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ISOLATION AND CHARACTERISATION OF THREE MUCOR CIRCINELLOIDES GENES FOR ISOPRENOID BIOSYNTHESIS

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Carotenoids are widely distributed coloured compounds present all around the living kingdoms. Some of them are of economic importance, since they are used in the food, feed and cosmetic industries. Indeed, it has been shown during the last decade that they could play a preventive role against several diseases, including cancer. Recently, the two Mucor circinelloides structural genes for carotenoid biosynthesis (carB and carRP) have been isolated and characterised. The metabolic steps preceding the synthesis of phytoene (the first carotenoid), although belonging to the more general isoprenoid biosynthesis pathway, are of equal importance for a prospective metabolic engineering of the carotenoid pathway, because they constitute the source of precursors, i.e. the prenyl diphosphates (FPP and GGPP). Here we present the isolation and analysis of three isoprenoid biosynthesis genes from M. circinelloides. A new structural gene (carG), was isolated by heterologous hybridisation using a probe derived from the Gibberella fujikuroi ggs1 gene. Functional analyses in E. coli showed that the encoded protein had geranylgeranyl pyrophosphate (GGPP) synthase activity. The analysis of carG mRNA accumulation after blue light irradiation showed that the expression of this gene is up regulated by blue light, as it happens with carB and carRP. The possibility that the carG gene played a key role in the regulation of carotenoid biosynthesis, despite it belongs to a more general metabolic pathway, is discussed. Two other genes involved in isoprenoid biosynthesis were isolated and characterised. The isoA gene encoded a typical eukaryotic FPP synthase, whereas the isoB was shown to be a solanesyl diphosphate synthase, which is the first fungal enzyme reported having this specificity.
In addition, a M. circinelloides one-marker-per-chromosome map has been completed by CHEF localisation of five isoprenoid/ carotenoid biosynthesis genes to individual chromosomes.

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TRANSCRIPTION FACTOR CPCR1 REGULATES CEPHALOSPORIN C BIOSYNTHESIS IN ACREMONIUM CHRYSOGENUM

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Winged helix transcription factors including the forkhead and the RFX subclass are characteristic for the eukaryotic domains in animals and fungi, but seem to be missing in plants. Using Acremonium chrysogenum as an experimental system we recently have determined the functional role of the RFX transcription factor CPCR1 in cephalosporin C biosynthesis [1, 2].
Promoter scanning analysis and gel retardation experiments with selected oligonucleotides led to the identification of two CPCR1 binding sites in the promoter of the cephalosporin C biosynthesis pcbAB/pcbC genes. The in vivo relevance of the two sequences for gene activation was demonstrated using pcbC promoter-lacZ fusions in A. chrysogenum. The deletion of both CPCR1 binding sites resulted in a severe reduction of reporter gene activity in transgenic strains.
Furthermore, results from the analysis of cpcR1 multicopy transformants and cpcR1 knockout strains clearly indicate the involvement of CPCR1 in the regulation of cephalosporin C biosynthesis. Significant differences in transcript levels of the pcbC gene were obtained with the knockout transformants. The overall production of cephalosporin C was identical in transformed and non-transformed strains, however, the knockout strains showed striking differences concerning the accumulation of the biosynthesis intermediate penicillin N. The complementation of the cpcR1 gene in the knockout strains results in pcbC transcript levels and penicillin N yields comparable to the control [3]. However, the complexity of the data points to a well-controlled or even functional redundant network of transcription factors with CPCR1 being only one player within this process.
We further have investigated the subcellular localization of different transcription factors from A. chrysogenum using confocal laser microscopy.

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CHARACTERIZATION OF PIPECOLIC ACID METABOLISM IN *Penicilium chrysogenum*. DISRUPTION OF THE *lys*7 GENE LEADS TO ACCUMULATION OF THE SECONDARY METABOLITE PRECURSORS PIPERIDEINE-6-CARBOXYLIC ACID AND PIPECOLIC ACID

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Pipecolic acid is an important compound that serves as substrate of some non-ribosomal peptide and polyketide synthetases, resulting in the formation of secondary metabolites with interesting pharmacological activities, e.g. immunosuppressors and antitumor agents. The formation of this compound is related to lysine metabolism in various organisms including plants, mammals, fungi and bacteria. In this work we showed that, in *P. chrysogenum,* pipecolic acid is converted into lysine. The conversion of pipecolic acid into lysine proceeds through the conversion of pipecolic acid into piperideine-6-carboxylic acid, saccharopine and lysine, by the consecutive action of pipecolate oxidase, saccharopine reductase and saccharopine dehydrogenase (Naranjo *et al*., 2001). On other hand, we showed that *P. chrysogenum* is able to synthesize pipecolic acid (Naranjo *et al*., 2003). The *lys*7 gene encoding saccharopine reductase of *P. chrysogenum* was target-inactivated by the double recombination method. Analysis of disrupted strain (named *P. chrysogenum* SR1⁻) showed the presence of a mutant *lys*7 gene lacking about 1000 bp in the 3´-end region. The *P. chrysogenum* SR1⁻ strain lacked saccharopine reductase activity, was a lysine auxotroph and accumulated P6C. The saccharopine reductase activity of this mutant was recovered after transformation with the intact *lys*7 gene in an autonomous replicating plasmid. When the *P. chrysogenum* SR1⁻ mutant was grown with L-lysine and D,L-α-aminoadipic acid as nitrogen sources, a high levels of P6C and pipecolic acid were accumulated intracellularly. A comparison of the SR1⁻ strain with a *lys*2 defective mutant (TDX195) provided evidence showing that *P. chrysogenum* synthesizes pipecolic acid from α-aminoadipic acid and not from lysine catabolism. In this work we report for the first time that disruption of the *lys*7 gene encoding saccharopine reductase leads to the accumulation of P6C a cyclic form of αaminoadipic acid semialdehyde, that is later converted into pipecolic acid. This recombinant strain may have

important potential industrial application for the production of pipecolate-derived products with interesting pharmacological properties.

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APPLICATION OF T-RFLP ANALYSIS TO FUNGAL CELLULASE GENES IN SOIL COMMUNITIES.

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Abstract
Cellulolytic organisms are key components of terrestrial and aquatic communities and may be potential indicators of ecosystem health. Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis was explored as a way to detect and monitor fungal cellobiohydrolase I (CBHI) genes within soil communities. Nine primers were designed to conserved regions for PCR amplification of 600-800 bp within the CBHI gene. One set of primers consistently amplified two amplicons of 625 base pairs and 750 base pairs. Sequence and Southern blot analysis confirmed that major amplicons contained CBHI DNA. The primers were used to PCR amplify DNA extracted from two soil samples taken from a petroleum degradation Land Treatment Unit (LTU) located in California followed by CBHI functional T-RFLP analysis using HpaII and HaeIII enzymes. Fungal ribosomal ITS T-RFLP analysis was also performed. The two soil communities yielded overlapping yet unique T-RFLP patterns resulting in 13-18 CBH1 peaks and 2-4 ribosomal ITS peaks which translated into an observed CBHI:ITS T-RFLP peak ratio of approximately 6:1. Genbank and soil clone library CBHI sequence data were used to determine whether observed T-RFLP peaks were due to CBHI-specific amplification and to identities could be assigned to fungi in the soil communities. One observed T-RFLP profile partially matched predictions made from Genbank sequence data, three profiles matched predictions from soil library clones identified as either Phanerochaete or Aspergillus CBHI sequences and an additional nine profiles matched predictions from nine unidentified soil clones. An additional three clones from the soil library contained CBHI inserts from an additional two fungal genera whose predicted T-RFLP profiles were not observed. In conclusion, a partial description of the fungal cellulolytic community was obtained using CBHI TRF data. Future work will include further PCR and primer optimization and increased analysis of CBHI soil libraries and other sequence data for a more complete description of these communities. This method shows good potential for describing and screening for the functional diversity of complex microbial communities.

EQUISETIN BIOSYNTHESIS FROM FUSARIUM HETEROSPORUM

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Equisetin is representative of the tetramic acid family of natural products which has shown a wide range of bioactivity. Equisetin inhibits HIV-1 integrase and is produced by the filamentous fungi Fusarium heterosporum ATCC74349 and F. equiseti NRRL5537. There are two distinct biosynthetic elements in this compound, a partially reduced polyketide and a serine residue. From an F. heterosporum cosmid library the equisetin biosynthetic pathway was identified and sequenced. This cluster marks the first reported tetramic acid synthetase and the first report of a polyketide synthase-nonribosomal peptide synthetase hybrid in fungi.

A PROTEIN COMPLEX BINDS TO SPECIFIC SEQUENCES IN THE PROMOTER REGION OF THE Phycomyces blakesleeanus carRA AND carB GENES ONLY IN DARK CONDITIONS.

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Blue light regulates transcription of the Phycomyces blakesleeanus carB and carRA genes. We are interested in the identification of the sequences involved in the perception of the light signal in the promoters of the light regulated genes as well as in the identification of the regulatory proteins which interact with those sequences and determine the activation of carotenogenesis under light conditions or its repression under darkness conditions.
To this end a battery of gel retardation experiments were carried out with several radioactively labeled DNA fragments derived from the carRA-carB promoter region and with total extracts of P. blakesleeanus nuclear proteins. In these experiments a DNA-protein complex was detected in those assays carried out with protein extracts derived from mycelia grown in darkness and a 300 bp fragment located in the central region of the carRA-carB promoter. These results indicate that a putative regulatory protein/s bind/s to the central region of the carRA-carB promoter under darkness conditions, possibly acting as a repressor of the carRA and carB genes transcription in darkness.
Gel retardation experiments were also carried out with protein extracts from mutants disturbed in the synthesis of carotenoids, either in structural genes (carRA, carB) or in putative regulatory genes (carC, carD, carS, picA, madA, madB, madD, madE, madJ), to check if the protein encoded by the gene altered in each of these mutants was or not essential to the formation of the complexes detected in the gel retardation experiments. The results obtained demonstrate that the products encoded by genes carRA, carB, carS, carD and carC are essential for the formation of a carotenogenesis regulatory complex which binds to specific sequences of the carRA-carB gene promoter region. These data support the carotenogenesis regulatory model by blue light previously proposed in this organism.

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HETEROLOGOUS EXPRESSION OF THE FUSARIUM GRAMINEARUM TRI4 GENE IN F. VERTICILLIOIDES PROVIDES EVIDENCE FOR A MULTIFUNCTIONAL OYGENASE IN TRICHOTHECENE BIOSYNTHESIS

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The biosynthesis of trichothecene mycotoxins by Fusarium sporotrichioides and F. graminearum involves a complex biochemical pathway that begins with the cyclization of farnesyl pyrophosphate to the sesquiterpene hydrocarbon trichodiene and continues with multiple oxygenation, cyclization and esterification reactions. In previous studies using F. sporotrichioides, disruption of the P450 monooxygenase-encoding gene Tri4 blocked trichothecene production and lead to the accumulation of trichodiene. Therefore, the TRI4 protein must act on trichodiene. To further elucidate the function of the TRI4 protein, we heterologously expressed the F. graminearum Tri4 (FgTri4) in F. verticillioides, which does not produce trichothecenes. Transgenic F. verticillioides carrying FgTri4 under the control of a fumonisin biosynthetic gene (FUM8) promoter converted exogenous trichodiene to isotrichodermin. Conversion of trichodiene to isotrichodermin requires seven biochemical reactions (trichodiene → 2-hydroxytrichodiene → 12,13-Epoxy-9,10-trichoene-2-ol → isotrichodiol → isotrichotriol → trichotriol → isotrichodermol → isotrichodermin). Previous studies indicate that two of these reactions (isotrichotriol → trichotriol → isotrichodermol) are non-enzymatic, and feeding studies done here indicate that wild-type F. verticillioides can convert isotrichodermol to isotrichodermin. Together, these results indicate that the FgTri4 protein is a multifunctional monooxygenase that catalyzes the four oxygenation reactions required for the conversion of trichodiene to istrichotriol during trichothecene biosynthesis.

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A MYSTERIOUS, PLEIOTROPIC GROWTH DEFECT CAUSED BY DELETION OF A GLYOXAL OXIDASE GENE IN BOTRYTIS CINEREA

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A Botrytis cinerea gene was cloned encoding a glyoxal oxidase homologue (BcGLYOX1). The gene product is presumably secreted and contains a chitin-binding domain homologous to plant lectins. BcGLYOX1 was anticipated to be required for production of oxalic acid and for virulence of this plant pathogenic fungus. Mutants constructed by gene replacement retained the ability to produce oxalic acid, indicating that BcGLYOX1 is not essential for oxalate production. Unexpectedly however, BcGLYOX1-deficient mutants lost the ability to germinate and grow on minimal media containing a range of carbon sources. The mutants were consequently non-pathogenic on a range of host tissues. The growth defect was fully restored by supplementing the medium with arginine at concentrations as low as 50 mM. Spontaneous suppressor mutants that regain the ability to grow on simple sugars in the absence of arginine were obtained at a frequency of about 10^{-6}. Reintroduction of the wild type gene into the mutant only partly restored the wild type growth phenotype. We conclude that the BcGLYOX1 mutation results in a severe defect in primary or secondary metabolism, which can be overcome by arginine supplementation. The mechanisms underlying the behaviour of the mutants, the relief of the phenotype by arginine, as well as the emergence of spontaneous suppressors remain elusive.

THE GIBBERELLA FUJIKUROI GLUTAMINE SYNTHETASE PLAYS AN IMPORTANT ROLE IN NITROGEN METABOLITE REPRESSION

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In Gibberella fujikuroi, the gibberellin (GA) and bikaverin biosynthesis are under control of nitrogen metabolite repression mediated by the general transcription factor, AREA. However, the signalling components acting upstream of AREA, and regulatory proteins, affecting AREA activity by protein-protein interaction, are still unknown. We investigated the role of glutamine synthetase (GS) as enzyme synthesizing glutamine, the key intermediate in nitrogen metabolism, and as a possible regulator in the nitrogen metabolite repression system.
The gene glnA-Gf was cloned and shown to be highly expressed under conditions of nitrogen starvation and nitrogen excess. Deletion mutants grow only in the presence of glutamine, but with a phenotype different from the wild-type. Unexpectedly, the mutants were unable to express nitrogen-repressed GA- and bikaverin-biosynthetic genes even under nitrogen starvation conditions. Complementation with the glnA-Gf wild-type copy fully restored GS activity, the expression of nitrogen-regulated secondary metabolism genes, and the production of GAs and the red pigment, bikaverin. In order to find more genes which are under control of GS, differential cDNA-screening and differential hybridization of macroarrays were performed with cDNA from the wild-type and ΔglnA mutant as probes. We found several genes, dramatically up- or down-regulated in the mutant. Among them are genes encoding uricase, homologues of the yeast DDR48 protein and the multiprotein bridging factor MBF1, a small protein, CipC, with unknown function, several translation initiation and elongation factors, a ribosomal 40S protein, a polyubiquitin homologue, as well as a putative histone acetyltransferase. Some of these genes are also under control of AREA (uricase, GA and bikaverin genes), others not. Treatment with the GS inhibitor L-methionine sulfoximine (MSX) resulted in similar expression patterns as in the glnA mutant with ammonium as nitrogen source, whereas glutamine can overcome the up-and down-regulation of most of the target genes. However, the expression of cipC is strictly repressed by MSX also in the presence of glutamine. The findings that the GA-and bikaverin biosynthetic genes are not expressed in the glnA deletion mutant despite the absolute starvation for nitrogen, and that cipC is expressed with glutamine, but not with MSX and glutamine, suggest that not only glutamine, but also GS itself plays an important role in nitrogen metabolite repression and transcriptional and translational control.

IDENTIFICATION AND RESTORATION OF TWO MAJOR ENZYMATIC BLOCKS IN GIBBERELLIC ACID BIOSYNTHESIS IN FUSARIUM PROLIFERATUM (GIBERELLA FUJIKUROI MP-D)

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Gibberella fujikuroi is a species complex with at least eight different biological species, termed mating populations (MPs) A to H. These species are known to produce many secondary metabolites, such as mycotoxins, pigments and plant hormones. So far, the ability to produce gibberellins (GAs) was restricted to G. fujikuroi MP-C (Fusarium fujikuroi) and some members of MP-A (F. verticillioides). We have previously characterized all seven genes responsible for GA biosynthesis in MP-C and have shown that these genes are organized in a gene cluster. Moreover, we have also shown by Southern blot analysis that most of the MPs consist of the genes for GA biosynthesis. In this study, we could clearly determine that members of MP-D (F. proliferatum) consist of all seven GA biosynthesis genes with a high degree of sequence homology to the corresponding genes of MP-C. However, GAs are not produced. Transformation experiments were performed to characterize functionality of single GA biosynthesis genes of MP-D in genetic backgrounds of both, MP-C as well as MP-D. Some of the genes of MP-D (P450-1, des) encode functional enzymes (in both backgrounds), which are able to metabolize radiolabelled precursors to some extent into the expected products. Despite the functionality of these enzymes, expression level was low. Other genes of MP-D, e.g. P450-4 and ggs2 /cps/ks, do not encode functional proteins due to several amino acid exchanges. Feeding experiments in both genetic backgrounds did not result in any conversion of [^{14}C]-ent-kaurene into [^{14}C]-ent-kaurenoic acid. Interestingly, P450-4 from MP-D showed a higher expression level than P450-1, which could be demonstrated by a GUS reporter gene assay. Moreover, transformation of the whole GA gene cluster from MP-C into MP-D resulted in fully restored GA production. Thus, regulatory background in MP-D seems to be functional.
Therefore, we demonstrate that members of MP-D lost the ability to produce GAs as a result of an accumulation of several mutations in the coding and 5' non-coding regions of the seven GA biosynthetic genes, although at least two genes encode functional enzymes. Moreover, we were finally able to restore GA production by targeted complementation of the investigated enzymatic blocks.

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MOLECULAR GENETICS OF THE ALKALOID PATHWAY IN CLAVICEPS PURPUREA

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Claviceps purpurea is an ubiquitous phytopathogenic ascomycete which produces ergot alkaloids during the dormant phase of its lifecycle. These cyclol structured indol derivatives are secondary metabolites that are only produced in sclerotial cells. They can be devided into two main groups. One group includes the lysergic acid derivatives and the structurally more complex ergopeptines. The other group are the clavine alkaloids. Due to their structural homology to neurotransmitters like serotonin and dopamine, ergot alkaloids act as agonists and antagonists at the receptor site of these biogenic amines. They find application in the treatment of a variety of clinical conditions, including postpartum hemorrhage, migraine, senile cerebral insufficiency and Parkinson´s disease. A vast quantity of detailed information concerning the biochemical aspects of the biosynthetic pathway is available, but up to now there exists only a limited knowledge of the participating genes.
We focussed on the isolation and cloning of genes which could mediate pathway-specific steps of the alkaloid biosynthesis. By means of chromosome walking combined with cDNA screening we were able to detect a 58000 bp cluster which exhibits a correlation between postulated protein function and the enzymes involved in the alkaloid biosynthesis pathway. Expression studies showed that the cluster genes are coregulated and that they are activated only under alkaloid producing conditions.
We characterized the gene cpd1 which encodes the key enzyme dimethylallyltryptophane-synthase (DMATS) [1], mediating the first pathway-specific step of the alkaloid biosynthesis. Furthermore four modular peptide synthetases (non ribosomal peptide synthetases NRPS), were identified. One of them (cpps2) was knocked out and showed to encode a monomodular lysergyl-peptide-synthetase (LPS 2) responsible for the activation of D-lysergic acid. The inactivation of cpps2 led to an ergopeptine-nonproducing mutant which – unlike the parent producer strain – accumulated D-lysergic acid [2]. Other identified genes are oxygenases and oxidoreductases. Targeted inactivation of these genes (e. g. cpP450-1) will reveal their function in the biosynthetic pathway of alkaloids.
Another aim is to compare different strains of Claviceps particularly with respect to their potential to produce different types of alkaloids. To clarify if these differences are due to different types of NRPS or to the availability of different amino acid, the corresponding NRPS Genes of two different C. purpurea strains were compared in detail.

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CLONING OF CYTOCHROME P450 MONOOXYGENASE GENES INVOLVED IN PATULIN BIOSYNTHESIS IN PENICILLIUM EXPANSUM.

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Patulin is a mycotoxin which can be produced by several species of *Aspergillus* and *Penicillium* which are found naturally on bruised fruits. High levels of patulin can be detected in fruit juices made from contaminated fruits and may pose a risk to the consumer. The enzymes involved in the biosynthesis of patulin have been well studied but many of the genes involved in the pathway have yet to be cloned and characterised. In order to identify some of these genes a suppression subtractive hybridisation-PCR (SSH-PCR) approach was taken using mRNA from patulin producing and non-producing conditions. A pool of transcripts which were expressed in higher concentrations during patulin production was obtained and a sub-set of these transcripts were subsequently cloned and sequenced. These gene sequences were then compared to sequences in the NCBI database and several clones displaying significant similarity to previously cloned cytochrome p450 monooxygenase genes were identified. Previous biochemical work has implicated the involvement of cytochrome p450 monooxygenases in the pathway leading to the production of patulin from the polyketide 6-methylsalicylic acid; particularly in the hydroxylation of m-cresol to m-hydroxybenzyl alcohol (1). and in the conversion of m-hydroxybenzyl alcohol to m-hydroxybenzaldehyde (2,3). Expression of the genes encoding these cytochrome p450 monooxygenases is up-regulated during patulin production in P. expansum indicating their involvement in the biosynthesis of this mycotoxin in the fungus.

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PATHWAY-SPECIFIC REGULATION OF GIBBERELLIN BIOSYNTHESIS IN GIBBERELLA FUJIKUROI–A NEW TYPE OF ZINC-FINGER TRANSCRIPTION FACTORS

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The gibberellins (GAs) are a group of phytohormones, which are produced by the rice pathogen Gibberella fujikuroi. Recently we cloned and characterized all seven biosynthetic genes which are organized in a gene cluster (1-3). However, in contrast to some secondary metabolite gene clusters of other fungi, no putative regulatory genes were found in the GA gene cluster or in the neighbourhood.

GA biosynthesis in G. fujikuroi is highly depended on nitrogen availability. We have shown by promoter studies using the GUS reporter system that AREA is required for the activation of the GA biosynthetic genes (4). Beside two double GATA sequence elements we found a 30 bp sequence stretch which is a putative binding site for a yet unknown pathway-specific transcription factor. Deletion or mutation of this motif resulted in a 80% reduction of uidA gene expression. In order to identify the corresponding transcription factor, this 30 bp element was used as “bait” in an one-hybrid approach. After several control experiments, only two positive yeast clones were obtained. Both clones contained vectors with fragments of the same gene. Gel retardation experiments using total protein extracts of a positive and a negative (control) yeast clone, and the 30 bp oligonucleotide as labeled probe confirmed that only the protein from the positive clone specifically interacted with the oligonucleotide resulting in the expected band shift. The newly identified gene was shown to be a single copy gene which is constitutively expressed on a low level. Sequence analysis revealed the highest homology to the Mus musculus Kruppel-like zinc-finger protein (30% sequence identity). So far, no fungal genes of this group of transcription factors are known.
To confirm that the identified gene encodes indeed a GA pathway-specific transcription factor, gene replacement-and–overexpression vectors were constructed. The transformation experiments are in preparation.

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Genetic and molecular aspects of sulfate transport in Aspergillus nidulans

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The newly isolated A. nidulans mutant impaired in the promoter region of the sulfate permease gene (sB^{pr}) was transformed with three different A. nidulans gene libraries to clone the sB gene. The sB gene was not found but three genes suppressing the sB^{pr} mutation were identified.
The first one, designated astA (alternative sulfate transporter), encodes a protein belonging to an extensive and poorly characterised family of fungal organic anion transporters. Interestingly, the astA gene was found in the A. nidulans IAM 2006 strain isolated in Japan, while in the standard strains of Glasgow origin it was shown to be a pseudogene containing a transposable element. The astA gene appears to be regulated by the sulfur metabolite repression system and the ASTA protein substitutes for the sulfate permease in sulfate transport.
In the second case suppression is brought about by overexpression of the metR gene encoding a sulfur specific transcription factor (Natorff et al., 2003).

The third gene (temporarily designated neo) complementing the sB^{pr} mutation encodes a transmembrane protein of unknown function, apparently specific for filamentous fungi. The protein shows weak similarity to calcineurin and G protein coupled receptors. The gene is not regulated by sulfur sources at least on the transcriptional level. Characteristics of the astA and neo genes will be presented.

VIIp-15

GENE-TARGETING IN ACREMONIUM CHRYSOGENUM

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Currently, genetic transformation of *Acremonium chrysogenum* is mainly based on ectopic integration of the gene of interest due to a low homologous recombination rate (about 0.5%) in this fungus. The resulting transformants show a broad range of expression of the gene of interest, which can be explained by locus-specific effects of the different integration loci. Nevertheless, for many studies it is important to avoid such locus-specific variation. Based on an *A. chrysogenum* mutant strain defective in *cefG* (encoding deacetylcephalosporin C acetyltransferase), which is essential for biosynthesis of the ß-lactam antibiotic cephalosporin, different gene targeting strategies have been established. Thereby, the cephalosporin biosynthetic pathway was complemented by re-introduction of *cephG* and the effect of genetic transformation was analysed by scoring Cephalosporin C production. Surprisingly, preliminary results suggest that genotypically identical transformants display an up to three-fold variation of Cephalosporin C production possibly indicating effects of the transformation process *per se*.

VIIp-16

PUTATIVE NADPH - CYTOCHROME P450 REDUCATSE GENES OF THE FILAMENTOUS FUNGUS COCHLIOBOLUS LUNATUS

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The filamentous fungus Cochliobolus lunatus is an important phytopathogen, capable of 11b-hydroxylation of steroids, which is a key step in the production of corticosteroids. The enzymes responsible for steroid bioconversion by filamentous fungi belong to the cytochrome P450 superfamily. These are part of ER electron transport systems and accept electrons from NADPH – cytochrome P450 reductase (CPR).

We designed degenerate oligonucleotide polymerase chain reaction primers for amplifiying CPR using the CODEHOP (Consensus - Degenerate Oligonucleotide Primers) method. The program offers an alternative primer design strategy, for amplifying unkown target genes, distantly related to multiply aligned protein sequences.
With this approach, we obtained fragments covering a substantial part of the coding sequence of two different putative CPR genes. The BLAST database search against the contigs, assembled from these fragments, revealed high homology with fungal CPRs. The CPR fragments will be used as probes in screening genomic or cDNA libraries, to obtain the entire sequence of cytochrome P450 reductase(s).

The obtained CPR sequences will also be used in further attempts to clone the steroid 11β-hydroxylase of C. lunatus, which has not yet been identified, inspite of its biotechnological importance.

FILAMENTOUS FUNGAL PHOSPHOPANTETHEINYL TRANSFERASES INVOLVED IN PRIMARY AND SECONDARY METABOLISM

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The genomes of filamentous fungi contain a large number of phosphopantetheine (ppant)-dependent pathways of primary and secondary metabolism, all requiring the post-translational addition of ppant into the carrier protein (CP) domains of multienzyme complexes by 4′-phosphopantetheinyl transferases (PPTases). While many PPTases were identified some time ago in prokaryotes, only a few have been identified in eukaryotes. In *Aspergillus nidulans npgA* is required for conidial pigment formation (**1, 2**), penicillin and siderophore biosynthesis (**2, 3**). We speculated that *npgA* encoded a broad-specificity PPTase essential for phosphopantetheinylation of carrier proteins of primary metabolism such as the α-aminoadipate reductase (AAR), a step in lysine biosynthesis. The full length NpgA was overexpressed in *Escherichia coli* as hexahistidine (6xHis) fusion and purified by nickel affinity chromatography. Recombinant NpgA and protein extract from *S. cerevisiae* strain YGL154c (*lys2*) were assayed for AAR activity in the presence of 200 µM CoA. The sample exhibited a significant AAR activity, demonstrating that NpgA is a functional PPTase that accepts a heterologous CP as substrate. The overexpression of 6xHis tagged *Candida albicans* Lys2 in *E. coli* provided a purified substrate to assay the PPTase activity of recombinant NpgA *in vitro* (**4**). The coupled approach led to a 9 fold increase in AAR activity. This experiment shows that NpgA is a functional PPTase which efficiently recognises and modifies a CP involved in primary metabolism. Another substrate for a PPTase is the small mitochondrial protein Acp1. In *S. cerevisiae*, a specific PPTase named Ppt2 activates Acp1 (**5**). When Ppt2 was used as a probe for a Blast search of *A. nidulans* and *A. fumigatus* genomes, no homologues were detected suggesting that NpgA was the only PPTase present in Aspergillus (**2**). With the completion of *A. nidulans* genome sequence project, we searched again using the deduced amino acid sequence of *Clostridium acetobutylicum acpS* and an acpS consensus sequence as probes. We identified an amino acid sequence containing conserved motifs among the acpS type PPTases, that has been preliminarily named *ppt2*-like gene. Fungal databases were searched for homologues using the same probes and deduced amino acid sequences of the *A. nidulans* Ppt2. We also found an acpS-type PPTase in *C. albicans* genome; the deduced amino acid sequence of this PPTase resembles the sequence of *S. cerevisiae* Ppt2 (24% identical amino acids). The deduced amino acid sequences of the filamentous fungal Ppt2 lack significant similarities with *S. cerevisiae* and *C. albicans* Ppt2. The filamentous fungal Ppt2 might be involved in ppant modification of acyl CPs of the primary metabolism. We suggest that the target for filamentous fungal Ppt2 is the mitochondrial Acp1 whereas NpgA and its homologues provide the catalytic ppant for Lys2 of primary metabolism and for all CPs of secondary metabolism. Progress on the generation and analysis of *npgA* and *"ppt2"* conditional mutants will be presented.

References

(**1**) Kim et al. (1999) *XX Fungal Genetics Conference.*

THE *PENICILLIUM PAXILLI* GENE CLUSTER AND BIOSYNTHETIC PATHWAY FOR THE PRODUCTION OF THE INDOLE-DITERPENE PAXILLINE.

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The indole-diterpenes are a large, structurally diverse group of natural products principally found in filamentous fungi of the genera *Penicillium*, *Apergillus*, *Claviceps* and *Neotyphodium*. Many of these compounds are potent mammalian tremorgens while others are known to confer anti-insect activity. The production of the indole-diterpene paxilline by *Penicillium paxilli* has served as a key experimental system in the elucidation of the genetics and biochemistry behind the biosynthesis of this important and interesting class of secondary metabolites. The *P. paxilli paxG* gene, which encodes for a geranylgeranyl pyrophosphate (GGPP) synthase, was previously shown to be required for paxilline production and has been proposed to represent the first committed step of paxilline biosynthesis (Young *et al*., 2001). It was also evident that *paxG* is located within a paxilline biosynthetic gene cluster on chromosome V of *P. paxilli* (Young *et al*., 2001).

Subsequent detailed analysis of this region, presented here, using various techniques including targetted gene deletions has confirmed five adjacent genes, *paxG*, *paxM*, *paxC*, *paxP* and *paxQ*, to be required for paxilline biosynthesis. PaxM, a FAD-dependent monooxygenase, and PaxC, a prenyl transferase, are required for the production of paspaline from GGPP. The conversion of paspaline to PC-M6 requires the cytochrome P450 monooxygenase, PaxP (McMillan *et. al.,* 2003). The pathway may branch at this point with PC-M6 converted to β-paxitriol or 13-desoxypaxilline via the activity of a second P450 enzyme, PaxQ, and an as yet unidentified protein, respectively. PaxQ, is also thought to be required for the conversion of 13-desoxypaxilline to paxilline as the deletion of *paxQ* results in the accumulation of 13-desoxypaxilline (McMillan *et. al.,* 2003). Analysis of a putative dehydrogenase encoding gene located 12 kb downstream from *paxG* and a candidate for a role in the latter steps of paxilline biosynthesis is also presented.

Young, C., McMillan, L., Telfer, Scott, B (2001) Mol. Microbiol. 39: 754-764

VIIp-19

A GENETIC STUDY TO CHARACTERISE THE RELEASE OF VOLATILE THIOLS BY SACCHAROMYCES CEREVISIAE DURING WINE FERMENTATION

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Abstract
Volatile thiols are an important contributor to wine varietal aroma. A non-volatile precurser for one of the most potent thiols, 4-mercapto-4-methylpentant-2-one (4MMP) has been identified as a cysteine-bound conjugate which is released by S. cerevisiae during the alcoholic fermentation of grape juice. Cleavage of the precurser by an unknown yeast enzymatic mechanism results in the volatile 4MMP being released. A method to measure 4MMP was developed by gas chromatography- atomic emission detection (GC-AED), which provides a sensitive and selective method for measuring sulfur compounds. In this study, S. cerevisiae deletion strains were assayed for their ability to release 4MMP from the synthesised non-volatile conjugate. Four of the deletion strains showed reduced capacity to release 4MMP, and the corresponding genes were cloned into yeast expression plasmids for overexpression. Involvement of these genes in the release of the thiols in an industrial wine yeast was investigated by replacement of the wild type gene with a KanMX4 deletion cassette. These modified wine yeast were assayed for their ability to release 4MMP. Purification of the corresponding enzymes will clarify the role that each specific enzyme has in the release of 4MMP.

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CATALYTIC REACTION OF BASIDIOMYCETE LENTINULA EDODES CYTOCHROME P450 ENZYME PRODUCED IN YEAST

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We isolated three cytochrome P450 genes, named Le.cyp1, Le.cyp2, and Le.cyp3 from Lentinula edodes. Differently from Le.cyp1 and Le.cyp2, Le.cyp3 was found to be a defective gene. The deduced amino acid (aa) sequences of Le.CYP1 (CYP510A1, 500 aa) and Le.CYP2 (CYP510A3, 499 aa) were highly similar to each other (87% identical) and they had 32–33% identities to that of the basidiomycete Coprinus cinereus P450 (CYP502) and 27–28% identities to those of two Aspergillus P450s (CYP64 family). Quantitative RT-PCR analysis of the transcripts of Le.cyp1 and Le.cyp2 genes showed that these genes are not constantly transcribed in the course of fruiting-body development and in parts of fruiting body of L. edodes; primordium appeared to contain larger amounts of these transcripts, and the transcript levels of these genes in the stipe of the premature fruiting body were higher than those in the whole pileus and gill tissue. To investigate the catalytic property of L. edodes P450, we, as a first step, functionally expressed Le.CYP1 in S. cerevisiae, of which gene Le.cyp1 showed higher transcript level than Le.cyp2. The microsomal fraction containing Le.CYP1 was prepared from the recombinant yeast and the Le.CYP1 was analyzed. The 7-ethoxycoumarin and benzo(a)pyrene were found to be the substrate of Le.CYP1 enzyme. Le.CYP1 converted 7-ethoxycoumarin to 7-hydroxycoumarin.

MOLECULAR ANALYSIS OF GLYCOLIPID PRODUCTION IN USTILAGO MAYDIS

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Under conditions of nitrogen starvation, Ustilago maydis secretes large amounts of amphipathic glycolipids. These surface active compounds can be grouped into two classes: ustilipids, derived from mannosylerythritol framework and ustilagic acid, derived from cellobiose. The ustilipids consist of a 4-O-β-D-mannopyranosyl D-erythritol which is esterified with palmitoic acid and shorter acyl groups. Ustilagic acid consists of a cellobiose moiety O-glycosidically linked to the ω-hydroxyl group of 15,16-dihydroxy-hexadecanoic-acid. Ustilagic acid has antibiotic properties and shows some hemolytic activity on blood agar plates. Whereas ustilagic acid forms needlelike cristals in liquid culture, the mannosylerythritol lipids are secreted as an extracellular oil. The oil production can be followed on charcoal containing agar plates where the spreading of ustilipids can be observed around U. maydis colonies.

To elucidate the biological function of these amphipathic substances, we screened for UV mutants which were defected in glycolipid production. After UV treatment cells were grown on blood agar plates and analyzed for their hemolytic activity and for the presence of oil rings around colonies on charcoal plates. We were able to identify mutants defective in mannosylerythritol lipid production. We could demonstrate the complete loss of mannositolerythritol-lipid production by thin layer chromatography. We were able to complement one of the mutants using a cosmid library.
In addition, we used a reverse genetic screen and tested potential glycosyltransferases which have been identified in the genomic sequence of Ustilago maydis. We could generate a mutant defective in ustilipid production by deleting a glycosyl transferase which shows similarity with macrolide glycosyltransferases of prokarytic origin. We are currently trying to identify additional genes involved in this secondary metabolism.

DISCOVERY OF NOVEL POLYKETIDE SYNTHASES IN FILAMENTOUS FUNGI

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Polyketides (PK) constitute a large class of natural compounds of which several have found use as high value pharmaceuticals. Additional structural diversification of PKs is likely to yield new products with new or improved capabilities. One way to obtain altered PKs is to engineer the polyketide synthase (PKS) genes that produce PKs in both bacteria and fungi. Relatively few fungal PKSs have been investigated, perhaps due to the lack of genetic techniques to isolate and manipulate gene clusters from different strains. In the search for novel valuable compounds, we use selected fungi from a strain collection of more than 30.000 fungal strains of which the Aspergillus and Penicillium species constitute the main part. In order to identify strains that produce PKs, metabolite profiles resulting from growth on various solid media are determined using HPLC DAD analysis. This procedure, reveals the prescence of PK compounds and the conditions necessary for production. Once identified, the strains are further screened by PCR using degenerated primers designed to anneal to conserved PKS sequences. The resulting PCR products are used as probes to identify the chromosomes hosting the putative PKS genes and ultimately to probe a strain specific BAC library of the relevant chromosomes. The BAC clones will eventually be expressed and characterized in surrogate hosts like Aspergillus nidulans or Aspergillus niger.

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INVESTIGATING NON-RBOSOMAL PEPTIDE SYNTHETASE GENES AND THEIR FUNCTION IN THE WHEAT PATHOGEN MYCOSPHAERELLA GRAMINICOLA

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The ascomycete fungus Mycosphaerella graminicola is the causative agent of Septoria Leaf Blotch on bread and durum wheat. Its significance has risen in recent years, due to the alteration of post-harvest farming practices. Today, it is an economically important pathogen, as infection results in reduced grain filling and yield loss.

M. graminicola penetrates the plant mainly through stomatal openings of the leaves, where it grows intracellularly, seemingly undetected by the plant. When a critical mass is reached, pycnidia formation is triggered accompanied by simultaneous plant mesophyll cell breakdown, causing chlorosis and necrosis of the leaf. Only a limited amount is known about the genes and their products controlling the complex molecular mechanisms involved in the infection process.

Recently, non-ribosomal peptide synthetase (NRPS) genes have been found in the M. graminicola genome. NRPS genes encode large multi-modular enzymes that synthesize small biologically active peptides. NRPS genes are significant pathogenicity determinants in other fungal pathogens, since they often encode enzymes required for toxin synthesis and nutrient uptake. Examples include synthetase genes for HC toxin from Cochiobolus carbonum, AM toxin of Alternaria sp. and the ferrichrome synthetase of Ustilago maydis.

Individual modules of NRPS genes contain areas of high homology and so can be amplified using degenerate PCR techniques. In our laboratory we have isolated eight unique NRPS modules using these techniques, in addition to three EST sequences found in infected leaf material cDNA libraries. Here, we present our research on the characterisation of these amplified modules and the genes corresponding to the EST sequences. In addition, we also evaluate the use of an Agrobacterium tumefaciens-mediated transformation system to disrupt NRPS genes in M. graminicola.

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REAL-TIME PCR FOR MONITORING ASPERGILLUS CARBONARIUS COLONIZATION IN SAMPLE OF GRAPE IN EUROPE

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Secondary metabolism
Species of Aspergillus belonging to section Nigri (Gams *et al*., 1985) are commonly known as black aspergilli and have a significant impact on modern society for their capability to produce different hydrolytic enzymes (amylases, lipases) and organic acid (citric acid, gluconic acid) useful for the fermentation industry and to cause food spoilage as well as to produce dangerous mycotoxins (Bennet and Klich, 1992; Kozakiewicz, 1989). In this respect A. carbonarius (Bainier) Thom. and A. niger aggregate Al Musallam were reported as Ochratoxin A (OTA) producers (Abarca *et al*., 1994; Téren *et al*., 1996; Varga *et al*., 1996) a higly harmful metabolite classified in 1993 by the International Agency for Research on Cancer as a possible human carcinogenic toxin (group 2B) (IARC, 1993). OTA has been shown to be genotoxic, teratogenic, immunotoxic, cytotoxic and nephrotoxic (Kuiper-Goodman, 1996). Recently, the contamination of grape and by-products by OTA has emerged as a deep problem for the health risk related to the consumption of such products by humans (Otteneder and Majerus, 2000; Zimmerli and Dick, 1996; Visconti et al 1999). A review on the presence of Ochratoxin A in grapes and wine underlined the importance of A. carbonarius as the main responsible for the OTA accumulation in wine (Battilani and Pietri, 2002). For the estimation of food quality or for the monitoring of the influence of hygienic measures upon the amount of fungi present in a food samples, a reliable rapid quantification system is important. A real time PCR assay was set up using primers designed from calmodulin gene for the quantification of *Aspergillus carbonarius* in sample of grape by measuring the *A. carbonarius* DNA content in grapes. To calculate the recovery rate of DNA isolation procedure, DNA from grape were inoculated with known concentrations of spores of A. carbonarius and analysed by real time PCR. The real time data were compared to the spore number and to the amount of ergosterol of mould. 150 natural grape samples from six different European countries, were collected in at least 10 representative vineyards, in 3 growth stages, starting from grape setting until ripening, and during storage for table grapes. After removing stem and crushing, total DNA was extracted from 300 mg of skin and flesh of fruit. The amount of *A. carbonarius*-DNA ranged from not detectable to 70ng/mg.

GENETIC AND BIOCHEMICAL STUDIES ON THE MECHANISMS OF AFLATOXIN BIOSYNTHESIS

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Aflatoxins B_1, B_2, G_1 and G_2 are secondary metabolites produced primarily by the filamentous fungi *Aspergillus flavus* and *A. parasiticus*. These toxins are the most carcinogenic and toxic natural compounds that contaminate foods and feed. Due to the health risk of aflatoxins posed on human and livestock, research on the genetics and biochemistry of aflatoxin biosynthesis has been carried out in great detail. The biosynthesis of aflatoxins is a multi-enzymatic process consisting of at least 16 structurally defined intermediates. Studies on the molecular mechanism of aflatoxin biosynthesis in this laboratory have identified an aflatoxin pathway gene cluster of 70 kilobase pairs in length consisting of at least 25 identified genes including a positive regulatory gene as transcription activator. In addition, a sugar utilization gene cluster consisting of four genes and a nitrogen utilization gene cluster consisting of two genes have also been cloned. The completed DNA sequence of the aflatoxin gene cluster has been determined and the genes involved in aflatoxin formation have been systematically renamed from *aflA* to *aflY* according to the gene convention in *Aspergillus*. They are: *aflA* (*fas-2*), *aflB* (*fas-1*), *aflC* (*pksA*), *aflD* (*nor-1*), *aflE* (*norA*), *aflF* (*norB*), *aflG* (*avnA*), *aflH* (*adhA*), *aflI* (*avfA*), *aflJ* (*estA*), *aflK* (*vbs*), *aflL* (*verB*), *aflM* (*ver-1*), *aflN* (*verA*), *aflO* (*omtB*), *aflP* (*omtA*), *aflQ* (*ordA*), *aflR* (*aflR*), *aflS* (*aflJ*), *aflT* (*aflT*), *aflU* (*cypA*), *aflV* (*cypX*), *aflW* (*moxY*), *aflX*, *aflY*. Gene profiling studies in *A. flavus* by Expressed Sequenced Tag (EST) and microarray have identified many genes that are potentially involved in aflatoxin formation among over 7,000 unique ESTs sequenced. These include genes directly involved in aflatoxin biosynthesis; regulation and signal transduction; genes that have the potential to contribute to fungal virulence or pathogeneicity; and genes involved in fungal development. Functional genomics studies using microarray under different conditions are underway. The goal of this research is aimed at providing information for developing new strategies for control of aflatoxin contamination of agricultural commodities. The functions of these pathway genes, their expressional regulation and cluster organization in comparison with the sterigmatocystin pathway gene cluster in *A. nidulans* will be presented.

CHARACTERISING OXYLIPINS SYNTHESIS AND FLAVOUR BIOGENESIS IN MUSHROOMS.

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The production of 8-carbon volatiles production occurs in many fungi, resulting in their characteristic odour. However very little is known on the biochemical mechanisms of 8-carbon volatiles synthesis, or its regulation. The oxylipin 1-octen-3-ol, a major 8-carbon volatile, is derived from first the oxygenation and then the cleavage of a polyunsaturated fatty acid: linoleic acid. This cleavage reaction has similarities to the plant system, but also major differences. There are two possible types of enzymes to perform the reaction of oxygenation: lipoxygenases (non-heme dioxygenases), present in plants, fungi and mammals, or heme-dioxygenases, present in mammals (the cyclooxygenase family) and in fungi, with the recent discovery of the enzyme linoleate diol synthase in the fungus *Gaeumannomyces graminis.* Although both enzymes are candidates to catalyse the reaction, an examination of the enzymic mechanisms and fatty acid chemistry suggests that a heme-dioxygenase is more likely to be involved in the production of 1-octen-3-ol.
Using a range of techniques (PCRs, bioinformatics, chemical analysis and functional genetics), we are characterising the 8-carbon volatile synthesis pathway in *Agaricus bisporus* (the white button mushroom) to gain a better understanding of mushroom flavour biogenesis and lipid metabolism.

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GENETIC ENGINEERING OF THE ZYGOMYCETE BLAKESLEA TRISPORA FOR IMPROVED CAROTENOID BIOSYNTHESIS – TRANSFORMATION USING AGROBACTERIUM TUMEFACIENS

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Carotenoids fulfil essential biological functions and have important beneficial effects on human health, e.g., as antioxidants or as potential antitumor agents. They are used commercially as food colours and animal feed supplements. To date, carotenoids are mainly produced by chemical synthesis, but due to increasing preference of natural products, the extraction of carotenoids from natural sources is becomimg an interesting alternative. The zygomycete Blakeslea trispora is used as natural source for beta-carotene production. A major drawback of B. trispora is the lack of a transformation system until now which allowed both to increase the production of beta-carotene and to produce interesting derivatives. In general, stable transformation of zygomycetes was rarely described and appears to be difficult due to unknown biological reasons. Consistently, all efforts to transform B. trispora via electroporation or protoplast formation with exogenous DNA were not successful. Here, we report the transformation of B. trispora using the gram-negative bacterium Agrobacterium tumefaciens. T-DNA mediated gene transfer in B. trispora via A. tumefaciens strain LBA4404 was achieved by placing the Escherichia coli hygromycin resistance gene (hygromycin B phosphotransferase gene hph) under control of the Aspergillus nidulans gpdA promoter. A. tumefaciens was able to introduce DNA into intact fungal cells such as hyphae and germinating spores which circumvents protoplast generation. In an additional approach the B. trispora tef1 promoter was used to control the expression of the crtZ gene of the green algae Haematococcus pluvealis leading to production of zeaxhantin in transgenic B. trispora cultures.

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ABSCISIC ACID BIOSYNTHESIS IN BOTRYTIS CINEREA

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The ascomycete Botrytis cinerea is the causive agent of the grey mould disease in more than 200 plant species. Like several other phytopathogenic fungi, it is known to produce different kinds of phytohormones in axenic culture, among them the sesquiterpenoid abscisic acid (ABA). Although the impact of this capacity is still unclear, it has been postulated that it could play a role in plant-pathogen interaction.
In higher plants, ABA is derived from cleavage products of carotenoids, whereas fungi seem to synthesise this hormone directly from farnesyl diphosphate via different oxidative steps.

In the ABA producing B. cinerea strain ATCC 58025, inactivation of the NADPH-cytochrome P450 oxidoreductase encoding gene bccpr1 drastically reduced ABA production, strongly suggesting the involvement of P450 monooxygenases in its biosynthetic pathway. Therefore, 28 different putative P450 monooxygenase encoding genes were cloned and characterised. Two of them were differentially expressed in ABA producing mycelium and induced after addition of the ABA precursor mevalonic acid to the medium. While inactivation of one of them (P450-12) did not affect ABA biosynthesis, knocking out the second one (bcaba1) completely abolished ABA production. Thus, bcaba1 represents the first fungal gene identified so far which is involved in biosynthesis of abscisic acid.
Neighbourhood analysis of bcaba1 revealed at least two genes that are possible further candidates for ABA biosynthetic genes indicating a putative gene cluster.

GLUCOSE REGULATION AT THE pacC1-creA1-pacC2 SITE OF THE PENICILLIUM CHRYSOGENUM pcbAB PROMOTER IS INFLUENCED BY ALKALINE pH. INITIAL EVIDENCE FOR INTERACTING GLUCOSE AND pH CONTROL IN PENICILLIN BIOSYNTHESIS.

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In Penicillium chrysogenum, the penicillin biosynthesis pathway is strongly regulated by carbon source. The first two genes of this pathway, pcbAB and pcbC, are expressed from a 1.1 kb bi-directional promoter region. In this region, there are several creA and pacC sequences that are believed to be involved in the regulation of both genes. CreA is a transcriptional factor involved in the regulation by carbon source in filamentous fungi. In the promoter region, there are six putative creA sites. It is unknown if all of these creA sites are functional or not.
Deletion of the creA-1 site showed that the reporter-gene expression level was about 300% higher than in the entire promoter when glucose is used as carbon source. Point mutations were made to test if this effect was due to the binding of CreA-protein to this site or to a conformational change in DNA which affects the binding of other factor, like the PacC protein.
There are two PacC sites very close to the creA-1 box, in both sides of this sequence. Point creA-1 mutations showed a similar effect to the deletion of creA-1 site, so the creA-1 site is involved in the pcbAB gene regulation. Further experiments were performed to study the behaviour of these two types of mutation (deletion and point mutation) in pH fermentor cultures. Our results showed that there is a carbon source regulation by glucose and a pH regulation stronger than the first one at this site. At alkaline pH, the carbon source effect at creA-1 site is reversed by the positive action of PacC.

GENETIC TRANSFORMATION OF MONASCUS RUBER KCTC6122

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The transformation protocol was established for Monascus ruber KCTC6122 as the first step for genetic dissection of biosynthesis of secondary metabolites. Monascus sp. produces various useful secondary metabolites such as pigments, monakolin K, and blood pressure-lowering substance etc, but also nephrotoxic citrinin, which limit application of Monascus for production of valuable substances. To develop the strains over producing useful substances but not producing citrinin, optimal conditions for protoplast formation and regeneration, and transformation by electroporation were established.
Conidia collected from cultures grown on C medium for 7-8 days at 28°C were germinated until over 80% germ tube reached to 3-5 conidia length. Over 80% of germlings were converted to protoplasts after 2 h treatment of 50 mg/ml glucanex in 0.6 M glycerol, 0.1 M citrate buffer. However, protoplast regeneration and transformation efficiency were the best with 30-40% protoplast formation rate. Proptoplasts were successfully transformed into hygromycin B resistance with 20 μg of linear DNA of hygromycin B phosphotransferase fused between the Aspergillus nidulans trpC promoter and terminator by electroporation yielding more than 700 transformants. This working transformation protocol will enable us to study M. ruber genetically for understanding of biosynthesis of secondary metabolites.

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CONSTRUCTION OF α-aminoadipyl-cysteinyl-valine (ACV) HYBRID SYNTHETASES

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Penicillium chrysogenum ACV synthetase, encoded by the pcbAB gene is a modular protein involved in the first step of the penicillin biosynthetic pathway, catalysing the non-ribosomal synthesis of the tripeptide δ(L-α-aminoadipyl)-L-cysteinyl-L-valine (ACV). The ACV synthetase consists of three modules involved in activation of a-aminoadipic, L-cysteine and L-valine. To define the linker regions between modules and to obtain hybrid ACV synthetases, two plasmids were constructed, pACV-A and pACV-CV. pACV-A, is an autonomous replicating plasmid carrying the a-aminoadipyl module and pACV-CV is an integrative plasmid that contains the cysteinyl and valine modules.
Co-transformation of P. chrysogenum T33 (a strain without the pcbAB gene) was performed with these plasmids. 125 transformants were tested by bioassay and 24 showed different levels of penicillin production. E. coli DH10B strain was transformed with DNA genomic obtained from penicillin production co-transformants. Then, several plasmid were selected: pACV-A and four types of recombinants plasmids, called I, II, III and IV. All of them contained a recombinant pcbAB gene.
The T33 strain was re-transformed with I, II and III type plasmids and the transformants obtained produced penicillin, indicating that these plasmids are responsible for antibiotic biosynthesis. However, the assay with type IV plasmid was negative. Penicillin production was obtained only when T33 strain was co-transformed with both plasmids type IV and pACV-A. An heterodimeric complementation of ACV synthetase system appears to be functional in this case.

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ANTISENCE INHIBITION OF XYLITOL DEHYDROGENASE GENE, XDH1, FROM *TRICHODERMA REESEI*

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Session: Biotechnology

Xylitol dehydrogenase secreted by the cellulolytic filamentous fungus *Trichoderma reesei* is one of the key enzymes responsible for converting D-xylose into D-xylulose. Here we examined the effectiveness of *xdh* antisense RNA strategies for metabolic engineering of *Trichoderma reesei.*

Using *gpdA* promoter and *trpC* terminator from *Aspergillus nidulans*, the *xdh1* antisense RNA expression vector pGTA-xdh was constructed (Fig.1). Co-transformation of pGTA-xdh and pAN7-1 into *T. reesei* protoplasts was carried out. Of 16 transformants screened on 100μg/ml Hygromycin B plates, one transformant, designated Rut LT15, showed distinctly reduction (about 29 percent) of xylitol dehydrogenase activity compared with Rut C30. The result of southern hybridization indicated that expression cassette of antisense DNA of *xdh* integrated into its genome as two copies. After 4 days incubation in 2% xylose MM medium, Xylitol accumulation by Rut LT15 was 2.37mg/ml, enhanced 5 times than control exhibited. The xylitol accumulation by mutated strain was achieved without affecting growth parameters of the fungus. The results indicated that the antisense technique was successfully employed to suppress the gene expression of xylitol dehydrogenase in *T. reesei.*

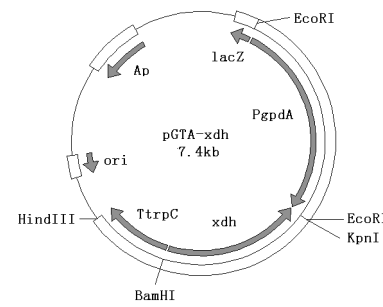

Fig. 1. The physical map of pGTA-xdh. The promoter of *A. nidulans gpdA* and the terminator of *trp*C from
plasmid pAN7-1 were inserted into pUC19 backbone to construct the antisense expression vector. A 1.4 kb fragment containing the *T. reesei xdh1* gene from *T. reesei* was inserted at reverse direction between the promoter of *gpdA* and the terminator of *trpC.*

sFungal secondary metabolites from the Anhydro-fructose Pathway operative under stress conditions show antioxidant and antimicrobial activities

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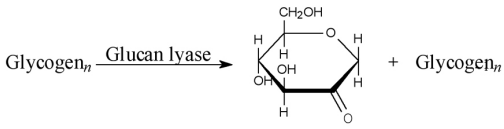
At Danisco Innovation in Copenhagen, we have discovered a new glycogen degrading pathway in fungi, namely, ***the Anhydrofructose Pathway***. In this pathway glycogen is broken to 1,5-anhydro-D-fructose by a novel polymer degrading enzyme called α-1,4-glucan lyase (EC 4. 2. 2. 13)(see figure below).



Where n *indicates the number of glucose units in the glycogen molecule.*

We have purified and cloned several glucan lyase genes from filamentous fungi, such as the morells *Morchella vulgaris* and *M. costata*. We have succeeded in expressing the glucan lyase gene in *Aspergillus niger* and *Hansenula polymorpha*. Furthermore, the 1,5-anhydrofructose produced enzymatically by the glucan lyase was used as substrate to produce a series of fungal metabolites, such as ascopyrone P. Ascopyrone P showed both antioxidant activity and antimicrobial activity in *in vitro* tests and in food model tests. In the meeting presentation on the alternative glycogen degrading pathway, i.e., the Anhydrofructose Pathway and its physiological importance in the filamentous fungi will be discussed.

KEY WORDS: Anhydrofructose Pathway, glucan lyase, anhydrofructose, ascopyrone P, antioxidant, antimicrobial, glycogen degradation.

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REGULATION OF THE CAROTENOID GENE CLUSTER OF FUSARIUM FUJIKUROI

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The fungus Fusarium fujikuroi (Gibberella fujikuroi, mating group C), has a rich secondary metabolism. Among other compounds, this fungus produces terpenoids, such as gibberellins and carotenoids, or polyketides, such as fusarins and bikaverin. Gibberellins are growth–promoting plant hormones produced upon nitrogen starvation. Carotenoids are pigments whose synthesis is induced in moderate amounts by light and in large amounts by mutations in the gene carS. The products of the genes carRA and carB are responsible of the biosynthesis of torulene, precursor of the main product of the pathway, the xanthophyll neurosporaxanthin. The gene coding for the enzyme achieving the conversion of torulene to neurosporaxanthin has not been identified.

Transcription of the genes carRA and carB is induced by light, and is deregulated in overproducing mutants. Contiguous to carRA and carB we found a gene with similarity to opsins, called carO, which is subject of a similar transcriptional regulation. Because the ability of opsins to detect light through a retinal prostetic group, a possible role of carO on carot-enoid regulation was presumed. Targeted mutagenesis of carO, did not produce any apparent phenotype in carotenoid biosynthesis, indicating a role independent of the carotenoid pathway.

Complementation analysis between 18 carotenoid overproducing mutants showed that all of them are affected in a single locus, that we call carS. Mutant searches for albino strains led to the isolation of several deletion mutants affecting the region of the carRA/carB gene cluster. Complementation between a carS strain and at least one deletion mutant suggests the linkage of carS to carRA and carB. Experiments to identify carS in the surrounding DNA are in progress. A search of genes regulated by carS has been done by differential display of gene expression in the wild type and a carS mutant.

Using a PCR approach, an internal segment of a gene of F. fujikuroi coding for a protein highly similar to the WC-1 photoreceptor of Neuro-spora crassa has been isolated. Northern blot and gene disruption experiments to determine the eventual role of this gene in the regulation of the carotenoid genes of F. fujikuroi are under way.