, M., M. Kesarwani, S. Chakraborty, K. Natarajan and A. Datta (2002). "Cloning and characterization of the 5'-flanking region of the oxalate decarboxylase gene from *Flammulina velutipes*." Biochem J **367**(Pt 1): 67-75.

Balmforth, A. J. and A. Thomson (1984). "Isolation and characterization of glyoxylate dehydrogenase from the fungus *Sclerotium rolfsii*." Biochem J **218**(1): 113-8.

Barnes, S. E., M. J. Alcocer and D. B. Archer (2008). "siRNA as a molecular tool for use in *Aspergillus niger*." Biotechnol Lett **30**(5): 885-90.

Baron, C., N. Domke, M. Beinhofer and S. Hapfelmeier (2001). "Elevated temperature differentially affects virulence, VirB protein accumulation, and T-pilus formation in different *Agrobacterium tumefaciens* and *Agrobacterium vitis* strains." J Bacteriol **183**(23): 6852-61.

Bateman, D. F. and S. V. Beer (1965). "Simultaneous Production and Synergistic Action of Oxalic Acid and Polygalacturonase During Pathogenesis by *Sclerotium Rolfsii*." Phytopathology **55**: 204-11.

Batra, K. K., J. H. Nordin and S. Kirkwood (1969). "Biosynthesis of the beta-D-glucan of *Sclerotium rolfsii* Sacc. Direction of chain propagation and the insertion of the branch residues." Carbohydrate Research **9**(2): 221-229.

Baumgartner, W. A., K. Bretonnel Cohen, L. Fox, M., G. Acquaah-Mensah and a. L. Hunter (2007). "Manual curation is not sufficient for annotation of genomic databases." Bioinformatics **2007**: 141–148.

Bautista-Gallego, J., F. N. Arroyo-Lopez, M. C. Duran-Quintana and A. Garrido-Fernandez (2008). "Individual effects of sodium, potassium, calcium, and magnesium chloride salts on *Lactobacillus pentosus* and *Saccharomyces cerevisiae* growth." J Food Prot **71**(7): 1412-21.

Bautista, L. F., A. Aleksenko, M. Hentzer, A. Santerre-Henriksen and J. Nielsen (2000). "Antisense silencing of the creA gene in *Aspergillus nidulans*." Appl Environ Microbiol **66**(10): 4579-81.

Beauvais, A., J. M. Bruneau, P. C. Mol, M. J. Buitrago, R. Legrand and J. P. Latge (2001). "Glucan synthase complex of *Aspergillus fumigatus*." J Bacteriol **183**(7): 2273-9.

Beijersbergen, A., A. D. Dulk-Ras, R. A. Schilperoort and P. J. Hooykaas (1992). "Conjugative Transfer by the Virulence System of *Agrobacterium tumefaciens*." Science **256**(5061): 1324-1327.

Berks, B. C., S. J. Ferguson, J. W. Moir and D. J. Richardson (1995). "Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions." Biochim Biophys Acta **1232**(3): 97-173.

Bernstein, E., A. A. Caudy, S. M. Hammond and G. J. Hannon (2001). "Role for a bidentate ribonuclease in the initiation step of RNA interference." Nature **409**(6818): 363-6.

Betts, M. F., S. L. Tucker, N. Galadima, Y. Meng, G. Patel, L. Li, N. Donofrio, A. Floyd, S. Nolin, D. Brown, M. A. Mandel, T. K. Mitchell, J. R. Xu, R. A. Dean, M. L. Farman and M. J. Orbach (2007). "Development of a high throughput transformation system for insertional mutagenesis in *Magnaporthe oryzae*." Fungal Genet Biol **44**(10): 1035-49.

Blackmore, M. A., and J. R. Quayle. (1970). "Microbial growth on oxalate by a route not involving glyoxylate carboligase." Biochem. J. **118**: 53-59.

Blackwood, C. B., M. P. Waldrop, D. R. Zak and R. L. Sinsabaugh (2007). "Molecular analysis of fungal communities and laccase genes in decomposing litter reveals differences among forest types but no impact of nitrogen deposition." Environ Microbiol **9**(5): 1306-16.

Blanco, F. A. and H. S. Judelson (2005). "A bZIP transcription factor from Phytophthora interacts with a protein kinase and is required for zoospore motility and plant infection." Mol Microbiol **56**(3): 638-48.

Bluhm, T., Deslandes, Y., Marchessault, R.H., Perez, S. and Rinaudo, M., (1982). "Solid-state and solution conformation of Scleroglucan." Carbohydrate Research **100**: 117-130.

Boels, I. C., A. Ramos, M. Kleerebezem and W. M. de Vos (2001). "Functional analysis of the *Lactococcus lactis* galU and galE genes and their impact on sugar nucleotide and exopolysaccharide biosynthesis." Appl Environ Microbiol **67**(7): 3033-40.

Boeuf, S., E. Steck, K. Pelttari, T. Hennig, A. Buneb, K. Benz, D. Witte, H. Sultmann, A. Poustka and W. Richter (2008). "Subtractive gene expression profiling of articular cartilage and mesenchymal stem cells: serpins as cartilage-relevant differentiation markers." Osteoarthritis Cartilage **16**(1): 48-60.

Bousfield, I. G., PN (1985). "Reclassification of bacteria of the genus Protomonas Urakami and Komagata 1984 in the genus Methylobacterium (Patt, Cole, and Hanson) Emend. Green and Bousfield 1983." International Journal of Systematic Bacteriology **35**(2): 209.

Breakspear, A. and M. Momany (2007). "The first fifty microarray studies in filamentous fungi." Microbiology **153**(Pt 1): 7-15.

Brenner, V. C. and J. C. Sherris (1972). "Influence of different media and bloods on results of diffusion antibiotic susceptibility tests." Antimicrob Agents Chemother **1**(2): 116-22.

Brigand, G. (1993). Scleroglucan, Academic Press, New York, USA

Bromley, M., C. Gordon, N. Rovira-Graells and J. Oliver (2006). "The *Aspergillus fumigatus* cellobiohydrolase B (cbhB) promoter is tightly regulated and can be exploited for controlled protein expression and RNAi." FEMS Microbiol Lett **264**(2): 246-54.

Bundock, P., A. den Dulk-Ras, A. Beijersbergen and P. J. Hooykaas (1995). "Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*." Embo J **14**(13): 3206-14.

Burda, P. and M. Aebi (1998). "The ALG10 locus of *Saccharomyces cerevisiae* encodes the alpha-1,2 glucosyltransferase of the endoplasmic reticulum: the terminal glucose of the lipid-linked oligosaccharide is required for efficient N-linked glycosylation." Glycobiology **8**(5): 455-62.

Burns, C., K. E. Gregory, M. Kirby, M. K. Cheung, M. Riquelme, T. J. Elliott, M. P. Challen, A. Bailey and G. D. Foster (2005). "Efficient GFP expression in the mushrooms *Agaricus bisporus* and *Coprinus cinereus* requires introns." Fungal Genet Biol **42**(3): 191-9.

Bussiere, F., S. Ledu, M. Girard, M. Heroux, J. P. Perreault and D. P. Matton (2003). "Development of an efficient cis-trans-cis ribozyme cassette to inactivate plant genes." Plant Biotechnol J **1**(6): 423-35.

Butaye, P., L. A. Devriese and F. Haesebrouck (2000). "Influence of different medium components on the in vitro activity of the growth-promoting antibiotic flavomycin against enterococci." J Antimicrob Chemother **46**(5): 713-6.

Çaliskan, M. (2000). "The Metabolism of Oxalic Acid." Turk J Zool **24**: 103–106.

Caracuel-Rios, Z. and N. J. Talbot (2008). "Silencing the crowd: high-throughput functional genomics in *Magnaporthe oryzae*." Mol Microbiol.

Cardoza, R. E., M. R. Hermosa, J. A. Vizcaino, F. Gonzalez, A. Llobell, E. Monte and S. Gutierrez (2007). "Partial silencing of a hydroxy-methylglutaryl-CoA reductase-encoding gene in *Trichoderma harzianum* CECT 2413 results in a lower level of resistance to lovastatin and lower antifungal activity." Fungal Genet Biol **44**(4): 269-83.

Carpenter, M. A., A. Stewart and H. J. Ridgway (2005). "Identification of novel *Trichoderma hamatum* genes expressed during mycoparasitism using subtractive hybridisation." FEMS Microbiol Lett **251**(1): 105-12.

Casas-Flores, S., T. Rosales-Saavedra and A. Herrera-Estrella (2004). "Three decades of fungal transformation: novel technologies." Methods Mol Biol **267**: 315-25.

Castro, O., F. Movsichoff and A. J. Parodi (2006). "Preferential transfer of the complete glycan is determined by the oligosaccharyltransferase complex and not by the catalytic subunit." Proc Natl Acad Sci U S A **103**(40): 14756-60.

Cederlund, E., Y. Lindqvist, G. Soderlund, C. I. Branden and H. Jornvall (1988). "Primary structure of glycolate oxidase from spinach." Eur J Biochem **173**(3): 523-30.

Cerning, J. (1990). "Exocellular polysaccharides produced by lactic acid bacteria." FEMS Microbiol Rev **7**(1-2): 113-30.

Chabaud, M., F. de Carvalho-Niebel and D. G. Barker (2003). "Efficient transformation of *Medicago truncatula* cv. Jemalong using the hypervirulent *Agrobacterium tumefaciens* strain AGL1." Plant Cell Rep **22**(1): 46-51.

Chakraborty, B. N., N. A. Patterson and M. Kapoor (1991). "An electroporation-based system for high-efficiency transformation of germinated conidia of filamentous fungi." Can J Microbiol **37**(11): 858-63.

Chang, P. K. (2008). "A highly efficient gene-targeting system for *Aspergillus parasiticus*." Lett Appl Microbiol **46**(5): 587-92.

Chavan, M., T. Suzuki, M. Rekowicz and W. Lennarz (2003). "Genetic, biochemical, and morphological evidence for the involvement of N-glycosylation in biosynthesis of the cell wall beta 1,6-glucan of *Saccharomyces cerevisiae*." Proc Natl Acad Sci U S A **100**(26): 15381-6.

Chen, X., M. Stone, C. Schlagnhaufer and C. P. Romaine (2000). "A fruiting body tissue method for efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*." Appl Environ Microbiol **66**(10): 4510-3.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald and W. J. Rutter (1979). "Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease." Biochemistry **18**(24): 5294-9.

Choudhary, S., H. C. Lee, M. Maiti, Q. He, P. Cheng, Q. Liu and Y. Liu (2007). "A double-stranded-RNA response program important for RNA interference efficiency." Mol Cell Biol **27**(11): 3995-4005.

Clausen, C. A., F., Green lll (1996). "Characterization of polygalacturonase from the brown-rot fungus *Postia placenta*." Appl Microbiol Biotechnol **45**: 750-754.

Coe, F. L., A. Evan and E. Worcester (2005). "Kidney stone disease." J Clin Invest **115**(10): 2598-608.

Cogoni, C., J. T. Irelan, M. Schumacher, T. J. Schmidhauser, E. U. Selker and G. Macino (1996). "Transgene silencing of the al-1 gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation." Embo J **15**(12): 3153-63.

Conesa, A. P., P. J. van Luijk and N. van den Hondel, C. A. (2001). "The secretion pathway in filamentous fungi: a biotechnological view." Fungal Genet Biol **33**(3): 155-71.

Cottrel, I. A. (1980). "Industrial Potential of Fungal and Bacterial Polysaccharides." Fungal Polysaccharides, ACS Symposium Series **126**(P.A. Sandford, I. Matsuda (Eds.), ACS, Washington, USA): 251–27.

Cramton, S. E., M. Ulrich, F. Gotz and G. Doring (2001). "Anaerobic conditions induce expression of polysaccharide intercellular adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis*." Infect Immun **69**(6): 4079-85.

da Silva Ferreira, M. E., M. R. Kress, M. Savoldi, M. H. Goldman, A. Hartl, T. Heinekamp, A. A. Brakhage and G. H. Goldman (2006). "The akuB (KU80) mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in *Aspergillus fumigatus*." Eukaryot Cell **5**(1): 207-11.

Damicone, J. (1997). "Fungicide Resistance Management." Oklahoma Cooperative Extension Service EPP-7663.

David, H., I. S. Ozcelik, G. Hofmann and J. Nielsen (2008). "Analysis of *Aspergillus nidulans* metabolism at the genome-scale." BMC Genomics **9**: 163.

Davidse, L. C. and W. Flach (1978). "Interaction of thiabendazole with fungal tubulin." Biochim Biophys Acta **543**(1): 82-90.

Davies, D. D. and H. Asker (1983). "Synthesis of Oxalic Acid by Enzymes from Lettuce Leaves." Plant Physiol **72**(1): 134-138.

Davies, J. and A. Jimenez (1980). "A new selective agent for eukaryotic cloning vectors." Am J Trop Med Hyg **29**(5 Suppl): 1089-92.

de Groot, M. J., P. Bundock, P. J. Hooykaas and A. G. Beijersbergen (1998). "*Agrobacterium tumefaciens*-mediated transformation of filamentous fungi." Nat Biotechnol **16**(9): 839-42.

de Jong, J. F., H. J. Deelstra, H. A. Wosten and L. G. Lugones (2006). "RNA-mediated gene silencing in monokaryons and dikaryons of *Schizophyllum commune*." Appl Environ Microbiol **72**(2): 1267-9.

Deed, A. and R. Seviour (1989). "Formation and regeneration of protoplasts of *Sclerotium glucanicum*." Applied Microbiology and Biotechnology **31**: 259–264.

Dempski, R. E., Jr. and B. Imperiali (2002). "Oligosaccharyl transferase: gatekeeper to the secretory pathway." Curr Opin Chem Biol **6**(6): 844-50.

Deshpande, M. V., H. Balkrishnan, P. K. Ranjekar and V. Shankar (1987). "Isolation and immobilization of *Sclerotium rolfsii* protoplasts " Biotechnology Letters **9**(1): 49-52.

Diatchenko, L., Y. F. Lau, A. P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E. D. Sverdlov and P. D. Siebert (1996). "Suppression subtractive hybridisation: a method for generating differentially regulated or tissue-specific cDNA probes and libraries." Proc Natl Acad Sci U S A **93**(12): 6025-30.

Doster, M. S. N., A. J.; Christopher, C. A. (1984). "Recovering petroleum from underground formations." U.S. Patent 1984, 4,457,372; Chem. Abstr. 1984, 101, 113713g. **101**.

Douglas, C. M. (2001). "Fungal beta(1,3)-D-glucan synthesis." Med Mycol 39 Suppl 1: 55-66.

Dowzer, C. E. and J. M. Kelly (1991). "Analysis of the creA gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*." Mol Cell Biol **11**(11): 5701-9.

Duc, A. N. C. (1982). "Glucosylglucans and their use in gastroenterology, especially in the treatment of colon disorders." Chem. Abstr. **96**(Eur. Patent 45,338): 168769p.

Dudasova, Z., A. Dudas and M. Chovanec (2004). "Non-homologous end-joining factors of *Saccharomyces cerevisiae*." FEMS Microbiol Rev **28**(5): 581-601.

Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola, I. Lafontaine, J. De Montigny, C. Marck, C. Neuveglise, E. Talla, N. Goffard, L. Frangeul, M. Aigle, V. Anthouard, A. Babour, V. Barbe, S. Barnay, S. Blanchin, J. M. Beckerich, E. Beyne, C. Bleykasten, A. Boisrame, J. Boyer, L. Cattolico, F. Confanioleri, A. De Daruvar, L. Despons, E. Fabre, C. Fairhead, H. Ferry-Dumazet, A. Groppi, F. Hantraye, C. Hennequin, N. Jauniaux, P. Joyet, R. Kachouri, A. Kerrest, R. Koszul, M. Lemaire, I. Lesur, L. Ma, H. Muller, J. M. Nicaud, M. Nikolski, S. Oztas, O. Ozier-Kalogeropoulos, S. Pellenz, S. Potier, G. F. Richard, M. L. Straub, A. Suleau, D. Swennen, F. Tekaia, M. Wesolowski-Louvel, E. Westhof, B. Wirth, M. Zeniou-Meyer, I. Zivanovic, M. Bolotin-Fukuhara, A. Thierry, C. Bouchier, B. Caudron, C. Scarpelli, C. Gaillardin, J. Weissenbach, P. Wincker and J. L. Souciet (2004). "Genome evolution in yeasts." Nature **430**(6995): 35-44.

Dumas, B., G. Freyssinet and K. E. Pallett (1995). "Tissue-Specific Expression of Germin-Like Oxalate Oxidase during Development and Fungal Infection of Barley Seedlings." Plant Physiol **107**(4): 1091-1096.

Dutton, M. V., & Evans, C. S. (1996). "Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment." Canadian Journal of Botany **42**: 881-895.

Dyer, P. S. and M. Paoletti (2005). "Reproduction in *Aspergillus fumigatus*: sexuality in a supposedly asexual species?" Med Mycol **43** Suppl 1: 7-14.

Edens, L., P. Dekker, R. van der Hoeven, F. Deen, A. de Roos and R. Floris (2005). "Extracellular prolyl endoprotease from *Aspergillus niger* and its use in the debittering of protein hydrolysates." J Agric Food Chem **53**(20): 7950-7.

El-Khoury, R., C. H. Sellem, E. Coppin, A. Boivin, M. F. Maas, R. Debuchy and A. Sainsard-Chanet (2008). "Gene deletion and allelic replacement in the filamentous fungus *Podospora anserina*." Curr Genet **53**(4): 249-58.

Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl (2001a). "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells." Nature **411**(6836): 494-8.

Elbashir, S. M., W. Lendeckel and T. Tuschl (2001b). "RNA interference is mediated by 21- and 22-nucleotide RNAs." Genes Dev **15**(2): 188-200.

Errakhi, R., P. Meimoun, A. Lehner, G. Vidal, J. Briand, F. Corbineau, J. P. Rona and F. Bouteau (2008). "Anion channel activity is necessary to induce ethylene synthesis and programmed cell death in response to oxalic acid." J Exp Bot **59**(11): 3121-9.

Escutia, M. R., L. Bowater, A. Edwards, A. R. Bottrill, M. R. Burrell, R. Polanco, R. Vicuna and S. Bornemann (2005). "Cloning and sequencing of two *Ceriporiopsis subvermispora* bicupin oxalate oxidase allelic isoforms: implications for the reaction specificity of oxalate oxidases and decarboxylases." Appl Environ Microbiol **71**(7): 3608-16.

Faijes, M. and A. Planas (2007). "In vitro synthesis of artificial polysaccharides by glycosidases and glycosynthases." Carbohydr Res **342**(12-13): 1581-94.

Farina, J. I., O. E. Molina and L. I. Figueroa (2004). "Formation and regeneration of protoplasts in *Sclerotium rolfsii* ATCC 201126." J Appl Microbiol **96**(2): 254-62.

Farina, J. I., F. Sineriz, O. Molina, E. and N. I. and Perotti (1998). "High scleroglucan production by *Sclerotium rolfsii*: Influence of medium composition." Biotechnology Letters **20**(9): 825-831.

Favaron, F., L. Sella and R. D'Ovidio (2004). "Relationships among endo-polygalacturonase, oxalate, pH, and plant polygalacturonase-inhibiting protein (PGIP) in the interaction between *Sclerotinia sclerotiorum* and soybean." Mol Plant Microbe Interact. **17**(12): 1402-9.

Fecke, W. U. N. a. H. W. (2008). An antisense RNA expression vector for *Neurospora crassa*. ECFG 9, ECFG 9 Poster Session 1.

Fedorova, N. D., N. Khaldi, V. S. Joardar, R. Maiti, P. Amedeo, M. J. Anderson, J. Crabtree, J. C. Silva, J. H. Badger, A. Albarraq, S. Angiuoli, H. Bussey, P. Bowyer, P. J. Cotty, P. S. Dyer, A. Egan, K. Galens, C. M. Fraser-Liggett, B. J. Haas, J. M. Inman, R. Kent, S. Lemieux, I. Malavazi, J. Orvis, T. Roemer, C. M. Ronning, J. P. Sundaram, G. Sutton, G. Turner, J. C. Venter, O. R. White, B. R. Whitty, P. Youngman, K. H. Wolfe, G. H. Goldman, J. R. Wortman, B. Jiang, D. W. Denning and W. C. Nierman (2008). "Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*." PLoS Genet **4**(4): e1000046.

Fincham, J. R. (1989). "Transformation in fungi." Microbiol Rev **53**(1): 148-70.

Fischer, R., N. Zekert and N. Takeshita (2008). "Polarized growth in fungi-interplay between the cytoskeleton, positional markers and membrane domains." Mol Microbiol **68**(4): 813-26.

Foreman, P. K., D. Brown, L. Dankmeyer, R. Dean, S. Diener, N. S. Dunn-Coleman, F. Goedegebuur, T. D. Houfek, G. J. England, A. S. Kelley, H. J. Meerman, T. Mitchell, C. Mitchinson, H. A. Olivares, P. J. Teunissen, J. Yao and M. Ward (2003). "Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*." J Biol Chem **278**(34): 31988-97.

Forrest, E. C., Cogoni, C., Macino, G. (2004). "The RNA-dependent RNA polymerase, QDE-1, is a rate-limiting factor in post-transcriptional gene silencing in *Neurospora crassa*." Nucleic Acids Res **32**(7): 2123-8.

Fu, J., F. J. Bailey, J. J. King, C. B. Parker, R. S. Robinett, D. G. Kolodin, H. A. George and W. K. Herber (1995). "Recent advances in the large scale fermentation of *Neisseria meningitidis*

group B for the production of an outer membrane protein complex." Biotechnology (N Y) **13**(2): 170-4.

Fullner, K. J. and E. W. Nester (1996). "Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*." J Bacteriol **178**(6): 1498-504.

Galagan, J. E., S. E. Calvo, C. Cuomo, L. J. Ma, J. R. Wortman, S. Batzoglou, S. I. Lee, M. Basturkmen, C. C. Spevak, J. Clutterbuck, V. Kapitonov, J. Jurka, C. Scazzocchio, M. Farman, J. Butler, S. Purcell, S. Harris, G. H. Braus, O. Draht, S. Busch, C. D'Enfert, C. Bouchier, G. H. Goldman, D. Bell-Pedersen, S. Griffiths-Jones, J. H. Doonan, J. Yu, K. Vienken, A. Pain, M. Freitag, E. U. Selker, D. B. Archer, M. A. Penalva, B. R. Oakley, M. Momany, T. Tanaka, T. Kumagai, K. Asai, M. Machida, W. C. Nierman, D. W. Denning, M. Caddick, M. Hynes, M. Paoletti, R. Fischer, B. Miller, P. Dyer, M. S. Sachs, S. A. Osmani and B. W. Birren (2005). "Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*." Nature **438**(7071): 1105-15.

Garcia-Rico, R. O., J. F. Martin and F. Fierro (2007). "The pga1 gene of *Penicillium chrysogenum* NRRL 1951 encodes a heterotrimeric G protein alpha subunit that controls growth and development." Res Microbiol **158**(5): 437-46.

Geertman, J. M., A. J. van Maris, J. P. van Dijken and J. T. Pronk (2006). "Physiological and genetic engineering of cytosolic redox metabolism in *Saccharomyces cerevisiae* for improved glycerol production." Metab Eng **8**(6): 532-42.

Gelvin, S. B. and L. L. Habeck (1990). "*vir* genes influence conjugal transfer of the Ti plasmid of *Agrobacterium tumefaciens*." J Bacteriol **172**(3): 1600-8.

Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin and S. G. Oliver (1996). "Life with 6000 genes." Science **274**(5287): 546, 563-7.

Goins, C. L., K. J. Gerik and J. K. Lodge (2006). "Improvements to gene deletion in the fungal pathogen *Cryptococcus neoformans*: absence of Ku proteins increases homologous recombination, and co-transformation of independent DNA molecules allows rapid complementation of deletion phenotypes." Fungal Genet Biol **43**(8): 531-44.

Goldberg, S. M., J. Johnson, D. Busam, T. Feldblyum, S. Ferriera, R. Friedman, A. Halpern, H. Khouri, S. A. Kravitz, F. M. Lauro, K. Li, Y. H. Rogers, R. Strausberg, G. Sutton, L. Tallon, T. Thomas, E. Venter, M. Frazier and J. C. Venter (2006). "A Sanger/pyrosequencing hybrid approach for the generation of high-quality draft assemblies of marine microbial genomes." Proc Natl Acad Sci U S A **103**(30): 11240-5.

Goldoni, M., G. Azzalin, G. Macino and C. Cogoni (2004). "Efficient gene silencing by expression of double stranded RNA in *Neurospora crassa*." Fungal Genet Biol **41**(11): 1016-24.

Goodell, b., Jellison, J., Liu, J., Daniel. G., Pasczynski. A., Fekete, F., Krishnamurthy, S., Jun, L. & Xu, G. (1995). "Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood." Jounal of Biotechnology **53**: 133-162.

Gowda, M., R. C. Venu, M. B. Raghupathy, K. Nobuta, H. Li, R. Wing, E. Stahlberg, S. Couglan, C. D. Haudenschild, R. Dean, B. H. Nahm, B. C. Meyers and G. L. Wang (2006). "Deep and comparative analysis of the mycelium and appressorium transcriptomes of *Magnaporthe grisea* using MPSS, RL-SAGE, and oligoarray methods." BMC Genomics **7**: 310.

Grant, J. E., L. M. Thomson, M. D. Pither-Joyce, T. M. Dale and P. A. Cooper (2003). "Influence of *Agrobacterium tumefaciens* strain on the production of transgenic peas (*Pisum sativum L.*)." Plant Cell Rep **21**(12): 1207-10.

Green, F. I., Clausen, C. A., Kuster, T. A. & Highley, T. L. (1995). "Induction of polygalacturonase and the formation of oxalic acid by pectin in brown-rot fungi." World Journal of of Microbiology and Biotechnology **11**: 519-524.

Green III, J. T., T.A. Kuster and T.L. Highley (1996). "Hydrolysis of bordered pits during colonization of conifers by brown-rot decay." The international research group on wood preservation Document No. : IRG/WP/ 95-10103.

Grimm, L. H., S. Kelly, R. Krull and D. C. Hempel (2005). "Morphology and productivity of filamentous fungi." Appl Microbiol Biotechnol **69**(4): 375-84.

Guigo, R., P. Agarwal, J. F. Abril, M. Burset and J. W. Fickett (2000). "An assessment of gene prediction accuracy in large DNA sequences." Genome Res **10**(10): 1631-42.

Ha, Y. S., S. F. Covert and M. Momany (2006). "FsFKS1, the 1,3-beta-glucan synthase from the caspofungin-resistant fungus *Fusarium solani*." Eukaryot Cell **5**(7): 1036-42.

Halleck, F. E. (1967). Polysaccharides and methods for the production thereof. US. 3,302,848.

Halleck, F. E. (1969). "Paint composition containing polysaccharides." Chem. Abstr. **100**(U.S. Patent 3,447,940): 51304a.

Halleck, F. E. (1970). "Wave set composition containing a polysaccharides." Chem. Abstr. **73**(U.S. Patent 3,507,290): 28775n.

Halliwell, B. and V. S. Butt (1974). "Oxidative decarboxylation of glycollate and glyoxylate by leaf peroxisomes." Biochem J **138**(2): 217-24.

Hamilton, I. R., P. J. Phipps and D. C. Ellwood (1979). "Effect of growth rate and glucose concentration on the biochemical properties of Streptococcus mutans Ingbritt in continuous culture." Infect Immun **26**(3): 861-9.

Hammel, K. E., M. D. Mozuch, K. A. Jensen, Jr. and P. J. Kersten (1994). "H_2O_2 recycling during oxidation of the arylglycerol beta-aryl ether lignin structure by lignin peroxidase and glyoxal oxidase." Biochemistry **33**(45): 13349-54.

Hammond, S. M., E. Bernstein, D. Beach and G. J. Hannon (2000). "An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells." Nature **404**(6775): 293-6.

Hammond, T. M. and N. P. Keller (2005). "RNA silencing in *Aspergillus nidulans* is independent of RNA-dependent RNA polymerases." Genetics **169**(2): 607-17.

Hara, S., R. F. Tsuji, O. Hatamoto and T. Masuda (2002). "A simple method for enrichment of uninucleate conidia of *Aspergillus oryzae*." Biosci Biotechnol Biochem **66**(3): 693-6.

Harris, D. M., Z. A. van der Krogt, W. M. van Gulik, J. P. van Dijken and J. T. Pronk (2007). "Formate as an auxiliary substrate for glucose-limited cultivation of *Penicillium chrysogenum*: impact on penicillin G production and biomass yield."Appl Environ Microbiol **73**(15): 5020-5.

Haynes, T. S., D. J. Klemm, J. J. Ruocco and L. L. Barton (1995). "Formate dehydrogenase activity in cells and outer membrane blebs of *Desulfovibrio gigas*." Anaerobe **1**(3): 175-82.

Hemschemeier, A., J. Jacobs and T. Happe (2008). "Biochemical and physiological characterization of the pyruvate formate-lyase Pfl1 of *Chlamydomonas reinhardtii*, a typically bacterial enzyme in a eukaryotic alga." Eukaryot Cell **7**(3): 518-26.

Henry, C., I. Mouyna and J. P. Latge (2007). "Testing the efficacy of RNA interference constructs in *Aspergillus fumigatus*." Curr Genet **51**(4): 277-84.

Herrmann, M., P. Sprote and A. A. Brakhage (2006). "Protein kinase C (*PkcA*) of *Aspergillus nidulans* is involved in penicillin production." Appl Environ Microbiol **72**(4): 2957-70.

Hinnen, A., J. B. Hicks and G. R. Fink (1978). "Transformation of yeast." Proc Natl Acad Sci U S A **75**(4): 1929-33.

Hoffmann, B., S. K. LaPaglia, E. Kubler, M. Andermann, S. E. Eckert and G. H. Braus (2000). "Developmental and metabolic regulation of the phosphoglucomutase-encoding gene, *pgmB*, of *Aspergillus nidulans*." Mol Gen Genet **262**(6): 1001-11.

Hood, E. E., G. L. Helmer, R. T. Fraley and M. D. Chilton (1986). "The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA." J Bacteriol **168**(3): 1291-301.

Hourton-Cabassa, C., F. Ambard-Bretteville, F. Moreau, J. Davy de Virville, R. Remy and C. C. Francs-Small (1998). "Stress Induction of Mitochondrial Formate Dehydrogenase in Potato Leaves." Plant Physiol **116**(2): 627-35.

Huang, X., X. L. Huang, W. Xiao, J. T. Zhao, X. M. Dai, Y. F. Chen and X. J. Li (2007). "Highly efficient *Agrobacterium*-mediated transformation of embryogenic cell suspensions of Musa acuminata cv. Mas (AA) via a liquid co-cultivation system." Plant Cell Rep **26**(10): 1755-62.

Inoue, H., H. Nojima and H. Okayama (1990). "High efficiency transformation of *Escherichia coli* with plasmids." Gene **96**(1): 23-8.

Isaacs, F. J., D. J. Dwyer and J. J. Collins (2006). "RNA synthetic biology." Nat Biotechnol **24**(5): 545-54.

Ishibashi, K., K. Suzuki, Y. Ando, C. Takakura and H. Inoue (2006). "Nonhomologous chromosomal integration of foreign DNA is completely dependent on MUS-53 (human Lig4 homolog) in *Neurospora*." Proc Natl Acad Sci U S A **103**(40): 14871-6.

Ishii, N., M. Robert, Y. Nakayama, A. Kanai and M. Tomita (2004). "Toward large-scale modeling of the microbial cell for computer simulation." J Biotechnol **113**(1-3): 281-94.

Jain, A., Y. Gupta and S. K. Jain (2007). "Perspectives of biodegradable natural polysaccharides for site-specific drug delivery to the colon." J Pharm Pharm Sci **10**(1): 86-128.

Jem, M. (1974). *Corticium rolfsii*. Commonwealth Mycological Institute description of pathogenic fungi and bacteria. CommonweaLTH, Commonwealth Mycological Institute, Kew. **41**.

Jones, A. R., M. Miller, R. Aebersold, R. Apweiler, C. A. Ball, A. Brazma, J. Degreef, N. Hardy, H. Hermjakob, S. J. Hubbard, P. Hussey, M. Igra, H. Jenkins, R. K. Julian, Jr., K. Laursen, S. G. Oliver, N. W. Paton, S. A. Sansone, U. Sarkans, C. J. Stoeckert, Jr., C. F. Taylor, P. L. Whetzel, J. A. White, P. Spellman and A. Pizarro (2007). "The Functional Genomics Experiment model (FuGE): an extensible framework for standards in functional genomics." Nat Biotechnol **25**(10): 1127-33.

Jones, M. G. (2007). "The first filamentous fungal genome sequences: *Aspergillus* leads the way for essential everyday resources or dusty museum specimens?" Microbiology **153**(Pt 1): 1-6.

Jong, S. C. and R. Donovick (1989). "Antitumor and antiviral substances from fungi." Adv Appl Microbiol **34**: 183-262.

Joosten, H. J., Y. Han, W. Niu, J. Vervoort, D. Dunaway-Mariano and P. J. Schaap (2008). "Identification of fungal oxaloacetate hydrolyase within the isocitrate lyase/PEP mutase enzyme superfamily using a sequence marker-based method." Proteins **70**(1): 157-66.

Kadotani, N., H. Nakayashiki, Y. Tosa and S. Mayama (2003). "RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*." Mol Plant Microbe Interact **16**(9): 769-76.

Kadotani, N., H. Nakayashiki, Y. Tosa and S. Mayama (2004). "One of the two Dicer-like proteins in the filamentous fungi *Magnaporthe oryzae* genome is responsible for hairpin RNA-triggered RNA silencing and related small interfering RNA accumulation." J Biol Chem **279**(43): 44467-74.

Karaoglu, D., D. J. Kelleher and R. Gilmore (1995). "Functional characterization of Ost3p. Loss of the 34-kD subunit of the *Saccharomyces cerevisiae* oligosaccharyltransferase results in biased underglycosylation of acceptor substrates." J Cell Biol **130**(3): 567-77.

Karnaukhova, E., Y. Ophir, L. Trinh, N. Dalal, P. J. Punt, B. Golding and J. Shiloach (2007). "Expression of human alpha1-proteinase inhibitor in *Aspergillus niger*." Microb Cell Fact **6**: 34.

Kaufman, G., B. A. Horwitz, R. Hadar, Y. Ullmann and I. Berdicevsky (2004). "Green fluorescent protein (GFP) as a vital marker for pathogenic development of the dermatophyte *Trichophyton mentagrophytes*." Microbiology **150**(Pt 8): 2785-90.

Kelkar, H. S., V. Shankar and M. V. Desphande (1990). "Rapid isolation and regeneration of *Sclerotium rolfsii* protoplasts and their potential application for starch hydrolysis." Enzyme and microbial technology **12**(7): 510-514.

Kelleher, D. J. and R. Gilmore (1994). "The *Saccharomyces cerevisiae* oligosaccharyltransferase is a protein complex composed of Wbp1p, Swp1p, and four additional polypeptides." J Biol Chem **269**(17): 12908-17.

Kersten, P. and D. Cullen (2007). "Extracellular oxidative systems of the lignin-degrading Basidiomycete *Phanerochaete chrysosporium*." Fungal Genet Biol **44**(2): 77-87.

Kersten, P. J. and T. K. Kirk (1987). "Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*." J Bacteriol **169**(5): 2195-201.

Kesarwani, M., M. Azam, K. Natarajan, A. Mehta and A. Datta (2000). "Oxalate decarboxylase from *Collybia velutipes*. Molecular cloning and its overexpression to confer resistance to fungal infection in transgenic tobacco and tomato." J Biol Chem **275**(10): 7230-8.

Khalaj, V., J. L. Brookman and G. D. Robson (2001). "A study of the protein secretory pathway of *Aspergillus niger* using a glucoamylase-GFP fusion protein." Fungal Genet Biol **32**(1): 55-65.

Khalaj, V., H. Eslami, M. Azizi, N. Rovira-Graells and M. Bromley (2007). "Efficient downregulation of *alb1* gene using an AMA1-based episomal expression of RNAi construct in *Aspergillus fumigatus*." FEMS Microbiol Lett **270**(2): 250-4.

Khatri, M. and M. V. Rajam (2007). "Targeting polyamines of *Aspergillus nidulans* by siRNA specific to fungal ornithine decarboxylase gene." Med Mycol **45**(3): 211-20.

Kim, H. M., S. Y. Paik, K. S. Ra, K. B. Koo, J. W. Yun and J. W. Choi (2006). "Enhanced production of exopolysaccharides by fed-batch culture of *Ganoderma resinaceum* DG-6556." J Microbiol **44**(2): 233-42.

Kim, K. S., J. Y. Min and M. B. Dickman (2008a). "Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development." Mol Plant Microbe Interact **21**(5): 605-12.

Kim, Y., M. P. Nandakumar and M. R. Marten (2007). "Proteomics of filamentous fungi." Trends Biotechnol **25**(9): 395-400.

Kim, Y., M. P. Nandakumar and M. R. Marten (2008b). "The state of proteome profiling in the fungal genus *Aspergillus*." Brief Funct Genomic Proteomic.

Kimmel, S. A., R. F. Roberts and G. R. Ziegler (1998). "Optimization of exopolysaccharide production by Lacto*bacillus delbrueckii* subsp. bulgaricus RR grown in a semidefined medium." Appl Environ Microbiol **64**(2): 659-64.

Kitamoto, N., S. Yoshino, K. Ohmiya and N. Tsukagoshi (1999). "Sequence analysis, overexpression, and antisense inhibition of a beta-xylosidase gene, xylA, from *Aspergillus oryzae* KBN616." Appl Environ Microbiol **65**(1): 20-4.

Klein, R. M., E. D. Wolf, R. Wu and J. C. Sanford (1992). "High-velocity microprojectiles for delivering nucleic acids into living cells. 1987." Biotechnology **24**: 384-6.

Kobayashi, T., K. Abe, K. Asai, K. Gomi, P. R. Juvvadi, M. Kato, K. Kitamoto, M. Takeuchi and M. Machida (2007). "Genomics of *Aspergillus oryzae*." Biosci Biotechnol Biochem **71**(3): 646-70.

Kooistra, R., P. J. Hooykaas and H. Y. Steensma (2004). "Efficient gene targeting in *Kluyveromyces lactis*." Yeast **21**(9): 781-92.

Korf, I., P. Flicek, D. Duan and M. R. Brent (2001). "Integrating genomic homology into gene structure prediction." Bioinformatics **17 Suppl 1**: S140-8.

Koukaki, M., E. Giannoutsou, A. Karagouni and G. Diallinas (2003). "A novel improved method for *Aspergillus nidulans* transformation." J Microbiol Methods **55**(3): 687-95.

Kouskoumvekaki, I., Z. Yang, S. O. Jonsdottir, L. Olsson and G. Panagiotou (2008). "Identification of biomarkers for genotyping Aspergilli using non-linear methods for clustering and classification." BMC Bioinformatics **9**: 59.

Kramer, C., J. J. Loros, J. C. Dunlap and S. K. Crosthwaite (2003). "Role for antisense RNA in regulating circadian clock function in *Neurospora crassa*." Nature **421**(6926): 948-52.

Krappmann, S., C. Sasse and G. H. Braus (2006). "Gene targeting in *Aspergillus fumigatus* by homologous recombination is facilitated in a nonhomologous end- joining-deficient genetic background." Eukaryot Cell **5**(1): 212-5.

Kritzman, G., Y. Okon, I. Chet and Y. Henis (1976). "Metabolism of L-threonine and its relationship to *sclerotium* formation in *Sclerotium rolfsii*." J Gen Microbiol **95**(1): 78-86.

Kritzman, G., I. Chet and I. Henis (1977). "The Role of Oxalic Acid in the Pathogenic Behavior of *Sclerotium rolfsii* Sacc." Experimental Mycology **1**: 280-285.

Krogh, B. O. and L. S. Symington (2004). "Recombination proteins in yeast." Annu Rev Genet **38**: 233-71.

Kuan, I. C. and M. Tien (1993). "Glyoxylate-supported reactions catalyzed by Mn peroxidase of Phanerochaete chrysosporium: activity in the absence of added hydrogen peroxide." Arch Biochem Biophys **302**(2): 447-54.

Kües, S. K. a. U. (2005). "Comparison of gpd genes and their protein products in basidiomycetes." Fungal Genetics Newsletter **52**: 18-23.

Kukuruzinska, M. A. and P. W. Robbins (1987). "Protein glycosylation in yeast: transcript heterogeneity of the ALG7 gene." Proc Natl Acad Sci U S A **84**(8): 2145-9.

Kummel, A., S. Panke and M. Heinemann (2006). "Systematic assignment of thermodynamic constraints in metabolic network models." BMC Bioinformatics **7**: 512.

Kuwahara, J. and Y. Sugiura (1988). "Sequence-specific recognition and cleavage of DNA by metallobleomycin: minor groove binding and possible interaction mode." Proc Natl Acad Sci U S A **85**(8): 2459-63.

Kuwazaki, S., N. Takaya, A. Nakamura and H. Shoun (2003). "Formate-forming fungal catabolic pathway to supply electrons to nitrate respiration." Biosci Biotechnol Biochem **67**(4): 937-9.

Lan, X., Z. Yao, Y. Zhou, J. Shang, H. Lin, D. L. Nuss and B. Chen (2008). "Deletion of the c*pku*80 gene in the chestnut blight fungus, *Cryphonectria parasitica*, enhances gene disruption efficiency." Curr Genet **53**(1): 59-66.

Lane, B. G., J. M. Dunwell, J. A. Ray, M. R. Schmitt and A. C. Cuming (1993). "Germin, a protein marker of early plant development, is an oxalate oxidase." J Biol Chem **268**(17): 12239-42.

Larraya, L. M., K. J. Boyce, A. So, B. R. Steen, S. Jones, M. Marra and J. W. Kronstad (2005). "Serial analysis of gene expression reveals conserved links between protein kinase A, ribosome biogenesis, and phosphate metabolism in *Ustilago maydis*." Eukaryot Cell **4**(12): 2029-43.

Le Lay, P., M. P. Isaure, J. E. Sarry, L. Kuhn, B. Fayard, J. L. Le Bail, O. Bastien, J. Garin, C. Roby and J. Bourguignon (2006). "Metabolomic, proteomic and biophysical analyses of *Arabidopsis thaliana* cells exposed to a caesium stress. Influence of potassium supply." Biochimie **88**(11): 1533-47.

Lee, D. G., I. Nishimura-Masuda, A. Nakamura, M. Hidaka, H. Masaki and T. Uozumi (1998). "Overproduction of alpha-glucosidase in *Aspergillus niger* transformed with the cloned gene aglA." J Gen Appl Microbiol **44**(3): 177-181.

Lee, K. (1998). Characterization of Scletroglucan Fermentation by *Sclerotium rolfsii* in terms of Cell, Scleroglucan and by-product, Oxalic Acid Concentrations, Viscosity and Molecular Weight Distribution. Biochemical Engineering. Irvine, California. Doctor of Philosophy: 222.

Lee, M. H. and R. M. Bostock (2006). "*Agrobacterium* T-DNA-mediated integration and gene replacement in the brown rot pathogen *Monilinia fructicola*." Curr Genet **49**(5): 309-22.

Lennarz, W. J. (2007). "Studies on oligosaccharyl transferase in yeast." Acta Biochim Pol **54**(4):673-7.

Lieske, J. C., D. S. Goldfarb, C. De Simone and C. Regnier (2005). "Use of a probiotic to decrease enteric hyperoxaluria." Kidney Int **68**(3): 1244-9.

Lindqvist, Y. and C. I. Branden (1980). "Structure of glycolate oxidase from spinach at a resolution of 5.5 A." J Mol Biol **143**(2): 201-11.

Lindqvist, Y. and C. I. Branden (1985). "Structure of glycolate oxidase from spinach." Proc Natl Acad Sci U S A **82**(20): 6855-6859.

Lindqvist, Y. and C. I. Branden (1989). "The active site of spinach glycolate oxidase." J Biol Chem **264**(6): 3624-8.

Lindqvist, Y. (1989). "Refined structure of spinach glycolate oxidase at 2 A resolution." J Mol Biol **209**(1): 151-66.

Lindqvist, Y., C. I. Branden, F. S. Mathews and F. Lederer (1991). "Spinach glycolate oxidase and yeast flavocytochrome b2 are structurally homologous and evolutionarily related enzymes with distinctly different function and flavin mononucleotide binding." J Biol Chem **266**(5): 3198-207.

Loftus, B. (2003). Genome Sequencing, Assembly and Gene Prediction in Fungi. Amsterdam, Elsevier.

Lombrana, M., F. J. Moralejo, R. Pinto and J. F. Martin (2004). "Modulation of *Aspergillus awamori* thaumatin secretion by modification of bipA gene expression." Appl Environ Microbiol **70**(9): 5145-52.

Lopez-Romero, E. and J. Ruiz-Herrera (1978). "Properties of beta-glucan synthetase from *Saccharomyces cerevisiae*." Antonie Van Leeuwenhoek **44**(3-4): 329-39.

Lopez, M. and L. Edens (2005). "Effective prevention of chill-haze in beer using an acid proline-specific endoprotease from *Aspergillus niger*." J Agric Food Chem **53**(20): 7944-9.

Lu, S., L. Lyngholm, G. Yang, C. Bronson, O. C. Yoder and B. G. Turgeon (1994). "Tagged mutations at the Tox1 locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration." Proc Natl Acad Sci U S A **91**(26): 12649-53.

Lugones, L. G., K. Scholtmeijer, R. Klootwijk and J. G. Wessels (1999). "Introns are necessary for mRNA accumulation in *Schizophyllum commune*." Mol Microbiol **32**(4): 681-9.

Ma, B., M. B. Mayfield and M. H. Gold (2001). "The green fluorescent protein gene functions as a reporter of gene expression in *Phanerochaete chrysosporium*." Appl Environ Microbiol **67**(2): 948-55.

Machida, M., K. Asai, M. Sano, T. Tanaka, T. Kumagai, G. Terai, K. Kusumoto, T. Arima, O. Akita, Y. Kashiwagi, K. Abe, K. Gomi, H. Horiuchi, K. Kitamoto, T. Kobayashi, M. Takeuchi, D. W. Denning, J. E. Galagan, W. C. Nierman, J. Yu, D. B. Archer, J. W. Bennett, D. Bhatnagar, T. E. Cleveland, N. D. Fedorova, O. Gotoh, H. Horikawa, A. Hosoyama, M. Ichinomiya, R. Igarashi, K. Iwashita, P. R. Juvvadi, M. Kato, Y. Kato, T. Kin, A. Kokubun, H. Maeda, N. Maeyama, J. Maruyama, H. Nagasaki, T. Nakajima, K. Oda, K. Okada, I. Paulsen, K. Sakamoto, T. Sawano, M. Takahashi, K. Takase, Y. Terabayashi, J. R. Wortman, O. Yamada,

Y. Yamagata, H. Anazawa, Y. Hata, Y. Koide, T. Komori, Y. Koyama, T. Minetoki, S. Suharnan, A. Tanaka, K. Isono, S. Kuhara, N. Ogasawara and H. Kikuchi (2005). "Genome sequencing and analysis of *Aspergillus oryzae*." Nature **438**(7071): 1157-61.

MacKenzie DA, J. D., Archer DB (2004). Filamentous fungi as expression systems for heterologous proteins, Springer-Verlag.

Maeda, H., M. Sano, Y. Maruyama, T. Tanno, T. Akao, Y. Totsuka, M. Endo, R. Sakurada, Y. Yamagata, M. Machida, O. Akita, F. Hasegawa, K. Abe, K. Gomi, T. Nakajima and Y. Iguchi (2004). "Transcriptional analysis of genes for energy catabolism and hydrolytic enzymes in the filamentous fungus *Aspergillus oryzae* using cDNA microarrays and expressed sequence tags." Appl Microbiol Biotechnol **65**(1): 74-83.

Maheshwari, R. (2005). "Nuclear behavior in fungal hyphae." FEMS Microbiol Lett **249**(1): 7-14.

Malonek, S. and F. Meinhardt (2001). "*Agrobacterium tumefaciens*-mediated genetic transformation of the phytopathogenic ascomycete *Calonectria morganii*." Curr Genet **40**(2): 152-5.

Maria, R., and Sullia, S. B. (2008). "Acquired resistance of *Alternaria solani* and *Sclerotium rolfsii* to Polyoxin-D." Journal of Phytopathology **116**(1): 60-66.

Martinez, D., R. M. Berka, B. Henrissat, M. Saloheimo, M. Arvas, S. E. Baker, J. Chapman, O. Chertkov, P. M. Coutinho, D. Cullen, E. G. Danchin, I. V. Grigoriev, P. Harris, M. Jackson, C. P. Kubicek, C. S. Han, I. Ho, L. F. Larrondo, A. L. de Leon, J. K. Magnuson, S. Merino, M. Misra, B. Nelson, N. Putnam, B. Robbertse, A. A. Salamov, M. Schmoll, A. Terry, N. Thayer, A. Westerholm-Parvinen, C. L. Schoch, J. Yao, R. Barbote, M. A. Nelson, C. Detter, D. Bruce, C. R. Kuske, G. Xie, P. Richardson, D. S. Rokhsar, S. M. Lucas, E. M. Rubin, N. Dunn-Coleman, M. Ward and T. S. Brettin (2008). "Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*)." Nat Biotechnol **26**(5): 553-60.

Martins, A. M., W. Sha, C. Evans, S. Martino-Catt, P. Mendes and V. Shulaev (2007). "Comparison of sampling techniques for parallel analysis of transcript and metabolite levels in *Saccharomyces cerevisiae*." Yeast **24**(3): 181-8.

Mashayekhi, F. and M. Ronaghi (2007). "Analysis of read length limiting factors in Pyrosequencing chemistry." Anal Biochem **363**(2): 275-87.

Mastromarino, P., R. Petruzziello, S. Macchia, S. Rieti, R. Nicoletti and N. Orsi (1997). "Antiviral activity of natural and semisynthetic polysaccharides on the early steps of rubella virus infection." J Antimicrob Chemother **39**(3): 339-45.

Matsumura, H., A. Ito, H. Saitoh, P. Winter, G. Kahl, M. Reuter, D. H. Kruger and R. Terauchi (2005). "SuperSAGE." Cell Microbiol **7**(1): 11-8.

Matsuzaki, F., M. Shimizu and H. Wariishi (2008). "Proteomic and metabolomic analyses of the white-rot fungus *Phanerochaete chrysosporium* exposed to exogenous benzoic acid." J Proteome Res **7**(6): 2342-50.

Maxwell, D. P. and D. F. Bateman (1965). "Influence of carbon source and pH on oxalate accumulation in culture filtrates of *S. rolfsii*." Phytopathology **58**: 1351-1355.

Mazur, P. and W. Baginsky (1996). "In vitro activity of 1,3-beta-D-glucan synthase requires the GTP-binding protein Rho1." J Biol Chem **271**(24): 14604-9.

McDonald, T., D. Brown, N. P. Keller and T. M. Hammond (2005). "RNA silencing of mycotoxin production in *Aspergillus* and Fusarium species." Mol Plant Microbe Interact **18**(6): 539-45.

Mehta, A. and A. Datta (1991). "Oxalate decarboxylase from *Collybia velutipes*. Purification, characterization, and cDNA cloning." J Biol Chem **266**(35): 23548-53.

Mendes, B. M. a. P. (2006). Bioinformatics Approaches to Integrate Metabolomics and Other Systems Biology Data, Springer Berlin Heidelberg.

Meyer, F., A. Goesmann, A. C. McHardy, D. Bartels, T. Bekel, J. Clausen, J. Kalinowski, B. Linke, O. Rupp, R. Giegerich and A. Puhler (2003). "GenDB an open source genome annotation system for prokaryote genomes." Nucleic Acids Res **31**(8): 2187-95.

Meyer, V. (2008). "Genetic engineering of filamentous fungi--progress, obstacles and future trends." Biotechnol Adv **26**(2): 177-85.

Meyer, V., M. Arentshorst, A. El-Ghezal, A. C. Drews, R. Kooistra, C. A. van den Hondel and A. F. Ram (2007). "Highly efficient gene targeting in the *Aspergillus niger kusA* mutant." J Biotechnol **128**(4): 770-5.

Meyer, V., M. Arentshorst, C. A. van den Hondel and A. F. Ram (2008). "The polarisome component SpaA localises to hyphal tips of *Aspergillus niger* and is important for polar growth." Fungal Genet Biol **45**(2): 152-64.

Meyer, V., D. Mueller, T. Strowig and U. Stahl (2003b). "Comparison of different transformation methods for *Aspergillus giganteus*." Curr Genet **43**(5): 371-7.

Meyer, V., M. Wedde and U. Stahl (2002). "Transcriptional regulation of the Antifungal Protein in *Aspergillus giganteus*." Mol Genet Genomics **266**(5): 747-57.

Micales, J. A. (1995). "Oxalate decarboxylase in the brown-rot wood decay fungus *Postia placenta*." Material und Organismen **29**: 177-186.

Micales, J. A. (1997). "Localization and Induction of Oxalate Decarboxylase in the Brown-Rot Wood Decay Fungus *Postia placenta*." International Biodeterioration & Biodegradation **39**(2-3): 125-132.

Michielse, C. B., M. Arentshorst, A. F. Ram and C. A. van den Hondel (2005a). "*Agrobacterium*-mediated transformation leads to improved gene replacement efficiency in *Aspergillus awamori*." Fungal Genet Biol **42**(1): 9-19.

Michielse, C. B., P. J. Hooykaas, C. A. van den Hondel and A. F. Ram (2005b). "*Agrobacterium*-mediated transformation as a tool for functional genomics in fungi." Curr Genet **48**(1): 1-17.

Migheli, Q., Friard, O., Tedesco, D., Del Musso ,M., M. R., Gullino, L. (2008). "Stability of transformed antagonistic *Fusarium oxysporum* strains in vitro and in soil microcosms." Molecular Ecology **5**(5): 641-649.

Mikio Shimada, Y. A., Toshiaki Tokimatsu, Kayoko Mii, Takefumi Hattori (1997). "Possible biochemical roles of oxalic acid as a low molecular weight compund involved in brown-rot and white-rot wood decays." Jounal of Biotechnology **53**: 103-113.

Monfort, A., L. Cordero, S. Maicas and J. Polaina (2003). "Transformation of Mucor miehei results in plasmid deletion and phenotypic instability." FEMS Microbiol Lett **224**(1): 101-6.

Moralejo, F. J., R. E. Cardoza, S. Gutierrez, M. Lombrana, F. Fierro and J. F. Martin (2002). "Silencing of the aspergillopepsin B (pepB) gene of *Aspergillus* awamori by antisense RNA expression or protease removal by gene disruption results in a large increase in thaumatin production." Appl Environ Microbiol **68**(7): 3550-9.

Morrison, N., G. Cochrane, N. Faruque, T. Tatusova, Y. Tateno, D. Hancock and D. Field (2006). "Concept of sample in OMICS technology." Omics **10**(2): 127-37.

Mouyna, I., C. Henry, T. L. Doering and J. P. Latge (2004). "Gene silencing with RNA interference in the human pathogenic fungus *Aspergillus fumigatus*." FEMS Microbiol Lett **237**(2): 317-24.

Mueller, D., U. Stahl and V. Meyer (2006). "Application of hammerhead ribozymes in filamentous fungi." J Microbiol Methods **65**(3): 585-95.

Mullins, E. D., X. Chen, P. Romaine, R. Raina, D. M. Geiser and S. Kang (2001a). "*Agrobacterium*-Mediated Transformation of *Fusarium oxysporum*: An Efficient Tool for Insertional Mutagenesis and Gene Transfer." Phytopathology **91**(2): 173-180.

Mullins, E. D. and S. Kang (2001b). "Transformation: a tool for studying fungal pathogens of plants." Cell Mol Life Sci **58**(14): 2043-52.

Munir, E., J. J. Yoon, T. Tokimatsu, T. Hattori and M. Shimada (2001a). "New role for glyoxylate cycle enzymes in wood-rotting basidiomycetes in relation to biosynthesis of oxalic acid." J. Wood Sci. 2001 **47**(5): 368-73.

Munir, E., J. J. Yoon, T. Tokimatsu, T. Hattori and M. Shimada (2001b). "A physiological role for oxalic acid biosynthesis in the wood-rotting basidiomycete *Fomitopsis palustris*." Proc Natl Acad Sci U S A **98**(20): 11126-30.

Nakayashiki, H. (2005). "RNA silencing in fungi: mechanisms and applications." FEBS Lett **579**(26): 5950-7.

Nakayashiki, H., S. Hanada, B. Q. Nguyen, N. Kadotani, Y. Tosa and S. Mayama (2005). "RNA silencing as a tool for exploring gene function in ascomycete fungi." Fungal Genet Biol **42**(4): 275-83.

Namekawa, S. H., K. Iwabata, H. Sugawara, F. N. Hamada, A. Koshiyama, H. Chiku, T. Kamada and K. Sakaguchi (2005). "Knockdown of LIM15/DMC1 in the mushroom *Coprinus cinereus* by double-stranded RNA-mediated gene silencing." Microbiology **151**(Pt 11): 3669-78.

Nayak, T., E. Szewczyk, C. E. Oakley, A. Osmani, L. Ukil, S. L. Murray, M. J. Hynes, S. A. Osmani and B. R. Oakley (2006). "A versatile and efficient gene-targeting system for *Aspergillus nidulans*." Genetics **172**(3): 1557-66.

Nevalainen, K. M., V. S. Te'o and P. L. Bergquist (2005). "Heterologous protein expression in filamentous fungi." Trends Biotechnol **23**(9): 468-74.

Ngiam, C., D. J. Jeenes, P. J. Punt, C. A. Van Den Hondel and D. B. Archer (2000). "Characterization of a foldase, protein disulfide isomerase A, in the protein secretory pathway of *Aspergillus niger*." Appl Environ Microbiol **66**(2): 775-82.

Nguyen, Q. B., N. Kadotani, S. Kasahara, Y. Tosa, S. Mayama and H. Nakayashiki (2008). "Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, *Magnaporthe oryzae*, using a high-throughput RNA-silencing system." Mol Microbiol **68**(6): 1348-65.

Nielsen, J. B., M. L. Nielsen and U. H. Mortensen (2008). "Transient disruption of non-homologous end-joining facilitates targeted genome manipulations in the filamentous fungus *Aspergillus nidulans*." Fungal Genet Biol **45**(3): 165-70.

Nierman, W. C., A. Pain, M. J. Anderson, J. R. Wortman, H. S. Kim, J. Arroyo, M. Berriman, K. Abe, D. B. Archer, C. Bermejo, J. Bennett, P. Bowyer, D. Chen, M. Collins, R. Coulsen, R. Davies, P. S. Dyer, M. Farman, N. Fedorova, N. Fedorova, T. V. Feldblyum, R. Fischer, N. Fosker, A. Fraser, J. L. Garcia, M. J. Garcia, A. Goble, G. H. Goldman, K. Gomi, S. Griffith-Jones, R. Gwilliam, B. Haas, H. Haas, D. Harris, H. Horiuchi, J. Huang, S. Humphray, J. Jimenez, N. Keller, H. Khouri, K. Kitamoto, T. Kobayashi, S. Konzack, R. Kulkarni, T. Kumagai, A. Lafon, J. P. Latge, W. Li, A. Lord, C. Lu, W. H. Majoros, G. S. May, B. L. Miller, Y. Mohamoud, M. Molina, M. Monod, I. Mouyna, S. Mulligan, L. Murphy, S. O'Neil, I. Paulsen, M. A. Penalva, M. Pertea, C. Price, B. L. Pritchard, M. A. Quail, E. Rabbinowitsch, N. Rawlins, M. A. Rajandream, U. Reichard, H. Renauld, G. D. Robson, S. Rodriguez de Cordoba, J. M. Rodriguez-Pena, C. M. Ronning, S. Rutter, S. L. Salzberg, M. Sanchez, J. C. Sanchez-Ferrero, D. Saunders, K. Seeger, R. Squares, S. Squares, M. Takeuchi, F. Tekaia, G. Turner, C. R. Vazquez de Aldana, J. Weidman, O. White, J. Woodward, J. H. Yu, C. Fraser, J. E. Galagan, K. Asai, M. Machida, N. Hall, B. Barrell and D. W. Denning (2005). "Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*." Nature **438**(7071): 1151-6.

Ninomiya, Y., K. Suzuki, C. Ishii and H. Inoue (2004). "Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining." Proc Natl Acad Sci U S A **101**(33): 12248-53.

Nyilasi, I., K. Acs, T. Papp, E. Nagy and C. Vagvolgyi (2005). "*Agrobacterium tumefaciens*-mediated transformation of Mucor circinelloides." Folia Microbiol (Praha) **50**(5): 415-20.

Nyilasi, I., T. Papp, A. Csernetics and C. Vagvolgyi (2008). "*Agrobacterium tumefaciens* -mediated transformation of the zygomycete fungus *Backusella lamprospora*." J Basic Microbiol **48**(1): 59-64.

O'Brien, H. E., J. L. Parrent, J. A. Jackson, J. M. Moncalvo and R. Vilgalys (2005). "Fungal community analysis by large-scale sequencing of environmental samples." Appl Environ Microbiol **71**(9): 5544-50.

Ogawa, Y. and M. Mii (2001). "Ti- and cryptic-plasmid-borne virulence of wild-type *Agrobacterium tumefaciens* strain CNI5 isolated from chrysanthemum (*Dendranthema grandiflora Tzvelev*)." Arch Microbiol **176**(5): 315-22.

Ohnishi, J., S. Mitsuhashi, M. Hayashi, S. Ando, H. Yokoi, K. Ochiai and M. Ikeda (2002). "A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant." Appl Microbiol Biotechnol **58**(2): 217-23.

Oliver, D. J. (1981). "Formate Oxidation and Oxygen Reduction by Leaf Mitochondria." Plant Physiol **68**(3): 703-705.

Oliver, S. G., M. K. Winson, D. B. Kell and F. Baganz (1998). "Systematic functional analysis of the yeast genome." Trends Biotechnol **16**(9): 373-8.

Orbach, M. J., E. B. Porro and C. Yanofsky (1986). "Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker." Mol Cell Biol **6**(7): 2452-61.

Orlean, P. A. (1982). "(1,3)-beta-D-Glucan synthase from budding and filamentous cultures of the dimorphic fungus *Candida albicans*." Eur J Biochem **127**(2): 397-403.

Ouriaghli, T. E., Francois, J., D. Sarazin, N.T. Dinh (1992). "Influence of nonionic surfactant on aggregation state of scleroglucan in aqueous solution." Carbohydr. Polym. **17**: 305–312.

Overkamp, K. M., B. M. Bakker, H. Y. Steensma, J. P. van Dijken and J. T. Pronk (2002). "Two mechanisms for oxidation of cytosolic NADPH by *Kluyveromyces lactis* mitochondria." Yeast **19**(10): 813-24.

Ozaki, H. and I. Shiio (1968). "Regulation of the TCA and glyoxylate cycles in Brevibacterium flavum. I. Ingibition of isocitrate lyase and isocitrate dehydrogenase by organic acids related to the TCA and glyoxylate cycles." J Biochem **64**(3): 355-63.

Pabuccuoglu, U. (2005). "Aspects of oxalosis associated with aspergillosis in pathology specimens." Pathol Res Pract **201**(5): 363-8.

Paintin, R. D. (1928). "Notes on the Parasitology of *Sclerotium Rolfsi* " Mycologia **20**(1): 22-26.

Paoletti, M., C. Rydholm, E. U. Schwier, M. J. Anderson, G. Szakacs, F. Lutzoni, J. P. Debeaupuis, J. P. Latge, D. W. Denning and P. S. Dyer (2005). "Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus*." Curr Biol **15**(13): 1242-8.

Papagianni, M., M. Mattey and B. Kristiansen (2003). "Design of a tubular loop bioreactor for scale-up and scale-down of fermentation processes." Biotechnol Prog **19**(5): 1498-504.

Patchen, L. B., P. (2000). "Mobilisation of peripheral blood precursor cells by beta (1,3)-glucan." The Collaborative Group, Ltd. (New York, USA) 2000, 6,117,850. US patent?

Patnaik, D. and P. Khurana (2001). "Germins and germin like proteins: an overview." Indian J Exp Biol **39**(3): 191-200.

Pedersen, H., C. Hjort and J. Nielsen (2000). "Cloning and characterization of *oah*, the gene encoding oxaloacetate hydrolase in *Aspergillus niger*." Mol Gen Genet **263**(2): 281-6.

Pel, H. J., J. H. de Winde, D. B. Archer, P. S. Dyer, G. Hofmann, P. J. Schaap, G. Turner, R. P. de Vries, R. Albang, K. Albermann, M. R. Andersen, J. D. Bendtsen, J. A. Benen, M. van den Berg, S. Breestraat, M. X. Caddick, R. Contreras, M. Cornell, P. M. Coutinho, E. G. Danchin, A. J. Debets, P. Dekker, P. W. van Dijck, A. van Dijk, L. Dijkhuizen, A. J. Driessen, C. d'Enfert, S. Geysens, C. Goosen, G. S. Groot, P. W. de Groot, T. Guillemette, B. Henrissat, M. Herweijer, J. P. van den Hombergh, C. A. van den Hondel, R. T. van der Heijden, R. M. van der Kaaij, F. M. Klis, H. J. Kools, C. P. Kubicek, P. A. van Kuyk, J. Lauber, X. Lu, M. J. van der Maarel, R. Meulenberg, H. Menke, M. A. Mortimer, J. Nielsen, S. G. Oliver, M. Olsthoorn, K. Pal, N. N. van Peij, A. F. Ram, U. Rinas, J. A. Roubos, C. M. Sagt, M. Schmoll, J. Sun, D. Ussery, J. Varga, W. Vervecken, P. J. van de Vondervoort, H. Wedler, H. A. Wosten, A. P. Zeng, A. J. van Ooyen, J. Visser and H. Stam (2007). "Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88." Nat Biotechnol **25**(2): 221-31.

Pettinger, R., Wolfe, RN., Hoehn, MM., Marks, PN., Dailey, WA., McGuire, JM. (1953). "Hygromycin I. Preliminary studies on the production and biological activity of a new antibiotic." Antibio. Chemother **3**: 1268-1278.

Pilz, F., Auling, Georg, Stephan, Dörthe, Rau, Udo and Wagner, Fritz (1991). "A High-Affinity Zn^{2+} Uptake System Controls Growth an Biosynthesis of an Extracellular, Branched p-1,3-(3-1,6-Glue *Sclerotium rolfsii* ATCC 15205." Experimental Mycology **15**: 181-192.

Pirog, T. P., M. A. Kovalenko and V. Kuz'minskaia Iu (2003). "Intensification of the exopolysaccharide synthesis by *Acinetobacter* sp. on an ethanol-glucose mixture: aspects related to biochemistry and bioenergetics." Mikrobiologiia **72**(3): 348-55.

Pirri, R. G. (1996). "Scleroglucan gel applied to the oil industry." S. N. E. Aquitaine. (France. Patent 5,555,936.).

Poggeler, S. and U. Kuck (2006). "Highly efficient generation of signal transduction knockout mutants using a fungal strain deficient in the mammalian ku70 ortholog." Gene **378**: 1-10.

Prets, H. E., H.; McNamee, R.; Jones, E.; Browder, I.; Williams, D. (1991). "Isolation, physicochemical characterisation and pre-clinical efficiacy evaluation of a soluble Scleroglucan." J. Pharmacol. Exp. Ther. **257**: 500.

Promega, C. (1996). Protocols and Applications Guide. Third Edition

Punt, P. J., R. P. Oliver, M. A. Dingemanse, P. H. Pouwels and C. A. van den Hondel (1987). "Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*." Gene **56**(1): 117-24.

Punt, P. J., N. van Biezen, A. Conesa, A. Albers, J. Mangnus and C. van den Hondel (2002). "Filamentous fungi as cell factories for heterologous protein production." Trends Biotechnol **20**(5): 200-6.

Raes, J., K. U. Foerstner and P. Bork (2007). "Get the most out of your metagenome: computational analysis of environmental sequence data." Curr Opin Microbiol **10**(5): 490-8.

Rapp, P. (1989). "1,3-b-glucanase, 1,6-b-glucanase and b-glucosidase activities of *Sclerotium glucanicum*: Synthesis and properties." J. Gen. Microbiol. **135** 2847–2858.

Rau, U. (2004). "Glucans secreted by fungi." Turkish electronical journal of Biotechnology **2**: 30-36.

Rautio, J. J., B. A. Smit, M. Wiebe, M. Penttila and M. Saloheimo (2006). "Transcriptional monitoring of steady state and effects of anaerobic phases in chemostat cultures of the filamentous fungus *Trichoderma reesei*." BMC Genomics **7**: 247.

Rekangalt, D., M. C. Verner, U. Kues, P. J. Walser, R. Marmeisse, J. C. Debaud and L. Fraissinet-Tachet (2007). "Green fluorescent protein expression in the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*." FEMS Microbiol Lett **268**(1): 67-72.

Requena, L. and S. Bornemann (1999). "Barley (*Hordeum vulgare*) oxalate oxidase is a manganese-containing enzyme." Biochem J **343 Pt 1**: 185-90.

Rho, H. S., S. Kang and Y. H. Lee (2001). "*Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus, *Magnaporthe* grisea." Mol Cells **12**(3): 407-11.

Richardson, K. E. and N. E. Tolbert (1961). "Oxidation of glyoxylic acid to oxalic acid by glycolic acid oxidase." J Biol Chem **236**: 1280-4.

Rio, M. C., B. V. de Oliveira, D. P. de Tomazella, J. A. Silva and G. A. Pereira (2008). "Production of calcium oxalate crystals by the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom disease of Cacao." Curr Microbiol **56**(4): 363-70.

Rodgers, N. E. (1973). "Scleroglucan." Industrial Gums, Academic Press, New York, USA 499–511.

Rogic, S., B. F. Ouellette and A. K. Mackworth (2002). "Improving gene recognition accuracy by combining predictions from two gene-finding programs." Bioinformatics **18**(8): 1034-45.

Ruijter, G. J. and J. Visser (1997). "Carbon repression in *Aspergilli*." FEMS Microbiol Lett **151**(2): 103-14.

Ruiz-Diez, B. (2002). "Strategies for the transformation of filamentous fungi." J Appl Microbiol **92**(2): 189-95.

Sahin, N., K. Isik, A. U. Tamer and M. Goodfellow (2000). "Taxonomic position of "*Pseudomonas oxalaticus*" strain ox14T (DSM 1105T) (Khambata and Bhat, 1953) and its description in the genus Ralstonia as Ralstonia oxalatica comb. nov." Syst Appl Microbiol **23**(2): 206-9.

Sakai, S., T. Nishide, E. Munir, K. Baba, H. Inui, Y. Nakano, T. Hattori and M. Shimada (2006). "Subcellular localization of glyoxylate cycle key enzymes involved in oxalate biosynthesis of wood-destroying basidiomycete *Fomitopsis palustris* grown on glucose." Microbiology **152**(Pt 6): 1857-66.

Sakai, Y., A. P. Murdanoto, T. Konishi, A. Iwamatsu and N. Kato (1997). "Regulation of the formate dehydrogenase gene, FDH1, in the methylotrophic yeast *Candida boidinii* and growth characteristics of an FDH1-disrupted strain on methanol, methylamine, and choline." J Bacteriol **179**(14): 4480-5.

Salvianti, F., P. P. Bettini, E. Giordani, P. Sacchetti, E. Bellini and M. Buiatti (2008). "Identification by suppression subtractive hybridisation of genes expressed in pear (Pyrus spp.) upon infestation with *Cacopsylla pyri* (Homoptera:Psyllidae)." J Plant Physiol.

Sambrook, J., Fritsch, E.F., Maniates, T. (1989). " Molecular Cloning: A laboratory manual, 2nd edition." Cold Spring Habour Laboratory Press.

San-Ei Chemical Industries Ltd., O., Japan (1982a). Improvement of frozen food quality with sclerogum. Kokai Tokkyo Koho, 57,163,451.

San-Ei Chemical Industries Ltd., O., Japan (1982b). Improvement of steamed food quality with sclerogum, Japanese Japane. Kokai Tokkyo Koho, 163,163,441.

San-Ei Chemical Industries Ltd., O., Japan (1982c). Rice cracker quality improvement with sclerogum. Japan. Kokai Tokkyo Koho, 57,163,440.

San-Ei Chemical Industries Ltd., O., Japan (1982d). Improvement of quality of Japanese cake with sclerogum. Japane. Kokai Tokkyo Koho, 57,163,442.

San-Ei Chemical Industries Ltd., O., Japan (1982e). Quality improvement of bakery products with sclerogum. Japane. Kokai Tokkyo Koho, 57,163,432.

Sanchez, A. M., G. N. Bennett and K. Y. San (2005). "Effect of different levels of NADH availability on metabolic fluxes of *Escherichia coli* chemostat cultures in defined medium." J Biotechnol **117**(4): 395-405.

Sandford, P. A. (1979). "Extracellular microbial polysaccharides" Adv. Carbohydr. Chem. Biochem. **36**: 265–312.

Sanford, J. C. K., T. M.; Wolf, E. D.; Allen, N. (1987). "Delivery of substances into cells and tissues using a microprojectile bombardment process." J. Particle Sci. Technol. **5**: 27-37.

Sawers, G. (1994). "The hydrogenases and formate dehydrogenases of *Escherichia coli*." Antonie Van Leeuwenhoek **66**(1-3): 57-88.

Schedel, H.-J. H. a. M. (1991). "Suitability of the shaking flask for oxygen supply to microbiological cultures " Bioprocess and Biosystems Engineering **7**(3): 123-131.

Schilling, B., M., Henning, A. and Rau U. (2000). "Repression of oxalic acid biosynthesis in the unsterile scleroglucan production process with *Sclerotium rolfsii* ATCC 15205." Bioprocess Engineering **22**: 51±55.

Schmid, F., B. A. Stone, R. T. Brownlee, B. M. McDougall and R. J. Seviour (2006). "Structure and assembly of epiglucan, the extracellular (1,3/1,6)-beta-glucan produced by the fungus *Epicoccum nigrum* strain F19." Carbohydr Res **341**(3): 365-73.

Schulze Gronover, C., C. Schorn and B. Tudzynski (2004). "Identification of *Botrytis cinerea* genes up-regulated during infection and controlled by the Galpha subunit BCG1 using suppression subtractive hybridisation (SSH)." Mol Plant Microbe Interact **17**(5): 537-46.

Schumann, J. and C. Hertweck (2007). "Molecular basis of cytochalasan biosynthesis in fungi: gene cluster analysis and evidence for the involvement of a PKS-NRPS hybrid synthase by RNA silencing." J Am Chem Soc **129**(31): 9564-5.

Shahinian, S., G. J. Dijkgraaf, A. M. Sdicu, D. Y. Thomas, C. A. Jakob, M. Aebi and H. Bussey (1998). "Involvement of protein N-glycosyl chain glucosylation and processing in the biosynthesis of cell wall beta-1,6-glucan of *Saccharomyces cerevisiae*." Genetics **149**(2): 843-56.

Sheth, P., L., L., (1967). "The coating of tablets." Chem. Abstr. 1967, 67,111442y(1,480,874).

Shih, I. L., B. W. Chou, C. C. Chen, J. Y. Wu and C. Hsieh (2008). "Study of mycelial growth and bioactive polysaccharide production in batch and fed-batch culture of *Grifola frondosa*." Bioresour Technol **99**(4): 785-93.

Shimada, M., Y. Akamatsu, T. Tokimatsu, M. Kayoko and H. Takefumi (1997). "Possible biochemical roles of oxalic acid as a low molecular weight compund involved in brown-rot and white-rot wood decays." Jounal of Biotechnology **53**: 103-113.

Shroff, R. A., S. M. O'Connor, M. J. Hynes, R. A. Lockington and J. M. Kelly (1997). "Null alleles of creA, the regulator of carbon catabolite repression in *Aspergillus nidulans*." Fungal Genet Biol **22**(1): 28-38.

Shulaev, V., D. Cortes, G. Miller and R. Mittler (2008). "Metabolomics for plant stress response." Physiol Plant **132**(2): 199-208.

Siliang Zhang , B.-C. Y., Ju Chu, Yingping Zhuang and Meijin Guo (2006). "From multi-scale methodology to systems biology: to integrate strain improvement and fermentation optimization." Journal of Chemical Technology & Biotechnology **81** (5): 734-745.

Singh, P. P., R. L. Whistler, R. Tokuzen and W. Nakahara (1974). "Scleroglucan, an antitumor polysaccharide from *Sclerotium glucanicum*." Carbohydr Res **37**(1): 245-7.

Singh, V., S. V. Satheesh, M. L. Raghavendra and P. P. Sadhale (2007). "The key enzyme in galactose metabolism, UDP-galactose-4-epimerase, affects cell-wall integrity and morphology in *Candida albicans* even in the absence of galactose." Fungal Genet Biol **44**(6): 563-74.

Smedsgaard, J. and J. Nielsen (2005). "Metabolite profiling of fungi and yeast: from phenotype to metabolome by MS and informatics." J Exp Bot **56**(410): 273-86.

Soga, T. (2007). "Capillary electrophoresis-mass spectrometry for metabolomics." Methods Mol Biol **358**: 129-37.

Spegel, C. F., A. R. Heiskanen, N. Kostesha, T. H. Johanson, M. F. Gorwa-Grauslund, M. Koudelka-Hep, J. Emneus and T. Ruzgas (2007). "Amperometric response from the glycolytic versus the pentose phosphate pathway in *Saccharomyces cerevisiae* cells." Anal Chem **79**(23): 8919-26.

Spirig, U., M. Glavas, D. Bodmer, G. Reiss, P. Burda, V. Lippuner, S. te Heesen and M. Aebi (1997). "The STT3 protein is a component of the yeast oligosaccharyltransferase complex." Mol Gen Genet **256**(6): 628-37.

Stahl, U., Leitner, E. and Esser, K (1987). "Transformation of *Penicillium chrysogenum* by a vector containing a mitochondrial origin of replication." Eur. J. of Appl. Microbiol. & Biotechnol. **26**: 237-241.

Sternberg, M. B. and S. Gepstein (2007). "Subtractive hybridisation techniques to study cellular senescence." Methods Mol Biol **371**: 289-305.

Stewart, C. S., S. H. Duncan and D. R. Cave (2004). "*Oxalobacter formigenes* and its role in oxalate metabolism in the human gut." FEMS Microbiol Lett **230**(1): 1-7.

Streit, W. R. and R. A. Schmitz (2004). "Metagenomics-the key to the uncultured microbes." Curr Opin Microbiol **7**(5): 492-8.

Sugui, J. A., Y. C. Chang and K. J. Kwon-Chung (2005). "*Agrobacterium tumefaciens*-mediated transformation of *Aspergillus fumigatus*: an efficient tool for insertional mutagenesis and targeted gene disruption." Appl Environ Microbiol **71**(4): 1798-802.

Survase, S. A., P. S. Saudagar and R. S. Singhal (2006). "Production of scleroglucan from *Sclerotium rolfsii* MTCC 2156." Bioresour Technol **97**(8): 989-93.

Survase, S. A., P. S. Saudagar, I. B. Bajaj and R. S. Singhal (2007a). "Scleroglucan: Fermentative Production, Downstream Processing and Applications." Food Technol. Biotechnol. **45**(2): 107-118.

Survase, S. A., P. S. Saudagar and R. S. Singhal (2007b). "Enhanced production of scleroglucan by *Sclerotium rolfsii* MTCC 2156 by use of metabolic precursors." Bioresour Technol **98**(2): 410-5.

Sutherland, I. W. (1989). "Microbial polysaccharides-a comparison with eukaryotic polymers." Symp Soc Exp Biol **43**: 389-402.

Suzuki, K., R. Itai, K. Suzuki, H. Nakanishi, N. K. Nishizawa, E. Yoshimura and S. Mori (1998). "Formate dehydrogenase, an enzyme of anaerobic metabolism, is induced by iron deficiency in barley roots." Plant Physiol **116**(2): 725-32.

Takahashi, T., T. Masuda and Y. Koyama (2006). "Enhanced gene targeting frequency in ku70 and ku80 disruption mutants of *Aspergillus sojae* and *Aspergillus oryzae*." Mol Genet Genomics **275**(5): 460-70.

Tanner, A. and S. Bornemann (2000). "*Bacillus subtilis* YvrK is an acid-induced oxalate decarboxylase." J Bacteriol **182**(18): 5271-3.

Taurhesia, S. and B. McNeil (1994). "Physicochemical factors affecting the formation of the biological response modifier scleroglucan." J Chem Technol Biotechnol **59**(2): 157-63.

Tentler, S., J. Palas, C. Enderlin, J. Campbell, C. Taft, T. K. Miller, R. L. Wood and C. P. Selitrennikoff (1997). "Inhibition of *Neurospora crassa* growth by a glucan synthase-1 antisense construct." Curr Microbiol **34**(5): 303-8.

Thompson, J. D., D. G. Higgins and T. J. Gibson (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." Nucleic Acids Res **22**(22): 4673-80.

Tilser, I., E. Breierova, M. Tichy, H. Skalska and E. Ettlerova (1996). "Effect of supplementation of University of Wisconsin solution with glycoproteins from psychrophilic strains of yeast on hypothermic liver storage of rats." Cryobiology **33**(3): 347-53.

Tokimatsu, T., Y. Nagai, T. Hattori and M. Shimada (1998). "Purification and characteristics of a novel cytochrome c dependent glyoxylate dehydrogenase from a wood-destroying fungus *Tyromyces palustris*." FEBS Lett **437**(1-2): 117-21.

Tooley, P., W., Leung, Hei and Leong, Sally A. (1992). "Meiotic and mitotic stability of transforming DNA in the phytopathogenic fungus *Magnaporthe grisea*." Current Genetics **21**(1): 55-60.

Torralba, S., M. Raudaskoski, A. M. Pedregosa and F. Laborda (1998). "Effect of cytochalasin A on apical growth, actin cytoskeleton organization and enzyme secretion in *Aspergillus nidulans*." Microbiology **144 (Pt 1)**: 45-53.

Uchimura, H., H. Enjoji, T. Seki, A. Taguchi, N. Takaya and H. Shoun (2002). "Nitrate reductase-formate dehydrogenase couple involved in the fungal denitrification by *Fusarium oxysporum*." J Biochem (Tokyo) **131**(4): 579-86.

Ueno, K., J. Uno, H. Nakayama, K. Sasamoto, Y. Mikami and H. Chibana (2007). "Development of a highly efficient gene targeting system induced by transient repression of YKU80 expression in *Candida glabrata*." Eukaryot Cell **6**(7): 1239-47.

Usaite, R., J. Wohlschlegel, J. D. Venable, S. K. Park, J. Nielsen, L. Olsson and J. R. Yates Iii (2008). "Characterization of global yeast quantitative proteome data generated from the wild-type and glucose repression *Saccharomyces cerevisiae* strains: the comparison of two quantitative methods." J Proteome Res **7**(1): 266-75.

Vallino, J. J. and G. Stephanopoulos (2000). "Metabolic flux distributions in Corynebacterium glutamicum during growth and lysine overproduction. Reprinted from Biotechnology and Bioengineering, Vol. 41, Pp 633-646 (1993)." Biotechnol Bioeng **67**(6): 872-85.

Van der Werf, M. J. (2005). "Towards replacing closed with open target selection strategies." Trends Biotechnol **23**(1): 11-6.

Van Dien, S. and C. H. Schilling (2006). "Bringing metabolomics data into the forefront of systems biology." Mol Syst Biol **2**: 2006 0035.

Verdoes JC, P. P., van der Hondel CAMJJ (1995). "Molecular genetic strain improvement for the overproduction of fungal proteins by filamentous fungi." Appl Microbiol Biotechnol **43**: 195-205.

Villalba, F., J. Collemare, P. Landraud, K. Lambou, V. Brozek, B. Cirer, D. Morin, C. Bruel, R. Beffa and M. H. Lebrun (2008). "Improved gene targeting in *Magnaporthe grisea* by inactivation of MgKU80 required for non-homologous end joining." Fungal Genet Biol **45**(1): 68-75.

Wachenheim, D. E. and J. A. Patterson (1992). "Anaerobic Production of Extracellular Polysaccharide by *Butyrivibrio fibrisolvens* nyx." Appl Environ Microbiol **58**(1): 385-391.

Walshaw, D. L., A. Wilkinson, M. Mundy, M. Smith and P. S. Poole (1997). "Regulation of the TCA cycle and the general amino acid permease by overflow metabolism in *Rhizobium leguminosarum*." Microbiology **143 (Pt 7)**: 2209-21.

Walti, M. A., C. Villalba, R. M. Buser, A. Grunler, M. Aebi and M. Kunzler (2006). "Targeted gene silencing in the model mushroom *Coprinus cinereus* by expression of homologous hairpin RNAs." Eukaryot Cell **5**(4): 732-44.

Wang, Y., B. McNeil, (1994). "Scleroglucan and oxalic acid formation by *Sclerotium glucanicum* in sucrose supplemented fermentation,." Biotechnol. Lett. **16**: 605–610.

Wang, Y. and B. McNeil (1996). "Scleroglucan." Crit Rev Biotechnol **16**(3): 185-215.

Wang, T. H., Y. H. Zhong, W. Huang, T. Liu and Y. W. You (2005). "Antisense inhibition of xylitol dehydrogenase gene, xdh1 from *Trichoderma reesei*." Lett Appl Microbiol **40**(6): 424-9.

Ward, M., C. Lin, D. C. Victoria, B. P. Fox, J. A. Fox, D. L. Wong, H. J. Meerman, J. P. Pucci, R. B. Fong, M. H. Heng, N. Tsurushita, C. Gieswein, M. Park and H. Wang (2004). "Characterization of humanized antibodies secreted by *Aspergillus niger*." Appl Environ Microbiol **70**(5): 2567-76.

Watanabe, T., S. Tengku, H. Takefumi and M. Shimada (2003). "A Role of Formate Dehydrogenase in the Oxalate Metabolism in the Wood-destroying Basidiomycete *Ceriporiopsis subvermispora*." Wood research **90**.

Watanabe, T., T. Hattori, S. Tengku and M. Shimada (2005). "Purification and characterization of NAD-dependent formate dehydrogenase from the white-rot fungus *Ceriporiopsis subvermispora* and a possible role of the enzyme in oxalate metabolism." Enzyme and Microbial Technology **37**: 68-75.

Watanabe, T., T. Fujiwara, T. Umezawa, M. Shimada and T. Hattori (2008). "Cloning of a cDNA encoding a NAD-dependent formate dehydrogenase involved in oxalic acid metabolism from the white-rot fungus *Ceriporiopsis subvermispora* and its gene expression analysis." FEMS Microbiol Lett **279**(1): 64-70.

Weckwerth, W., K. Wenzel and O. Fiehn (2004). "Process for the integrated extraction, identification and quantification of metabolites, proteins and RNA to reveal their co-regulation in biochemical networks." Proteomics **4**(1): 78-83.

Weidner, G., C. d'Enfert, A. Koch, P. C. Mol and A. A. Brakhage (1998). "Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus*

based on the pyrG gene encoding orotidine 5'-monophosphate decarboxylase." Curr Genet **33**(5): 378-85.

Weld, R. J., C. C. Eady and H. J. Ridgway (2006). "*Agrobacterium*-mediated transformation of Sclerotinia sclerotiorum." J Microbiol Methods **65**(1): 202-7.

Westergaard, S. L., A. P. Oliveira, C. Bro, L. Olsson and J. Nielsen (2007). "A systems biology approach to study glucose repression in the yeast *Saccharomyces cerevisiae*." Biotechnol Bioeng **96**(1): 134-45.

Willets, H. J. (1978). " *Sclerotium* Formation." The Filamentous Fungi **Vol. 3**(J.E. Smith, D.R. Berry (Eds.), Edward Arnold Publishers Ltd., London, UK): 197–221.

Wommack, K. E., J. Bhavsar and J. Ravel (2008). "Metagenomics: read length matters." Appl Environ Microbiol **74**(5): 1453-63.

Wydra, K. (1996). "Collection and determination of root and stem rot pathogens." Annual Report IITA, Ibadan. *Niger*ia: 68.

Xu, C. P., J. Sinha, J. T. Bae, S. W. Kim and J. W. Yun (2006). "Optimization of physical parameters for exo-biopolymer production in submerged mycelial cultures of two entomopathogenic fungi Paecilomyces japonica and Paecilomyces tenuipes." Lett Appl Microbiol **42**(5): 501-6.

Yamada, O., R. Ikeda, Y. Ohkita, R. Hayashi, K. Sakamoto and O. Akita (2007). "Gene silencing by RNA interference in the *koji* mold *Aspergillus oryzae*." Biosci Biotechnol Biochem **71**(1): 138-44.

Yanaki, T. K., T.; Norisuye, T. (1981). "Triple helices in dilute aqueous sodium hydroxide." Polymer J. **13**: 1135.

Yanaki, T. N., T. (1983). "Triple helix and random coil Scleroglucan in dilute solution." Polymer J. **15**: 389.

Yokota, A., S. Haga and S. Kitaoka (1985). "Purification and some properties of glyoxylate reductase (NADP+) and its functional location in mitochondria in *Euglena gracilis*" Biochem J **227**(1): 211-6.

Yoshihira, A. K. a. O. (1969). "Production and Properties of a-L-Arabinofuranosidase from *Corticium rolfsii*." Applied Microbiology **17**(6): 910-913.

Yu, X. M., X. D. Yu, Z. P. Qu, X. J. Huang, J. Guo, Q. M. Han, J. Zhao, L. L. Huang and Z. S. Kang (2008). "Cloning of a putative hypersensitive induced reaction gene from wheat infected by stripe rust fungus." Gene **407**(1-2): 193-8.

Zamore, P. D., T. Tuschl, P. A. Sharp and D. P. Bartel (2000). "RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals." Cell **101**(1): 25-33.

Zaragoza, O., C. J. Chrisman, M. V. Castelli, S. Frases, M. Cuenca-Estrella, J. L. Rodriguez Tudela and A. Casadevall (2008). "Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival." Cell Microbiol.

Zheng, X. F., Y. Kobayashi and M. Takeuchi (1998). "Construction of a low-serine-type-carboxypeptidase-producing mutant of *Aspergillus oryzae* by the expression of antisense RNA and its use as a host for heterologous protein secretion." Appl Microbiol Biotechnol **49**(1): 39-44.

Zhang, J., V. Carey and R. Gentleman (2003). "An extensible application for assembling annotation for genomic data." Bioinformatics **19**(1): 155-6.

Zhang, D. L., L. Ji and Y. D. Li (2004). "Analysis, identification and correction of some errors of model refseqs appeared in NCBI Human Gene Database by in silico cloning and experimental verification of novel human genes." Yi Chuan Xue Bao **31**(5): 431-43.

Zhou, F., Z. Zhang, P. L. Gregersen, J. D. Mikkelsen, E. de Neergaard, D. B. Collinge and H. Thordal-Christensen (1998). "Molecular characterization of the oxalate oxidase involved in the response of barley to the powdery mildew fungus." Plant Physiol **117**(1): 33-41.

8 Appendix

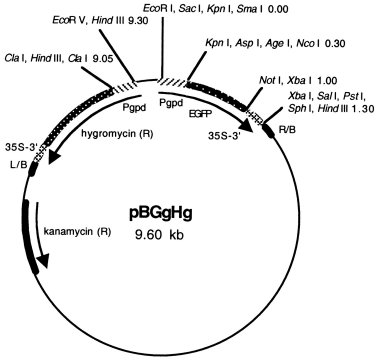

Figure A: Organization of binary vector pBGgHg. pBGgHg is 9.6 kb in size and consists of a pCAMBIA 1300 backbone containing the kanamycin resistance (R) gene and the right border (R/B) and left border (L/B) sequences of *Agrobacterium*-DNA. The hygromycin resistance and EGFP genes are located between the border sequences, and each is joined to the *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase promoter (*Pgpd*) and the cauliflower mosaic virus terminator (35S-39). Shown are restriction enzyme sites with map distances in kilo bases (Chen et al. 2000).

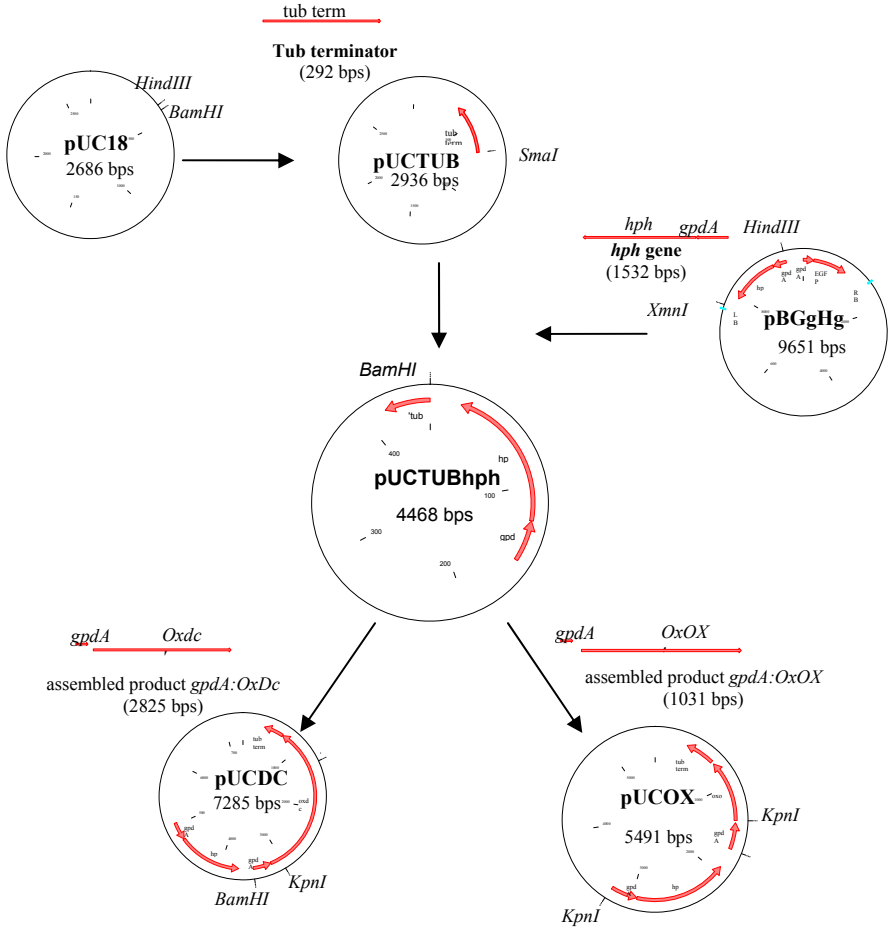

Figure B: Cloning strategy of the suitable transformation vectors for heterologous expression in *S. rolfsii*. Origin of both vectors was pUC 18, linearized by use of Hind III and BamHI. Tub-terminator of pAN-NC3 (obtained by primer pair (HI BT and BI BT) was introduced, resulting in the intermediate plasmid called pUCTUB. Linearization for further introduction was performed by use of *SmaI* and adjacent ligation of *hph* cassette obtained via PCR from pBGgHg. The resulting hygromycin resistance conferring intermediate was named pUCTUB hph. Linearization and further introduction of coding sequences of interest for successful metabolic engineering of S. *rolfsii* is performed by use of BamHI cognitive sequence for ligation. Oxalate oxidase as well as oxalate decarboxylase cDNA with corresponding BamHI sequence mediators was ligated, resulting in the both heterologous transformation vectors, named pUCDC for oxalate decarboxylase referring activity and pUCOX for oxalate oxidase referring activity respectively.

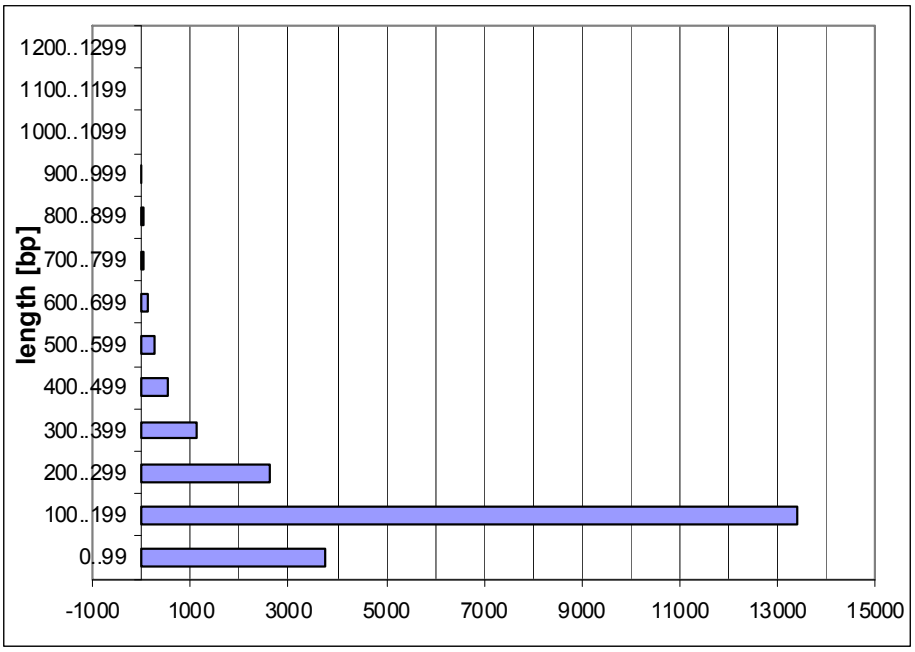

Figure C: Statistics of the single read length obtained by pyrosequencing. Length is given in basepairs with corresponding amount of contigs.

Table A: Long time storage of *S. rolfsii* mycelium within the different tested solvents. There were used 20 and 50% of glycerol and 10 and 20% DMSO. 1 cm^2 of mycelia was stored in the different solvents at 4 °C and -80 °C respectively. Germination events after given time up to 60 weeks are indicated by + for growth, ++ for good growth and +++ for strong growth. – indicates no growth and (+) indicates different results in the biological duplicates.

Solvent	Temperature	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 8
20% Glycerol	4 °C	+++	+++	++	+++	+++	+	-
	- 80 °C	+++	+++	++	+++	+++	+++	+++
50% Glycerol	4 °C	++	-	-	-	-	-	-
	- 80 °C	(+)	-	+	-	++(+)	++	++(+)
10% DMSO	4 °C	+++	+++	+++	+++	+++	+++	++(+)
	- 80 °C	+	+	++(+)	++	+	-	+
20% DMSO	4 °C	+++	++	+++	+++	+++	+	-
	- 80 °C	+	+	++(+)	-	-	+	-
Solvent	**Temperature**	**Week 9**	**Week 12**	**Week 16**	**Week 25**	**Week 30**	**Week 52**	**Week 60**
20% Glycerol	4 °C	-	-	-	-	-	-	-
	- 80 °C	+++	+++	+++	++	++	++	++
50% Glycerol	4 °C	-	-	-	-	-	-	-
	- 80 °C	-	++(+)	++	-	-	++	+(+)
10% DMSO	4 °C	++	++(+)	+++	-	-	-	-
	- 80 °C	-	-	++	-	-	-	-
20% DMSO	4 °C	-	-	-	-	-	-	-
	- 80 °C	-	-	+	-	-	-	-

Table B: Long time storage of *S. rolfsii* sclerotia within the different tested solvents. There were used 20% of and 10% of DMSO. Several sclerotia, harvested of the same plates were stored in the different solvents at 4 °C and -80 °C respectively. Germination events after given time up to 60 Weeks are indicated by + for growth, ++ for good growth and +++ for strong growth. – indicates no growth and (+) indicates different results in the biological duplicates.

Solvent	Temperature	Week 1	Week 2	Week 3	Week 4	Week 5)	Week 6	Week 8
20% Glycerol	4 °C	+++	+++	++	+++	(+)	+++	+++
	- 80 °C	+++	+++	++	+++	(+)	+++	++(+)
10% DMSO	4 °C	+++	++	+++	+++	(+)	+++	+++
	- 80 °C	+++	+++	+++	+++	(+)	+++	+++
Solvent	**Temperature**	**Week 10**	**Week 12**	**Week 16**	**Week 24**	**Week 36**	**Week 52**	**Week 60**
20% Glycerol	4 °C	+++	+	+(+)	++(+)	+	-	-
	- 80 °C	+++	(+)	+++	+++	+++	++	++
10% DMSO	4 °C	+++	(+)	+++	+++	(+)	-	-
	- 80 °C	+++	+(+)	+(+)	+++	+++	+++	+++

Table C: Contigs used for schematic metabolism in enhanced glucose based cultivation with corresponding fold changes as drawn in Figure 28.

TargetID	Min37 vs. Min61	Min61 vs. Max61	Max37 vs. Min61	Max37 vs. Max61	Min 37 vs. Max61	Min37 vs. Max37	Genes
E4.seq	0.00	0.00	0.00	0.00	1.40	0.00	Pyruvate decarboxylase
contig15326	1.43	0.00	0.00	-1.15	-1.56	0.00	Citrate synthase
contig02546	0.00	0.00	0.00	0.00	-1.34	0.00	Isocitrate dehydrogenase
contig00175	0.00	-3.19	0.00	-3.89	-4.03	0.00	Isocitrate Lyase
contig09153	0.00	0.00	0.00	-1.16	-1.42	0.00	Malate dehydrogenases
contig05630	0.00	-3.91	0.00	-4.46	-4.53	0.00	Oxaloacetate hydrolease
contig00773	0.00	-4.04	1.51	-2.50	-3.27	0.00	Glycolate oxidase subunit
G8.seq	0.00	0.00	0.00	0.00	1.03	0.00	Pyruvate decarboxylase
contig21272	0.00	0.00	0.00	-1.18	-1.00	0.00	Pyruvate carboxylase
contig18170	1.13	1.06	0.00	0.00	2.28	1.86	Squalene epoxidase
contig17971	1.06	1.24	0.00	1.88	2.64	0.00	Phosphoglycerate mutase
contig11078	0.00	-1.16	0.00	0.00	-1.16	0.00	6-phosphofructo-2-kinase
contig18170	1.12	1.05	0.00	0.00	2.28	1.86	Squalene epoxidase
contig10515	0.00	0.00	0.00	0.00	1.37	0.00	6 phosphogluconolactonase
C2.seq	0.00	0.00	-1.06	0.00	2.77	1.49	Glycogen phosphorylase