Molecular aspects of sex and trisporoid signaling in mucoralean fungi

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“Twenty years from now you will be more disappointed by the things that you didn’t do than by the ones you did so. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover.” - Mark Twain
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Abbreviations

APCI- Atmospheric pressure chemical ionisation
bp- base pairs
Bt- *Blakeslea trispora*
cDNA- complementary DNA
cm- centimeter
Ct- threshold cycle
°C- degree Celsius
DEPC- Diethylpyrocarbonate
DNA- Deoxy ribonucleic acid
dNTP- deoxy ribonucleotide diphosphate
dt- deoxy thymine nucleotides
DTT- Dithiothreitol
EDTA- Ethylene diamine tetra acetic acid
FSU- Friedrich Schiller University
g - Gravitational acceleration
LCQ- Liquid chromatography quadrupole
Mcl- *Mucor circinelloides f. lusitanicus*
µl- Microliter
µg/ml- microgram per milliliter
Ma- million years ago
mg- Milligram
mm- millimeter
mM- Millimolar
Mw- molecular weight
NCBI- National Center for Biotechnology Information
ng- Nanogram
OD- optical density
Pb- *Phycomyces blakesleeanus*
qRT-PCR- quantitative reverse transcription Polymerase chain reaction
RNA- Ribonucleic acid
rpm- revolutions per minute
TAE- Tris-acetate
UV- ultra violet light
V- Volt
Vis- Visible light
Wm²- Watts per square meter
w/v- weight per volume
Chapter 1. Introduction

1.1. Fungi foster terrestrial ecosystem

Life on Earth originated around 3.5 billion years ago as per the records on microfossils and thin microbial films/mats formed by photosynthesizing prokaryotes, trapped in mud that form layered rock structure over the years known as stromatolite (Awramik, 2006). In those harsh physical environments, the colonization of terrestrial habitat by eukaryotes probably had been facilitated by the symbiosis between a fungus and a phototroph (Heckman et al., 2001, Selosse & Le Tacon, 1998). Even though most primitive fungi and land plants are not well preserved, fossil records suggest evidence that arbuscular mycorrhizal fungus and land plants had associations dated back to Ordovician era 480 - 460 million years ago (Ma) (Itturiaga et al., 2000, Redecker et al., 2000). Based on well-studied ribosomal gene by the molecular clock data, evolutionary biologists estimated the origin of fungi in Precambrian era (600 Ma) (Heckman et al., 2001). Animals and fungi were grouped into eukaryotic super group Ophistokonta based on small subunit ribosomal RNA (SSUrRNA) evolutionary trees and were diverged from a common unicellular ancestor approximately 1 billion years ago. These multicellular heterotrophs possess uniflagellated reproductive structures (male reproductive cells of animals and zoospores in chytrid fungi) in common (Steenkamp et al., 2006). The early colonization of land by fungi (1000 Ma) and plants (700Ma) possibly enriched the climate and atmosphere habitable for other living forms on Earth, contributing to a rise in oxygen leading to a Cambrian explosion of animal kingdom (Knoll, 1992). Besides, fungi play a key role in biogeochemical cycling of elements, decomposition of inorganic and organic materials or even degrade pollutants in the natural ecosystem through mycoremediation.
1.2. Ecological interactions define the Chemistry in nature

In our day to day life numerous interactions are taking place as all living forms detect, emit and respond to diverse physical and chemical cues which underlie the biotic environment that we live in. “Chemical ecology is a discipline driven by the recognition that organisms of the most diverse kinds, ranging from microbes to mammals make use of chemicals to find mates, recruit symbionts, deter enemies and fend off pathogens” (Eisner & Berenbaum, 2002). Molecular biology exclusively complements this exciting research field as the signal molecules that convey information within or among organisms are genetically controlled through a cascade of molecular events by which the messages are systematically relayed and translated to behavioral, physiological or morphogenetic responses (Meinwald & Eisner, 2008). Understanding the molecular mechanisms of these chemical transmissions and characterization of signaling molecules enable us to infer, the vocabulary of the complex chemical communication in nature meant for the co-existence of diverse living forms. “Fungi are the best model organisms to study environmental sensing as they are simple, but their evolutionarily conserved signal transduction pathways are equivalent to those present in multicellular eukaryotes” (Bahn et al., 2007).

1.3. Chemical communication: a fungal perspective

Fungi comprising of approximately 1.5 million species contribute to a quarter of the total global biomass. They undergo a wide variety of symbiotic interactions with plants, animals and eukaryotic protozoa on a mutualistic, parasitic or lethal association. Social networking in fungi could be conceptualized as a survival strategy by the individual cells forming hyphae to mycelium in order to cope up and adapt to the diverse stimuli through an efficient communication system to maintain harmony in its biological niche.
1.3.1. Co-ordinated growth and development

Majority of the fungi belonging to both Dikarya (Ascomycetes and Basidiomycetes) and basal lineages (arbuscular mycorrhizal fungi, microsporidia, chytrids and zygomycetes) are saprophytic in nature feeding upon dead and decayed organic matter (Leeder et al., 2011). An asexual fungal spore germinates under favorable environmental conditions to a multicellular filament known as hypha. These haploid vegetative hyphae undergo chemotropic interactions and cell fusions to form interconnected networks forming a colony known as mycelium (Carlile et al., 2001). Hyphal network formation is a prerequisite for plant pathogen *Alternaria brassicicola* virulence (Craven et al., 2008) and infection of plant roots by *Fusarium oxysporum* (Rosales & Di Pietro, 2008).

The mitogen activated protein kinase (MAPK) signaling cascade is responsible for virulence (Silar, 2005) and cell fusion (Pandey et al., 2004) in ascomycetes. Nitrogen limitation leads to filamentous growth in yeast *Saccharomyces cerevisiae* and regulate germling and hyphal fusion in *F. oxysporum* (Lopez-Berges et al., 2010) through the protein kinase target of rapamycin (TOR). The circadian rhythm plays a key role in fungal development where a white collar protein WC-1 is the fungal photosensor identified in zygomycetes (Corrochano, 2007), basidiomycetes and ascomycetes (Baker et al., 2012). Quorum sensing enables fungi in its host interactions to maintain an optimal population density for the survival within host (Hogan, 2006). The apocarotenoid sex hormones trisporic acids, act as extracellular signaling molecule during mycoparasitism in zygomycetes while the sesquiterpene farnesol facilitates invasion of human cells by *A. nidulans* (Dinamarco et al., 2011).
1.3.2. Secondary metabolism

The discovery and development of Penicillin in the early 20\textsuperscript{th} century bring forth the potentials of fungal secondary metabolites to the world of biochemistry. Based upon the enzymes involved in their biosynthesis, secondary metabolites of fungal origin are classified to polyketides, non-ribosomal peptides, terpenes and indole alkaloids (Keller \textit{et al.}, 2005). The diterpenes and carotenoids derived from geranyl geranyl pyrophosphate had been extensively studied in the basal lineage of zygomycetes and in ascomycetes fungi. But the biochemical and genetic studies on the genes involved in these biosynthetic pathways are still in their infancy due to the minute quantities of the enzymes produced by the organism or the technical difficulties in carrying out classic genetic approaches with those fungal systems. Although the specific metabolic genes are known to have been clustered along with the regulatory gene for the whole pathway the mechanisms of transcription factor genes that co-regulate these clusters are unclear. \textit{Aspergillus} responds to environmental cues influencing secondary metabolism by signal transmission through Cys\textsubscript{2} His\textsubscript{2} zinc-finger transcription factors that mediate carbon (CreA) (Dowzer & Kelly, 1989), nitrogen (Wong \textit{et al.}, 2009) and pH (PacC) signaling (Tilburn \textit{et al.}, 1995).

1.3.3. Sex and signaling molecules in fungi

The sex in fungi is a very fascinating attribute as the probabilities for sexual interactions are much more diverse than in any other living forms (Dyer, 2008). Sexual development is orchestrated by a genetic locus known as MAT or mating type locus (Ni \textit{et al.}, 2011). In bread mold fungus \textit{Rhizopus} (zygomycetes) or ascomycete \textit{Aspergillus}, mating occurs through outcrossing or heterothallism between 2 members in a species, each having a complementary and defined mating type. Some among them are homothallic possessing both
mating types and hence are self-fertile. Mushrooms and toadstools belonging to basidiomycetes having a tetra polar multiallelic mating system need the presence of different alleles or idiomorphs among two mating loci (MAT A and MAT B) for the sexual phase (Skrede et al., 2013). For a successful mating the products of homeodomain transcription factors encoded by MAT A of one mating partner should interact with that of sex pheromones or receptors encoded by MAT B (Casselton, 2008, Brown & Casselton, 2001). Hence *Schizophyllum commune* has 28,000 different sexes based on diverse combination of alleles present in both mating loci.

Fig.1.3.3.1. Mating in inkcap mushroom *Coprinus cinereus* orchestrated by MAT loci
Coprinus cinereus (Fig.1.3.3.1) and Schizophyllum commune are unique exhibiting thousands of mating types as a pheromone can activate several different receptors and a receptor can be activated by many pheromones (Casselton & Kues, 2007). But pheromone sensing orchestrates only later events of mating like nuclear migration and clamp cell fusion. In the basidiomycete Cryptococcus neoformans, an opportunistic human pathogen, pheromones and their receptors contribute to both opposite and same sex mating (Bahn et al., 2007, Lee et al., 2010, Fraser et al., 2005). The first step in fungal mating is the recognition of two compatible mating partners which further undergo cell fusion or plasmogamy. A nuclear fusion or karyogamy is the next step. But in a few ascomycetes and in basidiomycetes the nuclear fusion extends for a longer time. Finally the dikaryon undergoes fusion and produce haploid recombinant progeny by meiosis (Lee et al., 2010). A cell to cell communication initiates the process of sexual communication in fungi. Complementary cells in S. cerevisiae undergo chemotrophic growth, when small peptide hormones released from male cells binds to G-protein coupled receptors evoking an MAPK cascade (Fig.1.3.3.2) resulting in fruiting body (Li et al., 2007, Brown & Casselton, 2001). The activated homeodomain transcription factor Ste12p induces mating gene expression. After mating, homeoproteins α1 and α1 in the diploid cell undergo dimerization and become a negative regulator ceasing pheromone signaling.

The first fungal pheromones discovered were from aquatic chytrid Allomyces macrogynus, sirenin in female and parisin from male (Kochert.G, 1978). Apocarotenoid trisporic acids and their precursors regulate sexual phase through hyphal fusion of complementary mating partners in basal fungal class of zygomycetes (Gooday, 1978). The variation in the chemical nature of pheromones from simple organic apocarotenoid molecules in basal lineages to
more complex peptide hormones in Dikarya, probably is an evolutionary adaptation of the fungal kingdom to get along with a diverging terrestrial ecosystem.

**Fig.1.3.3.2.** Mating in budding yeast, *Saccharomyces cerevisiae*. Binding of pheromones to the Ste receptors activates a heterodimer G-protein which in turn initiates phosphorylation through mitogen activated phospho kinase cascade signaling.
1.4. Basal zygomycetes fungi belonging to the order Mucorales

1.4.1. Evolution and ecology

As per the new monophyly based classification, the basal fungal lineage of phylum zygo-
mycota is replaced with an uncertain systematic position (*insertae cedis*) having the mono-
phyletic subphylum Mucoromycotina, Kickxellomycotina, Zoogomycotina and Ento-
mophthoromycotina (Hibbett et al., 2007). Three orders namely Mucorales, Endogonales
and Mortierellales constitute the subphylum Mucoromycotina. Microfossil records indicate
the presence of zygosporangium - gametangial complexes resembling to that of modern
Endogonales observed on Earth during the Precambrian era about 600 million years ago
(Krings et al., 2013). The zygomycetous fungi located in diverse terrestrial habitats con-
sists of approximately 1% of the true fungi. Mucorales are fast growing soil saprobes
which comprise about 205 species (Kirk et al., 2008). They feed on dead and decaying or-
ganic matter and lack the ability to invade healthy hosts possessing refractory substances
like cellulose or chitin. Some among them like *Mucor hiemalis* are known as sugar fungi
due to their unsurpassable ability to assimilate easily available nutrients like sugars from
substrates on which they grow (Carlile et al., 2001). *Rhizopus* sp. and *Choanephora* sp.
leads to flower and fruit rots while those growing on dung like *Syncephalis sphaerica* are
mycoparasites. *Mucor circinelloides*, *Rhizopus* sp. and *Lichthemia* sp. are causal organisms
or opportunistic pathogens of lethal mucormycosis on immune compromised human beings
(Gomes et al., 2011).

1.4.2. Life cycle and physiology

Asexual phase predominates their life cycle from a multinucleate sporangiospore, germi-
nating on a suitable substrate to form hyphae that grow and branches out in circle to a veg-
etative colony of mycelium in a day. The hyphae are multinucleated and coenocytic without cell walls. Mucorales prefer aerobic conditions for the hyphal development and growth at a temperature not higher than 25° C. The erect asexual hyphae bearing a spherical sporangium (Fig.1.4.2) possessing thousands of sporangiospores are known as sporangiophores. For example in *P. blakesleeanus* and *M. mucedo* the yellow sporangium having β-carotene later appear black in colour, due to the oxidative polymerization of carotene resulting in sporopollenin which is a component present in both pollen grains and fungal spores imparting resistance to chemical and biological degradation (Hocking, 1965, Gooday, 1973, Kawase & Takahashi, 1995). Besides, the sporangiophores respond to many sensory stimuli as anemotropism or phototropism positively and negatively towards geotropism or hydrotropism (Bahn et al., 2007). As the sporangium bursts out the spores are dispersed via air currents or rain splash towards diverse destinations and establish new colonies. A light intensity of $10^{-8}$ Wm$^{-2}$ induces macrophore development in *P. blakesleeanus* while blue light activates β-carotene production in both *M. circinelloides* and *Phycomyces* (Corrochano, 2007). The negative regulator of carotenogenesis CrgA, a member of ubiquitin ligases family, universally present in eukaryotes acts as an activator of asexual sporulation in *M. circinelloides* and *B. trispora* (Navarro et al., 2001, Quiles-Rosillo et al., 2005). Sexual phase in Mucorales takes place to overcome unfavorable environmental conditions, nutrient depletion or oxidative stress. In contrast to the large sex specific chromosome in animals, fungi possess a small region of the genome for sex determination encoded by high mobility group (HMG) domain transcription factor. The sex locus having HMG domain protein designated as SexP in (+) and SexM in (-) mating part-
ners were flanked by a triose phosphate transporter homolog (TPT) and a RNA helicase gene in *R. oryzae, P. blakesleeanus* and *M. circinelloides* (Idnurm *et al.*, 2008).

Fig.1.4.2. Asexual and sexual phases in life cycle of a typical heterothallic mucoralean fungus.

Unlike the idiomorphic nature of MAT loci in Dikarya encoding divergent genes, the sequences of genes encoding SexP and SexM are divergent but allelic in plus (+) and minus (-) mating types of heterothallic members (Lee *et al.*, 2010). Darkness stimulates sexual phase among Mucorales (Sutter *et al.*, 1973, Werkman & Vandennen.H, 1973). ΔsexM mutants of *M. circinelloides* failed to produce sexual zygospores in association to wild type (+) mating partners. Even then the *sexM* mutants produced larger sporangiospores alike the
wild type and exhibited similar virulence in wax moth larvae indicating sex locus has no role in virulence or sexP allele do have an inhibitory role (Li et al., 2011). M. mucedo and P. blakesleeanus had been extensively studied on their sexual phase as they have very distinct morphological modifications. When the (+) and (-) mating partners are in close proximity vegetative hyphae of both produce specialized short and stout aerial hyphae known as zygophores (Wurtz & Jockusch, 1975). The zygophores of opposite mating partners elongate and grow towards each other known as zygotropism and once they fuse swelling occurs immediately adjacent to the area of contact giving two multinucleate progametangia having thousands of nuclei (Fig.1.4.2). Each progametangium develop to a gametangium forming cross walls that delimits it from the region of zygophore which is then known as suspensor (Gooday & Carlile, 1997). Once the cross walls breaks down then the fused gametangia develops to a sexual zygospore. During the quiescent phase which may extend from a month to a year most of the nuclei degrade and the dikaryotic zygospore remains highly resistant with black warty ornamentations. In M. mucedo only one among the four recombinant progeny formed by meiosis, survives and subsequently multiplies (Carlile et al., 2001). About 80% of zygospores undergo germination in 80-120 days in P. blakesleeanus. It is reported in B. trispora that sporopollenin and lycopene (33% of total carotenoids) render resistance to zygospores against oxidative stress (Tereshina et al., 2003).

1.4.3. Biosynthesis of trisporic acid sex pheromones

A cocktail of apocarotenoid C_{18} trisporic acids (designated as A, B, C, D, E based on the substitution pattern at C_{15} and functional group attached to C_{4}) are the universal sexual pheromones that switch vegetative cycle to mating phase among Mucorales. In addition to
morphological differentiations, a series of biochemical events initiated by the cleavage of β-carotene leads to the generation of trisporic acids (Fig.1.4.3) in mated cultures where the exchange of trisporoid precursors takes place among the partners (Sutter et al., 1973). The sex pheromones were identified as stimulants of carotene production as they positively regulated a feedback loop. Considering that the amount of trisporic acids produced is 100% in mated cultures of *B. trispora*, the asexual phases in independent cultures produce only 0.001% in (+) strain and 0.1% in the (-) strain. "Trisporic acid C (C\textsubscript{18} H\textsubscript{26}O\textsubscript{4}, MW 306) is the most active compound inducing zygophores even at 14 ng, a lower limit for detecting ± response in *Mucor* bioassay" (Sutter et al., 1973) and trisporic acid B having oxygen instead of a hydroxyl group at C\textsubscript{13}, is also present in minute amounts. Two major enzymes involved in biogenesis of carotene (Itturiaga et al., 2000) are CarRA/RP (phytoene synthase and lycopene cyclase) and CarB (phytoene desaturase). But little is known about the genes involved in the carotenoid cleavage leading to trisporic acid biogenesis. Retinal, the C\textsubscript{20} apocarotenoid having a structural similarity to trisporic acids was considered to be the first cleavage product of β-carotene, in mucorales similar to that in carotene producing ascomycete *Fusarium oxysporum* (Prado-Cabrero et al., 2007). The functional characterization of CarS cloned in genetically engineered β-carotene overproducing *E. coli* lead to the detection of C\textsubscript{25} compound β-apo-12’-carotenal rather than retinal as the first cleavage product in *P. blakesleeanus*. Another enzyme designated as apocarotenoid cleavage oxygenase (AcaA) cleaved the C\textsubscript{13}-C\textsubscript{14} bond of β-apo-12’-carotenal to produce the first C\textsubscript{18} precursor of trisporic acid known as D’orenone or β-apo-13-carotenone (Medina et al., 2011). A series of biochemical transformations modify the C\textsubscript{18} precursor metabolite orienting a ‘chemical dialect’ among the opposite mating partners. All those 18-carbon com-
pounds involved in trisporic acid biogenesis ranging from D’orenone to methyl trisporic acid are known as trisporoids. Analyses of subsequent metabolites in trisporic acid pathway after supplementation with deuterium labeled D’orenone confirmed the fact that both mating partners in B. trispora in principle can produce full spectrum of compounds up to trisporols, but only (+) partners were capable of further oxidation towards their methyl trisporoids (Schachtschabel & Boland, 2007). Two additional functionally characterized enzymes in M. mucedo known to be involved in trisporic acid biogenesis are 4-dihydromethyl trisporate dehydrogenase (TSP1) and 4-dihydrotrisporin dehydrogenase (TSP2) (Wetzel et al., 2009). TSP1 in M. mucedo had a high amino acid position similarity with the xylose reductase (XYL1) of Pichia stipitis (Czempinski et al., 1996, Amore et al., 1991). Even though the transcription and translation were constitutively maintained in both mating types, TSP1 released from sexually stimulated (-) partner converted the (+) specific 4-dihydromethyl trisporate to methyl trisporate in M. mucedo (Schimek et al., 2005).
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Fig. 1.4.3. Schematic diagram of the putative biosynthetic pathway and genes encoding proteins involved in β-carotene metabolism. The enzymes depicted on left side of arrows lead to β-carotene biosynthesis while those on right are involved in trisporic acid biogenesis. CarRA is a bifunctional enzyme having 2 domains CarR and CarA leading to biogenesis of beta-carotene. The first apocarotenoid formed by carotenoid cleavage dioxygenase (TSP3/CarS) is further cleaved down to C18 trisporoid compounds. Double black arrow indicates more than one step involved in formation of the subsequent metabolite.
1.5. Aims of the project

Sex in Mucorales fungi belonging to subphylum Mucoromycotina is a morphologically complex and biochemically fascinating phenomenon interlinked with secondary metabolism. Little is known about the physiological functions of enzymes and products involved in sexual phase. We hypothesize a chemical dialect induced by each of the apocarotenoids during sex have a specific regulatory function upon the transcription of genes linked to sex hormone production varying with species and developmental phases. *Mucor circinelloides* having a sequenced genome and a well-developed genetic transformation protocol is a suitable model organism for unraveling the molecular aspects of gene regulation associated to carotenoid metabolism. The dynamic nature of biological processes in living beings especially in a metabolic grid makes it necessary to have time series experimental data towards a meaningful conclusion. The goals of this study are as follows:

1. How the gene expression of carotenoid metabolic structural genes vary with asexual and sexual phase in wild type strains of *B. trispora* and *M. circinelloides*. Do the early developmental phases influence gene transcription?

2. What are the apocarotenoid products formed by the activity of carotenoid/apocarotenoid cleavage dioxygenases in *B. trispora* and *M. circinelloides f. lusitanicus*?

3. How the *M. circinelloides* sex genes transcriptionally respond to mating under diverse genetic backgrounds?

4. Do the carotenogenesis repressor CrgA regulate sexual phase? Whether there is a variation in gene expression among different genera?
5. Does the apocarotenoid signaling impart a chemical dialect regulating the transcription of genes which varies with genes and genotypes?

6. Is it possible to localize sex hormones of *M. mucedo* by the *in vivo* imaging method of Coherent Anti-stokes Raman micro Spectroscopy?

7. Do the different sugar sources influence the transcription of *TSP1* belonging to aldo/keto reductase superfamily in *P. blakesleeanus*?
CHAPTER 2. MATERIALS AND METHODS

2.1. Strains

FSU331 (+) and FSU332 (-) of B. trispora FSU621 (+) and FSU620 (-) of M. mucedo in addition to FSU2486 (+) and FSU2487 (-) of P. blakesleeanus were obtained from the Jena Microbial Resource Collection (JMRC) at the Friedrich Schiller University and Hans Knoell Institute. The (-) and (+) mating types of Mucor circinelloides f. lusitanicus CBS 277.49 (-) and CBS852.71 (+) were purchased from the Centralbureau voor Schimmelcultures (CBS), the Netherlands. Mutants of Mucor circinelloides MU223 (Δ crgA), MU367 (Δ acaA) and MU366 (Δ crgAΔ acaA) were generated and acquired from the lab of Prof. Rosa M. Ruiz-Vázquez, University of Murcia, Spain. The E. coli strain JM 109 was used for plasmid transfection and pβ plasmid overproducing β-carotene was a kind gift from Dr. Salim Al-Babili, University of Freiburg, Germany (currently at KAUST, Saudi Arabia).

2.2. Growth conditions

Fungal pre-inoculum was prepared on solid agar plates (9 mm) of induction medium (IM)(Schimek et al., 2003)by plating a single disc of fungal mycelium (1 mm diameter) and growing the individual strains for 80 hours. Tab 2.2.1 is the recipe for preparing solid induction medium, while the ingredients ranging from I-III were autoclaved separately. Later the ingredients were mixed up under aseptic conditions and stored in petri dishes at 4º C. Spores were collected in distilled water and counted using a haemocytometer. Mating experiments were carried out under dark in solid induction medium using fungal agar discs placed 1 cm apart the complementary partners in the middle of the agar plate. 10^6 spores/ml of (+), (-) and (+/-) strains, grown in liquid induction broth upto 12/18 hours for wild types/mutants were used as pre-inoculum. For transcript analysis, strains of either
Mating types were inoculated with $10^8$ spores ml$^{-1}$ for faster growth in 50 ml IM at 23-24°C in the dark with a shaker speed of 220 rpm for 144 hours. The mated culture experiments were performed using a 1:1 ratio of spore inoculum. The photo induction experiments were done using a (Sylvania active) fluorescent lamp (36 W/m$^2$) as illumination source. After an initial dark phase for 12 hours, the cultures were subjected to alternate white light-dark stimuli upto 96 hours with an interval of 24 hours.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10g</td>
<td>Potassium nitrate (KNO$_3$)</td>
</tr>
<tr>
<td></td>
<td>5g</td>
<td>Potassium hydrogen phosphate (KH$_2$PO$_4$)</td>
</tr>
<tr>
<td></td>
<td>2.5g</td>
<td>Magnesium sulphate hepta hydrate (MgSO$_4$·7H$_2$O)</td>
</tr>
<tr>
<td></td>
<td>1.0g</td>
<td>Yeast extract</td>
</tr>
<tr>
<td></td>
<td>200ml</td>
<td>Distilled water</td>
</tr>
<tr>
<td>II</td>
<td>13.0g</td>
<td>Agar</td>
</tr>
<tr>
<td></td>
<td>700ml</td>
<td>Distilled water</td>
</tr>
<tr>
<td>III</td>
<td>20g</td>
<td>Maltose</td>
</tr>
<tr>
<td></td>
<td>100ml</td>
<td>Distilled water</td>
</tr>
</tbody>
</table>

**Tab.2.2.1. Recipe for preparing Induction medium (1 liter)**

**2.3. Gene expression studies**

**2.3.1. Gene sequences and primer design**

The gene sequences used for this work given in Tab 2.3.1 were obtained from JGI and GenBank. Primers ranging amplicon sizes with 91-261 basepairs were designed using Primer-Blast software from NCBI for real time qPCR. Vector NTI software was used to select
the best available primer pair with the least number of potential secondary structures in
amplicon and with a GC content of 46-70%.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene amplified</th>
<th>Tm (°C)</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon (bp)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bt Car-RA</td>
<td>Phytoene synthase&amp;lycopene cyclase</td>
<td>65.5</td>
<td>for: CCGTGCACGTGAGATGGGGC revive: GCTACGGGCGTTGCCTTGGCT</td>
<td>151</td>
<td>AY176663.1</td>
</tr>
<tr>
<td>Bt TSP3</td>
<td>Carotenoid cleavage dioxygenase</td>
<td>62.1</td>
<td>for: TCGGAACAAACGCTGTTTGC rev: AGTTCTCCACGCTTAGACCT</td>
<td>125</td>
<td>AM409182.1</td>
</tr>
<tr>
<td>Bt Act</td>
<td>β-actin</td>
<td>63.7</td>
<td>for: TCCCCGCCCTGAA-GCTCTCT rev: CGCTTCA TGATGGAG TCCTTGG</td>
<td>91</td>
<td>AJ287143.1</td>
</tr>
<tr>
<td>Bt Gpd</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>62.0</td>
<td>for: GGGGTGCAACGTA CGTAAATCA rev: AGCGAGATACCTGC GAGGCA</td>
<td>157</td>
<td>AJ278318.1</td>
</tr>
<tr>
<td>Bt Pyrg</td>
<td>Orotidine-5’-monophosphate decarboxylase</td>
<td>61.0</td>
<td>for: TGCTCACACAGTTCTGAG rev: GGCAGAGCCATATTGAAC AGAT</td>
<td>152</td>
<td>AY262090.1</td>
</tr>
<tr>
<td>Bt Tef</td>
<td>Translation elongation factor 1-α</td>
<td>63.1</td>
<td>for: CTCCCCGCGTACAA CTCCCCGGTGACAAC-GTGC rev: GAACCGGCTTCTTG</td>
<td>110</td>
<td>AF157235.1</td>
</tr>
<tr>
<td>Bt TSP1</td>
<td>4-dihydromethyl trisporate dehydrogenase</td>
<td>61.8</td>
<td>for: TGTCAGGTTGGTGCA rev: GCACAAACGTATCTGCGC</td>
<td>165</td>
<td>FSU331 JX470964</td>
</tr>
<tr>
<td>Bt CrgA</td>
<td>Negative regulator of carotenogenesis</td>
<td>55.0</td>
<td>for: ATGCTTTAAACGACACAGATCTTG rev: CTCGCACTACATA-GGGGGT</td>
<td>160</td>
<td>AJ585199</td>
</tr>
<tr>
<td>Mcl CarS</td>
<td>Carotenoid cleavage dioxygenase</td>
<td>59.4</td>
<td>for: CATCAGCCCCTCTGGCGT CAT rev: ATTGCGATGATGGAGA GGAAGAAAGCA</td>
<td>261</td>
<td>146755</td>
</tr>
<tr>
<td>Mcl AcaA</td>
<td>Apocarotenoid cleavage oxygenase</td>
<td>58.0</td>
<td>for: AGACGTGATGGCCGT CCTGAT rev: AGTTGACCTTTGATCGAGCA</td>
<td>208</td>
<td>141273</td>
</tr>
</tbody>
</table>
### Tab. 2.3.1. List of primers used for both qPCR and standard PCR reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mcl</strong>&lt;br&gt;CarRP</td>
<td>Phytoene synthase &amp; lycopene cyclase</td>
<td>for:AACGACCAACTGCCT GCCTCG</td>
<td>rev:AATCCCACTTGACCC GTCGA</td>
<td>AJ250827.1</td>
</tr>
<tr>
<td><strong>Mcl</strong>&lt;br&gt;SexP</td>
<td>Transcription factor (plus) sex gene</td>
<td>for:ATGTGAAGTGCGTTC GAAAATCT</td>
<td>rev:TACACCAATCGAGAT GTTTTTAAAAAT</td>
<td>FJ009107</td>
</tr>
<tr>
<td><strong>Mcl</strong>&lt;br&gt;SexM</td>
<td>Transcription factor (minus) sex gene</td>
<td>for:ATTACAAAACGACACC AGACGA</td>
<td>rev:AGCATGTGCTCGCT CTCTGTCAT</td>
<td>FJ009106</td>
</tr>
<tr>
<td><strong>Mcl</strong>&lt;br&gt;CrgA</td>
<td>Negative regulator of carotenogenesis</td>
<td>for:ATGGCATCCAGCAGA AGACGA</td>
<td>rev:AGCATGTGTTCTTGCT CTCTGTCAT</td>
<td>AJ250998.1</td>
</tr>
<tr>
<td><strong>Mcl</strong>&lt;br&gt;Act</td>
<td>β-actin</td>
<td>for:TCCTGTAGGGCAAGTC ATACTGT</td>
<td>rev:CGAAACTAAATGAA CAACCAACC</td>
<td>AJ287173.1</td>
</tr>
<tr>
<td><strong>Pb</strong>&lt;br&gt;TSP1</td>
<td>4-dihydromethyltrisporate dehydrogenase</td>
<td>for:AGCAGACTCCGTCCGT TGAGT</td>
<td>rev:TGTGGGAGCATGAGG TTATCAAGT</td>
<td>NRRL1555</td>
</tr>
<tr>
<td><strong>Pb</strong>&lt;br&gt;Act</td>
<td>β-actin</td>
<td>for:ATGTGCTCTTCGACTT TGAGCAGG</td>
<td>rev:GGCTGGAAGAGAGCT TCGGGA</td>
<td>AJ287184.1</td>
</tr>
<tr>
<td><strong>Bt</strong>&lt;br&gt;TSP3 full</td>
<td>Carotenoid cleavage dioxygenase</td>
<td>for:ATGAAATTGCTTGAA GGTGAAGAG</td>
<td>rev:TCAATTAACAGCTAT GCCTCTTC</td>
<td>AM409182.1</td>
</tr>
<tr>
<td><strong>Mcl</strong>&lt;br&gt;CarS full</td>
<td>Carotenoid cleavage dioxygenase</td>
<td>for:ATGATCACTCCGCTGA AGCC</td>
<td>rev:TTAATTGACGTCGATGCCTCT</td>
<td>146755</td>
</tr>
<tr>
<td><strong>Mcl</strong>&lt;br&gt;AcaA full</td>
<td>Apocarotenoid cleavage oxygenase</td>
<td>for:ATGATGTTAGGATTGC TTACAT</td>
<td>rev:TCACATGACCATGACATG</td>
<td>141273</td>
</tr>
</tbody>
</table>
2.4. RNA extraction and cDNA synthesis

2.4.1. RNA Isolation

Trizol reagent (Invitrogen, Karlsruhe, Germany) was used for isolating RNA from approximately 100 mg of the fungal biomass samples that were initially freeze dried in liquid nitrogen and stored at -80°C.

Protocol

1. Weigh 100 mg sample (fungal mycelium).

2. Transfer mycelium to a mortar. Add liquid nitrogen and grind thoroughly using a pestle.

3. Add 1ml TRIZOL reagent for homogenization

4. Incubate homogenized sample for 5 minutes at 15-30°C (to permit complete dissociation of nucleoprotein complexes).

5. Add chloroform 0.2ml/ml of TRIZOL reagent. Shake the tube vigorously by hand for 15 seconds and incubate them at 15 -30°C for 2-3 minutes.

6. Centrifuge the sample at no more than 12000 x g for 15 minutes at 2-8°C. Then the mixture separates into a lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase (having RNA) - phase separation.

7. Transfer the aqueous phase to a new tube and discard the organic phase. Add isopropyl alcohol at 0.5ml per ml of TRIZOL reagent initially used for homogenization. Incubate samples overnight at 20°C rather than at 15-30°C for 10 minutes to improve yield and centrifuge at no more than 12000 x g for 10 minutes at 2-8°C – RNA precipitation
8. Discard supernatant. Wash RNA pellet with 75% ethanol at 1.0ml per ml of TRIZOL reagent initially used for homogenization. Mix sample using a vortex and centrifuge at no more than 7500x g for 5 minutes at 2-8°C – **RNA wash**

9. Briefly dry the RNA pellet in air for 5-10 minutes. Dissolve RNA in 50 μl RNase free water by passing the solution a few times through a pipette tip and incubating for 10 minutes at 55-60°C - **redissolving RNA**

10. Store at -80°C in aliquots.

### 2.4.2. DNase treatment

Genomic DNA contamination of the isolated RNA was avoided by Turbo DNase treatment (Turbo DNA-free™ kit, Ambion, AM 1907, Carlsbad, USA) as prescribed by the manufacturer.

### 2.4.3. RNA clean up

DNase treated RNA was subjected to clean up using RNeasy-MinElute Cleanup kit (Qiagen, Hilden, Germany) for improving the purity of the DNase treated RNA as per the instructions given by the manufacturer. The concentration of each of the RNA sample was determined using a NanoVue™ (GE healthcare, Freiburg, Germany) UV/Vis spectrophotometer. RNA samples that had the OD$_{260}$/OD$_{280}$ value of 1.9-2.1 were chosen for further experimental processes.

### 2.4.4. Gel electrophoresis

The quality and integrity of RNA samples were ensured by a formaldehyde/TAE agarose gel run. Electrophoreses were carried out as per the protocols (Masek *et al.*, 2005, Sambrook *et al.*, 1989). Agarose (1% w/v, Roth, Karlsruhe) gel prepared after adding 0.5
µg/ml ethidium bromide from stock 10 mg/ml. RNA samples were denatured by heating for 5 minutes at 65°C, immediately chilled on ice for 5 minutes and loaded on gel using 6x loading dye along with DNA marker (Massruler, Thermo scientific, Darmstadt). Electrophoresis was performed in 1X TAE working solution made up of 40 mM Tris, 20 mM Acetate, 2 mM EDTA, pH (8.2) using an Agagel mini (BioRad GmbH, Göttingen) with EPS 301 (Amersham Pharmacia, Munich, Germany) with 4-7 V/cm and gel documentation (Bio Doc analyze, Biometra, Göttingen) was done.

2.4.5. cDNA synthesis

First-strand cDNA synthesis was done using SuperScript™ III reverse transcriptase (Invitrogen, Karlsruhe, Germany) as per the manufacturer’s instructions as follows. For a reaction volume of 20 µl, 1 µg/ml total RNA was mixed along with 1 µl of oligo (dt)₂₀ (50 µM), 1 µl dNTP mix (Invitrogen, Carlsbad, U.S.A) and DNase/RNase free distilled water (Gibco, Karlsruhe, Germany) upto 13 µl in a DNase/RNase free 0.5 ml micro tubes. The mixture was heated to 65°C for 5 minutes and incubated on ice for 1-2 minutes. A brief centrifugation of 1 minute was done to collect the contents in the tube and added 4 µl 5X first strand buffer, 1 µl 0.1 M DTT, 1 µl RNaseOUT recombinant RNase inhibitor along with 1 µl Superscript III reverse transcriptase. The mixture was further homogenized by pipetting gently up and down followed by heating at 50°C for an hour. Later the reaction was inactivated by heating upto 70°C for 15 minutes in a thermo mixer (Eppendorf, Hamburg, Germany) and briefly centrifuged to collect the contents before storing at -80°C until use. All the reactions were carried out quickly using DNAase/RNAase free pipette tips specifically meant for quantitative real time PCR analysis in all those experimental steps.
2.5. Polymerase Chain Reaction (PCR)

2.5.1. Standard PCR

PCR was performed using a Master cycler Pro (Eppendorf, Hamburg). The reaction mixture comprised of 5 µl 10X PCR buffer, 1 µl of 10 mM dNTP mix 1 µl each of 10 µM sense and antisense primers, 0.4 µl Taq/Accuprime Taq High Fidelity polymerase (Invitrogen, USA) besides x µl of template DNA/cDNA (1 µg/ml or 20 ng) in nuclease free distilled water made upto a volume of 50 µl. The initial denaturation was 94°C for 2 minutes, followed by 30-35 cycles of denaturation step at 94°C for 30 seconds. Annealing was performed at a temperature of 58-62°C for 30 seconds and extension for 68°C for 1 minute per kb of the PCR product.

2.5.2. Real time quantitative PCR

Real time PCR were carried out using a Stratagene Mx 3000P (Agilent, Karlsruhe, Germany) with an optimized thermal profile for all reactions as shown in Fig.2.5.2 The segment 1 indicated hot start phase, initial denaturation temperature necessary for the activation of polymerase which was chosen at 95°C for 10 minutes. Segment 2 consisted of 35 cycles of denaturation at 95°C for 30 seconds, 60°C to avoid nonspecific annealing and primer-dimer formation with an extension of 72°C for 1 minute. At the end of each cycle the fluorescence of cyanine dye SYBR II, which gets intercalated in the minor groove of double stranded DNA was measured.

The melting curve analysis or dissociation curve was performed in segment 3, at the end of PCR cycles and confirmed the specificity of primer annealing which was represented as a single sharp peak.
**Materials and Methods**

2.5.2. The thermal profile set up for qPCR as per Stratagene. Segment 2 had been modified to 35 cycles and plateau temperature optimized for 60°C to avoid unspecific binding.

qPCR reaction mixes were made in strips of 8 PCR tubes (4ttitude, Dorking, UK) containing 12.5 µl 2X Brilliant II SYBR green qPCR master mix, 1.25 µl each of sense and antisense primers (10 µM each), 2 µl cDNA, 0.375 µl of the passive reference dye ROX closed with optical strip cap and were briefly centrifuged to avoid air bubbles.

2.5.3. Transcript analysis

Independent liquid culture experiments using three biological replicates, each with (+), (-) and (+/-) cultures of *Blakeslea trispora*, *Mucor circinelloides* and *Phycomyces*
blakesleeanus were carried out for the transcript analysis of carotenoid metabolic genes. qPCR assays were performed using three technical replicates for each of the three independent biological replicates and a No RT control (NRT) for every sample, along with a No Template Control (NTC) for every primer pair used in each run using Brilliant II SYBR green qPCR kit (Agilent). PCR efficiency measures the rate at which the polymerase converts reagents like dNTP, oligonucleotides and template cDNA to amplicon. In order to estimate PCR efficiency, all selected primers (synthesized by Eurofins; Ebersberg, Germany) were run with pooled samples (from all time points) in 5-fold dilution series over five points (Nolan et al., 2006). Built-in software from Stratagene Mx 3000P was used to construct a standard curve for each primer pair and the efficiency was determined. The qRT-PCR assays relies on measuring the increase in fluorescence signal, which is proportional to the amount of DNA produced per cycle. Hence the data represents fluorescence intensity expressed as a function of number of cycles and thus they are log-linearly plotted. The Ct value is the cycle in which fluorescence rises significantly above the baseline or threshold level and hence inversely proportional to the expression level of the gene. Relative quantification has the choice of a calibrator sample, which was the untreated 12 hpi (hours post inoculation) sample in each of our experiments. The target gene signal was normalized to that of the house keeping gene β-actin, thereby avoiding the variations that may arise due to different amounts of total RNA and manual pipetting errors. Hence the expression of gene of interest or target genes in the sample were quantified relative to the house keeping or reference gene considering the PCR efficiency based on $2^{-\Delta Ct}$ method (Pfaffl, 2001).
2.6. cDNA cloning

The *in vivo* carotenoid cleavage assays were carried out in *Escherichia coli* JM 109 strain as it possesses a stable genotype. pβ-carotene plasmid engineered with a gene cluster of 4 enzymes of *Erwinia carotovora*, i.e., CrtB (Geranyl geranyl diphosphate), CrtE (phytoene synthase), CrtI (phytoene desaturase) and CrtY (lycopene cyclase) (von Lintig & Vogt, 2000, Bustin *et al.*, 2009, Campos-Takaki & Dietrich, 2009). The pβ-carotene plasmid is a pACYC177 low copy plasmid with constitutive expression and having a kanamycin resistance marker (Medina et al., 2011, Sahadevan *et al.*, 2013). The cDNA of TSP3 from *B. trispora*, CarS and AcaA from *Mucor circinelloides* f. *lusitanicus* were amplified with the full-length primers (Table 2.3.1) using Accuprime Taq polymerase (Invitrogen, Carlsbad, USA) and were subjected to 3’A overhang addition on post-amplified product following the instruction manual. The products were further ligated into an ampicillin resistant pBAD/Topo Thio vector (Invitrogen). The integrity of the products was verified by sequencing (MWG Eurofins, Ebersberg, Germany).

2.7. *In vivo* enzyme assay

The pβ-carotene plasmid was co-transformed with the pBAD-TSP3, pBAD-CarS and pBAD-AcaA independently in *E. coli* JM 109 chemically competent cells. Overnight cultures were grown at 28°C with a shaker speed of 250 rpm in Luria-Bertani (LB) broth, induced with 0.08% and 0.2% and 2% arabinose (Sigma) at an OD_{600} of 0.5. Samples were collected at 0, 4, 16 and 24 hours after arabinose induction. A positive control without pβ-carotene plasmid at 2% arabinose induction and a negative control without TSP3/CarS/AcaA were maintained along with a no arabinose control to check for leaky expression. Cell pellets were obtained by centrifugation at 2599 x g (rotor radius 92.79
mm) at 4°C for 20 minutes and re-suspended in HPLC grade acetone. After centrifugation, the supernatant was dried and dissolved in HPLC solvent mixture, followed by HPLC and LC-MS analysis.

2.8. Chromatography and Mass Spectrometry

High-performance liquid chromatography (HPLC) was conducted on a HP1100 system equipped with a photodiode array detector and an automatic sample injector. The separations were carried out using a Bischoff C-30 reverse-phase column 250×4.6 mm×3 μm using methanol (A) and methyl tertiary butyl ether (B) as solvents (Lacker et al., 1999). The column was developed at a flow rate of 1 ml/min at 20% B, initially up to 5 minutes. A gradient was maintained within 5 minutes to 90% B with a 1 minute hold time and then switched to the initial 20% B till the end of run time. The standard compound β-carotene was purchased from Sigma-Aldrich (Seelze, Germany) and β-apo-12’-carotenal from Carotenature (Lupsingen, Switzerland). Both standards and samples were treated with the same solvent mixture with an injection volume of 15 μl and monitored at three wavelengths of 420, 450 and 461.4 nm. The chromatographic spectra were acquired using the chemstation software package. Mass spectrometry was carried out using an LCQ mass spectrometer with an APCI interface (Finnigan MAT, Bremen, Germany). The capillary temperature was set at 160°C and vaporizer temperature of 450°C.

2.9. Gene transformation

The entire AcaA gene (protein ID 141273) and adjacent sequences were cloned by PCR amplification of M. circinelloides genomic DNA with primers acaA1 (5’-ATTATTCCGGGCATCCTGCTACTGTGTCCTGGACCC-3’) and acaA2 (5’-CAGCAGCAGCTCCAAGACTACATAGTGTTGTAC-3’), which contain SmaI
and SacI sites, respectively (underlined). The 3990 bp amplified fragment was digested with SmaI and SacI and cloned into pBluescript KS to give plasmid pMAT1095. This plasmid was used to construct a knockout vector designed to generate null mutants for acaA by gene replacement. Plasmid pMAT1095 was reverse PCR-amplified using oligonucleotides acaA3 (5’-AAGCCGGGATTCCGTAACGAGATCAATGTAAGCAATCC-3’) and acaA4 (5’-TGGTCAGGATTCCGCGTTACCTTCCATGGCTC-3’). These primers amplified a 5.12 kb fragment that included the vector sequence flanked by 1.15 kb and 1.0 kb of acaA upstream and downstream sequences, producing a deletion of the acaA coding region. The PCR product was ligated with a 3.4 kb BamHI fragment containing the complete PyrG gene, which was isolated from pEMP1 and blunt ended by Klenow treatment. The resulting plasmid was named pMAT1096 and contains the PyrG gene, used as selective marker, flanked by enough sequences of the AcaA adjacent regions to allow homologous recombination.

2.10. Raman and Coherent Anti-stokes Raman micro Spectroscopy

Raman spectroscopic measurements of pure standards of β-carotene, D’orenone, trisporic acids B and C were carried out using a near infra-red-Fourier transform Raman spectrometer with an excitation wavelength of 532 nm (Institute of Physical Chemistry, Friedrich Schiller University). Due to the large Raman scattering cross sections, the substances were illuminated for 0.5-15 s. M. mucedo(+) and (-) mating partners were pre-inoculated in a single solid agar plate with 4 biological replicates so that the asexual phase and sexual phase at mating zone coincided with 48-96 hpi at the time of CARS measurements at Institute of Photonic Technology, Jena. Asexual hyphae and sexual structures bearing progametangia, gametangia and zygospores were first observed under a bright field microscope to
cut off the respective mycelia mat from agar in 0.5-1 cm grids and transferred to glass slides having a droplet of water. The moisture prevents drying up of the sample under the cover slip after exposure to high energy laser beam. Raman shift or a difference in frequency of pump and stokes beams designated as $\omega_P-\omega_S$, was required for CARS imaging to specifically distinguish each of the standard compound in the sample. This was achieved using a pico second pulsed Ti: sapphire laser (Mira HP, Coherent) at a wavelength of 831-834 nm to obtain a spectral resolution in the order of magnitude 12-14 cm$^{-1}$. The pump laser wavelength from an optical parametric oscillator (OPO), was tuned to wavelength of 735 and 741 nm, to generate anti-stokes signal, with respect to the particular C=C Raman vibration for trisporic acid and β-carotene respectively. Both the pump and stokes beam were spatially and temporally overlapped by means of a dichroic beam splitter and an optical delay line, subsequently coupled to a laser scanning Zeiss microscope (Weissflog et al., 2010). The CARS signals were collected in forward direction for generating images and each pixel was averaged 4 times.

2.11. GC-MS analysis

Extraction of trisporic acids from mated cultures of *M. mucedo* and its derivatisation with diazomethane was done as reported earlier (Schachtschabel & Boland, 2007) from both solid and liquid cultures (Schachtschabel et al., 2010). 100 µl of diazomethane derivatised samples and standards were dissolved in dichloromethane and were kept in auto sampler. Analysis was done using a Finnigan mass spectrometer coupled to a gas chromatograph. The injection volume was programmed to 1 µl and initial oven temperature was 50°C at a hold time of 1 minute, with ramp at 15°C/min upto 300°C. Solvent delay or equilibration time was 0.30 minute. Helium was the carrier gas at a flow rate of 1.5 ml/min. Electron
ionisation mode was maintained at a detector voltage of 350 V where the mass spectrum was acquired in a full scan mode.

2.12. Software

Following software had been used in the work for graphical representations, statistical analyses and image acquisitions.

Origin 8G (Origin Lab corporation, Northampton, USA)

Adobe Photoshop and illustrator (CS5, Adobe systems, California, USA)

Sigma stat 2.03 ( Systat software Inc. California, USA)

Laser Scanning Microscope Image Browser (Zeiss Germany)

Axiocam HR (Carl Zeiss Germany)
CHAPTER 3. RESULTS

3.1. Sequence alignments and phylogenetic analyses

Based on amino acid sequence similarity and phylogenetic analyses, we had chosen two putative carotenoid cleavage oxygenases designated as CarS and AcaA of *M. circinelloides* for functional characterization. CarS [e_gw1.06.133.1] and AcaA [e_gw1.03.260.1] (JGI) shared high sequence similarity to homologs in *B. trispora, Rhizopus delemar* (BROAD institute, http://www.broadinstitute.org), *P. blakesleeanus* (GenBank Acc.no ADU04395) and *Umbelopsis ramanniana* (JGI http://www.jgi.doe.gov; Grigoriev et al. 2012)(Table 3.1.1) belonging to the same order Mucorales. The sequence identity in *M. circinelloides* ranged between 60-81% for CarS and 35-52% for AcaA (Fig.S2) to corresponding sequences of those fungi. Both genes are phylogenetically distinct (Fig. 3.1.1) with bootstrap support of 100% for each clade. Four conserved histidine and glutamate residues essential for the carotenoid cleavage dioxygenase function were missing in those two genes, designated as unknown (189974 and 114475) in *M. circinelloides* genome. AcaA from *Phycocystis* did possibly undergone gene duplication, but the function of the second copy is not yet verified (Medina et al., 2011). Multiple sequence alignments (Fig.S2) were generated using MAFFT v6.901b (Katoh & Standley, 2013). Alignment consists of 18 sequences (all BLAST hits for the five species) and 1296 characters. Phylogenetic tree (Fig. 3.1.1) was calculated using RAxML v.7.4.4 from the CIPRES portal (http://www.phylo.org; Miller et al. 2010) under the default settings, with 1000 bootstrap replications. A sequence similarity matrix was generated in BioEdit v7.0.9.0 (Hall 1999) based on all sequences. I sincerely acknowledge the great help from Dr. Kerstin Kaerger who did the sequence alignments and phylogenetic analyses for the study.
Tab.3.1.1. Details of apo/carotenoid cleavage dioxygenases identified in the genome of five species in the order Mucorales.
Fig.3.1.1. Phylogenetic tree of the apo/carotenoid cleaving enzymes designated as CarS (carotenoid cleavage dioxygenase) and AcaA (apocarotenoid cleavage oxygenase).

3.2. Selection of developmental phase and internal standards using *Blakeslea trispora* for quantitative gene expression analysis

The two strains FSU331 (+) and FSU332(-) were grown independently and as mated cultures in liquid induction medium in the dark at 23-24°C at 220 rpm on a shaker. No universal internal standard exists that reflects uniformly the gene expression in all tissues under all environmental cues (Bustin, 2000; Gibbs *et al.*, 2003). Hence, we evaluated the performance of four reference genes with different functions, namely the transcription elongation factor (*Tef1*), the glyceraldehyde phosphate dehydrogenase (*Gpd*), the orotidine-5’-
monophosphate decarboxylase (*PyrG*) and β-actin (*Act*) (Fig. 3.2.1) following the Minimum Information for Publication of Quantitative Real time PCR Experiments (Bustin *et al.*, 2009). Actin showed higher expression levels, lower Cq values and the least temporal variation over the growth period.

![Graph](image)

**Fig. 3.2.1.** Real time PCR cycle threshold values (Ct/Cq) of 4 different internal standards actin (*Act*), glyceraldehyde phosphate dehydrogenase (*Gpd*), orotidine monophosphate decarboxylase (*PyrG*) and translation elongation factor (*Tef-1*) plotted for *B. trispora* cross culture over six time points after inoculation. Central line is the median while the boxes are 25% and 75%. Whiskers indicate the range or variability over different time points.
A crucial factor observed was the role of the fungal developmental phase for the estimation of the gene transcription. Data analyses were performed with samples collected at 12, 18, 24, 36 and 48 hours of growth as zero hour or basal expression level as per the $2^{-\Delta Ct}$ method (Pfaffl, 2001) to evaluate the standard variations in the relative gene expression within the time points. The rate of conversion of reagents to amplicon by polymerase during a PCR is considered as 2-fold per cycle at 100% efficiency (Livak & Schmittgen, 2001). But in reality, artefacts like unspecific binding due to primer-dimers or manual errors results in a low or high efficiency in calculations (Taylor et al., 2010).

![Graph showing relative gene expression](image)

**Fig.3.2.2.** Relative gene expression of *CarRA* in mated culture of *Blakeslea trispora* (*Bt*+/−) on different time point chosen for data analysis. Data analyses were performed with samples collected at 12, 18, 24, 36 and 48 hours of growth as zero hour or basal expression level as per the $2^{-\Delta Ct}$ method to evaluate the standard variations in the relative gene expression within the time points.
Therefore it was important to plot standard curves using log of initial template quantity to the Cq values obtained. The coefficient of determination ($r^2$) from linear regression line equation was used to evaluate the qPCR assay optimisation. The $r^2$ values of calibration curves for genes CarRA (0.981), TSP3 (0.994), TSP1 (0.999) and Act (0.994) were obtained based on the stratagene software (MxPro-Mx3000P, version 4.10). There was a significant difference in the relative expression rates of CarRA in the mated partners at different growth phases (Fig. 3.2.2). Hence we optimized the growth conditions to obtain enough biomass for RNA isolation at an early growth phase of 12 hours (basal time point) and, furthermore, collected samples at 24 hours interval up to 144 hours. Suitable primer pairs for the amplicon chosen from the middle of the available sequence with an annealing temperature around 60°C were more reliable than those from 5’ or 3’ regions of the gene.

### 3.3. Time dependent transcript analyses of carotenoid metabolic gene expression in *Blakeslea trispora*

Unlike *M. mucedo*, *B. trispora* produces 1000 times more trisporic acids. A putative non-heme carotenoid cleavage dioxygenase (TSP3) had been reported as the first enzyme involved in sex hormone synthesis (Burmester *et al.*, 2007). Hence we performed transcript analysis in *B. trispora* for CarRA, TSP3 and TSP1 involved in three different stages of carotenoid metabolism in order to identify how developmental phases induce temporal trends in gene expression. The fold change in target gene normalized to internal standard β-actin, relative to expression at time zero (12 hrs post incubation) was carried out for the time series analysis up to 144 hours in 24 hours interval as per the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). CarRA (Fig. 3.3.1A) was significantly up regulated in *B. trispora* (-) with a 160-fold (P<0.001) increase after 144 h of incubation while low and constitutive transcript levels up
to 25-fold (P=0.002) was observed with (+) strain. There was no statistically significant difference in CarRA transcripts beyond 48 hours of growth in mated (+/-) and (-) cultures. The expression of TSP3 was 16000-fold at 48 hours in (+/-) cultures and declined to 7000-fold after 144 hours of incubation (Fig. 3.3.1B). In (+), the gene TSP3 was constitutively up-regulated for up to 72 hours and shows a progressive increase with a maximum of 400-fold at 120 hours, declining to 200-fold at 144 hours. A statistically significant difference exist for the 6 time points under investigation, among the 3 culture types ie., (+), (-) and (+/-) cultures based on their 3 independent biological replicates in TSP3 transcripts (n=18, P=0.028). Curiously, the mRNA level of TSP1, the penultimate enzyme proposed in trisporic acid biogenesis, exhibited down-regulation irrespective of asexual and sexual phase in B. trispora (Fig. 3.3.1C). Our results indicate that CarRA and TSP3 transcripts vary during asexual and sexual developmental phases of the fungal growth in submerged liquid induction medium based on their diverse physiological functions (Sahadevan et al., 2013).
Fig. 3.3.1. Real time qRT-PCR analyses on dynamics of CarRA (a), TSP3 (b) and TSP1 (c) in Blakeslea trispora (+), (-) and (+/−). The small letters indicate significant difference based on Tukey’s test (post hoc) at each time point among (+), (-) and (+/−) mating types and capital letters between different time points in a single mating type.
3.4. Transcript analysis of *CarRP, CarS, AcaA, SexM* and *SexP* in *Mucor circinelloides* f. *lusitanicus* (wild types)

The time dependent analyses of (+), (-) and (+/-) strains of *M. circinelloides* were necessary to identify how the transcription of genes involved in β-carotene metabolism vary with sexual and asexual developmental phases? Statistical analysis using two way ANOVA was conducted by log transformation of relative fold change values of gene expression for every target gene (dependent variable) with 2 independent variables viz., time and mating types. Hence the balanced design passed normality test (P=0.089) and equal variance test (P=0.449). A statistically significant difference was observed with time (F=76.461; P<0.001), mating types (F=49.55; P<0.001) and with time x mating type interactions (F=16.46; P<0.001). Tukey’s test was performed for multiple comparisons. A transient, mating type specific upregulation in gene expression was observed only for *CarS* in (+) and *AcaA* in (-) strains (Fig. 3.4.1a,b). Contrary to *Blakeslea* (Sahadevan et al., 2013) the mated phase in *M. circinelloides* maintained a steady mRNA level for all genes except *CarRP* (Fig. 3.3.1c). The *CarRP* transcripts culminated to a 6 fold by 72 hours post inoculation (hpi) in both (-) and (+/-) cultures. An upregulation of 3.5 fold was constitutively maintained except at 96 hpi in (+) partners.

Even though *CarRP* had a higher transcript abundance, no statistically significant difference was observed among (+), (-) and (+/-) cultures on a kinetic scale. These transcript data corroborate the phenotypic identity of the tested strains; an intense yellow pigmentation was observed only on exposure to (continuous) light (Fig.3.4.2). Meanwhile both sexual and asexual phases had a fluffy white phenotype growing in the dark. Appearance of
the wild type strains provoked us to ponder about the mating type specificity of these strains.

Fig.3.4.1. Differential gene expression in the plus, minus and mating phase of *M. circinelloides* grown in dark normalized with actin as internal standard. Graphs are plotted on the mean value of 3 biological replicates with error bars indicating standard error
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mean. Small letters indicate significant differences among (+), (-) and (+/-) strains at each time point. Capital letters indicate significant differences of single gene transcript abundance at different time points under investigation.

But PCR analyses for sex genes amplified only SexP in CBS852.71 (+) and SexM in CBS 277.49 (-) (Fig.S1). Hence in *M. circinelloides* “being fluffy” is not an indication of the unusual self-fertility unlike in *P. blakesleeanus*, but it is a common feature varying with the moisture of culture media and culture conditions.

![Image of agar plates with different conditions](image)

**Fig.3.4.2. Mucor circinelloides f. lusitanicus** exposed to continuous dark and light conditions in solid induction medium agar plates.

A temporal transcript abundance of 8 fold was observed in SexP during mated phase while for SexM it was a stable downregulation until 72 hpi shifting towards a 3 fold gene upregu-
ulation at 96 hpi (Fig. 3.4.3). The development dependent transient expression of sex genes could be an indication of their potential contribution to mating associated cellular morphogenesis or involvement in signal transduction cascades (Lee et al., 2010). The earlier gene transcription reports on light induced carotenogenesis in *M. circinelloides* were more focused upon *CrgA, CarG, CarRP* and *CarB* (phytoene desaturase) gene expression after exposure to light for a short interval of few minutes, by northern blot analysis (Velayos et al., 2000a, Velayos et al., 2003, Lorca-Pascual et al., 2004). The *CrgA* transcripts were downregulated at a steady state in (-) while, the (+) had high transcript abundance within a short time of incubation even higher than that of the mated condition (Fig. 3.3.4a).

Fig.3.4.3. Transcriptional dynamics of HMG domain mating type specific transcription factor sex genes *SexM* and *SexP* at asexual and sexual phases in *M. circinelloides.*

The graphs are based on data from three independent replicates and error bar indicates the standard error mean.
Contrarily in *Blakeslea*, *CrgA* transcripts were 100 fold more in the (+/-) mated phase compared to (+) or (-) strains (Fig. 3.3.4b). *CrgA* genes among both genera share similar domain characteristics and photo responses grown under absolute darkness (Quiles-Rosillo et al., 2005), however, our data imply a clear difference in transcriptional regulation of *CrgA* within the order.

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**Fig. 3.3.4b:**

- **a.**
  - *Mcl CrgA*
  - Relative fold change in gene expression over time (hours) with different strains labeled.

- **b.**
  - *Bt CrgA*
  - Relative fold change in gene expression over time (hours) with different strains labeled.
Fig. 3.4.4. The relative fold changes of CrgA gene expression in *M. circinelloides* and *B. trispora*. The notation +/- indicates sexual phase where the spore inoculum from both mating partners were inoculated to liquid broth in equal ratio.

As carotenogenesis is a highly dynamic and complex process in soil borne mucoralean fungi, we were interested to examine the variations in gene expression on a long term exposure to white light and how it influences apo/carotenoid cleavage? *M. circinelloides* (-) that exhibited a significant temporal trend of *AcaA* transcription (Fig. 3.3.1b) was subjected to an initial 12 hours dark, 12 hours light cycle followed by 24 hours of alternate dark and light cycles (Fig. 3.3.5).

![Graph](image)

**Fig. 3.4.5. Transcript levels of genes involved in carotenoid metabolism in CBS 277.49 (-) exposed to a dark and light regime during growth.** Small letters indicate significant differences at P<0.001 among *CarRP*, *CarS*, *AcaA* and *CrgA* at a particular time point and capital letters indicate significant differences at P<0.001 in a single gene at different time points investigated.
Time and genes were the two independent variables analyzed by two-way ANOVA. Relative fold change values of each gene expression were log transformed (log 10) to get a balanced design. Pairwise multiple comparisons were done by Bonferroni t-test. A statistically significant interaction between time and genes were observed.

3.5. Prediction of CarS and AcaA function by template based homology modeling

The protein fold recognition server Phyre2 (Protein Homology/analogy Recognition Engine) was used to predict the functions of CarS and AcaA (Kelley & Sternberg, 2009). Both CarS and AcaA sequences had 100% confidence interval to Synechocystis ACO, VP14 complex having 9 cis-epoxycarotenoid cleavage 1 and retinal pigment epithelium-specific 65 proteins. The final model of CarS protein (95% modeled at >90% confidence) had a single domain with 45% sequence identity having an immunoglobulin like beta-sandwich fold (Fig. S3, S4). The model of AcaA was based on VP 14 with 100% confidence and 78% residue coverage. AcaA structure has an additional glucocorticoid domain belonging to Zn finger family to differentiate it from CarS.

3.6. Heterologous expression and functional characterization of carotenoid cleaving enzymes in E.coli

In vivo enzyme assays were carried out in E. coli having the β-carotene over-producing pβ-carotene plasmid co-expressed with pBAD-TSP3 (Sahadevan et al., 2013). pBAD vector system is arabinose inducible. Among the co-expressed E. coli broth cultures, 0.08% and 0.2% arabinose induction indicated notable bleaching effects in the pelleted cells (Fig. 3.6.1). LC-MS analysis of cells harvested and re-suspended in acetone at 0 and 4 hours did not indicate the presence of β-carotene or any other apocarotenoid, while after 24 hours, the acetone extracts of pelleted cells showed the presence of C_{25}, β-12'-apocarotenal along
with the initial substrate, β-carotene by HPLC (Fig. 3.6.2). The maximum absorption wavelength ($\lambda_{\text{max}}$) was 422 nm for β-apo-12'-carotenal and 450 nm for β-carotene. The compounds were identified using authentic standards. β-apo-12'-carotenal showed the characteristic UV absorption and the expected molecular ion (M+H$^+$) at m/z = 351.10 (Fig. S5).

\[
\text{Beta carotene} \rightarrow \text{Beta-apo-12'-carotenal} + \text{Beta-apo-12-carotenal}
\]

**Fig.3.6.1.** β-carotene got bleached by carotenoid cleavage dioxygenase (TSP3) activity.

*Escherichia coli* cells harbouring both pβ-carotene plasmid and TSP3 gene inserts at 0.08% (B) and 0.2% (C) arabinose grown at 28°C for 24 hours were centrifuged and pellets were decolorized by the enzyme that actively cleaved β-carotene to apocarotenoids. The negative control (E) had pβ-carotene plasmid and empty pBAD vector induced with 0.08% arabinose and (A) was the same without arabinose induction to check for leaky expression. The positive control (D) was pBAD-TSP3 treated with 0.2% arabinose. Four replicates for test samples (B, C) and two controls were maintained for *in vivo* experiments.

The coding region of the designated carotenoid cleaving enzymes CarS and AcaA were PCR amplified, putative positive clones were sequenced and inserted to pBAD vector fused with thioredoxin. *In vivo* enzyme assays were carried out in *E.coli* having the β-carotene over-producing pβ-carotene plasmid co-expressed independently with pBAD-
CarS and pBAD-AcaA. Unlike the homologs in *Phycomyces* (Medina et al., 2011) and *Blakeslea* (Sahadevan et al., 2013) CarS protein in *M. circinelloides*, did not display its expected function of \(\beta\)-carotene cleavage and hence no apocarotenoid products were detected in chromatographic analysis. The inability of AcaA to cleave \(\beta\)-carotene provides strong evidence that like its characterized homolog in *Phycomyces*, the appropriate substrate would be any other carotenoid like lycopene or apocarotenoids.

**Fig.3.6.2.** HPLC chromatogram of *in vivo* enzyme assay with TSP3 co-expressed along \(\beta\)-carotene producing plasmid after 24 hours and EV- empty pBAD vector. Insets show the Ultraviolet-Visible spectra of \(\beta\)-carotene substrate and \(\beta\)-apo-12\(^{+}\)-carotonal.
3.7. Individual trisporoids differentially regulate gene expression in *B. trispora* and *M. circinelloides f. lusitanicus* mating partners

Until now, all research on biological functions of trisporic acids and trisporins were limited either on carotenogenesis or the potential for the development of sexual structures known as zygophores in *M. mucedo* or *P. blakesleeanus*. We speculate that the prominent fluctuations of TSP3 transcripts in *B. trispora* during sexual phase implies the synergistic effects of *de novo* trisporoids regulating a positive feedback metabolic loop (Fig.1.1). In order to understand the genetic potential of individual trisporoids as sexual stimulants mimicking opposite mating partners in *Blakeslea*, real-time PCR transcript analysis of *CarRA* and TSP3 were conducted in (+) and (-) after treating with methyl trisporate C /MTSPC (C\textsubscript{19}H\textsubscript{28}O; 320Da), D’orenone (C\textsubscript{18}H\textsubscript{26}O; 258Da) and trisporin C/TSPC (C\textsubscript{18}H\textsubscript{28}O; 276Da) that are formed at early and late stages of trisporic acid biogenesis.

The trisporoids were treated at 12 hours post inoculation and temporal trends of relative fold change in gene expression was evaluated up to 60 hours of incubation as mentioned above but here the time interval of sample collection is shortened to 12 hours (Fig 3.7.1 & 2). Ethanol, methanol and isopropanol are active solvent stimulants of microbial carotenogenesis (Bhosale, 2004). Therefore the sparingly water soluble trisporoids were dissolved in acetone (50 µM final concentration) and supplemented on both mating partners grown independently in 50 ml liquid induction medium at 12 hours after incubation. The three tested trisporoids induced statistically significant differences in transcripts at each of the 4 given time points in (+) partners (P<0.001, N=4x3), whereas in (-) partners, transcript levels differed significantly among three treatments at 24 hours (P=0.002), at 48h (P=0.001) and 60h (P=0.007). The *CarRA* gene expression varied with each of the trisporoid treat-
mements among the 4 time points (N=4x3) with D’orenone (P=0.016), MTSPC (P<0.001), TSPC (P=0.043) in (-), and D’orenone (P<0.001), MTSPC (P=0.003) and TSPC (P=0.033) in (+) (Fig. 3.7.1a). However, MTSPC and D’orenone imposed unique temporal trends (Fig. 3.7.1b) of differential expression in (-) strains.

The first C_{18} trisporoid D’orenone and methyl trisporate C induced the carotene cleavage in the (+) mating type (Fig. 3.7.2). The impact of trisporins was negligible in both mating partners in comparison to that of D’orenone or β-apo-13-carotenone. At 48 hours after supplementation (60 h after incubation) with D’orenone, the TSP3 transcript abundance in *B. trispora* (+) was 325 fold (Fig. 3.7.2a). All treatments induced statistically significant differences (P=0.009, N=4x3) after 24 h, (P=0.003), 36 h (P=0.027), 48 h (P=0.029). The influence of each trisporoid on the *B. trispora* (+) TSP3 transcript differed significantly among different time points (N=4x3) i.e., D’orenone (P=0.003), MTSPC (P=0.001) and TSPC (P<0.001). Contrary to its impact on CarRA, MTSPC was the major activator in TSP3 expression, in both mating types with a maximum of 1200-fold in (+) partners at 60 hours after incubation (Fig.3.7.2a). The maximum expression in *B. trispora* (-) was at 36 hours both by MTSPC and D’orenone, with 17.5-fold and 4.8-fold increase, respectively (Fig. 3.7.2b). The TSP3 gene expression significantly varied among the treatments including control only in *B. trispora* (-), considering the different time points as a single variable (ANOVA, P=0.018, N=4x4x3).
Fig. 3.7.1. Dose-response transcriptional data of *B. trispora* plus (a) and minus (b) partners on treatment with early and late trisporoids. Letters indicate the statistically significant differences among the treatments namely, D’orenone, MTSPC and TSPC along with control solvent acetone at each time point (small) and among different time points for each treatment (capital).
Fig. 3.7.2. Transcriptional dynamics of carotenoid cleaving dioxygenase (*TSP3*) in *B. trispora* plus (a) and minus (b) mating types after treatment with trisporoids at 12 hours post inoculation. The difference in scale of graphics should be noted. Notations using letters indicate statistically significant difference as previously mentioned.
The variations in transcriptional dynamics in *B. trispora* with different trisporoids motivated us to do similar experiments in the (-) mating partner of *M. circinelloides*, which had a high transcript turnover for genes under natural conditions than (+) partner. Due to its limited availability and lack of knowledge about its biological functions, β-apo-12’-carotenal was treated at 72 hpi and both treated and untreated samples were collected by 84hpi (12 hours after induction). Interestingly, Trisporin C, the compound that was ineffective in *B. trispora*, was the most efficient effector contributing a distinct temporal trend, for *CarRP*, *AcaA* (Fig.3.7.3) and *CarS* transcripts (Fig. 3.7.4a). β-apo-12’-carotenal (*C*₂₅), triggered *AcaA* expression 5 times more and *CarRP* a few folds than the untreated control (Fig.3.7.4b) for the same time point of analysis ie., 84hpi. Therefore the chemical dialect endorsing a successful communication among partners during sex, also depends upon the precursor metabolites that acts as appropriate signal molecules, interacting with genes and genotypes in a specific mode.
Fig. 3.7.3. Transcriptional dynamics of CarRP and AcaA in minus mating type of M. circinelloides after treatment with two trisporoids namely D’orenone and trisporin C. Acetone is the solvent control. The letters indicate significant statistical differences as described previously.
Fig.3.7.4. Transcriptional dynamics of carotenoid metabolic genes in (-) *M. circinelloides* treated with different apocarotenoids. (a) *CarS* transcript after being treated with D’orenone, TSPC and acetone at 12 hpi; (b)*AcaA*, *CarS* and *CarRP* gene expression after β-apo-12’-carotenal treatment at 72 hpi. Hence 72 hpi expression is time zero or basal expression level. Consider 84 hpi (untreated) and 84 apo-12’ (treated) for comparison.
3.8. Generation of $AcaA$ gene knockout mutants

The mating type specific trend in $AcaA$ transcripts motivated us to characterize its biological function adopting the classical approach of genetic transformation feasible in (-) mating type in collaboration with the lab of Prof. RosaRuiz-Vázquez, University of Murcia, Spain. The gene transformation experiments were carried out in her lab and I received the mutants. $acaA$ null mutants were generated by gene replacement, designing a knockout fragment that contains the $pyrG$ gene as a selection marker, flanked by sufficient sequences from adjacent regions of $acaA$ to allow homologous recombination (see Materials and Methods). The 5.6 kb disruption fragment was released from plasmid pMAT1096 by $SmaI$-$SacI$ double digestion for the transformation of a $leuA$ $pyrG$ mutant MU402, (Nicolas et al., 2007) and a $crgA$ $pyrG$ MU223 (Navarro et al., 2001), strain. The $pyrG$ gene included in the disruption fragment complements the uracil auxotrophy of those strains. Forty two and 176 Ura$^+$ transformants were obtained from MU402 and MU223 strains, respectively. As initial transformants are heterokaryons due to the presence of several nuclei in the protoplasts, they were grown in selective medium for several vegetative cycles to obtain homokaryotic transformants. Many transformants with high proportion of transformed nuclei were PCR analyzed to identify homologous integrations, using primers acaA1 and acaA2. Those primers will amplify a 4 kb fragment from the wild type $acaA$ gene that turns to a 5.6 kb fragment if homologous integration of the disruption fragment at the $acaA$ locus had occurred (Fig.3.8.1). Two out of seven Ura$^+$ transformants obtained in the MU223 strain amplified the expected fragment; nevertheless, only one was homokaryotic for homologous integration. This $acaA$ $crgA^-$ strain was named MU366. Four out of nine Ura$^+$ transformants obtained in strain MU402 also amplified the expected PCR fragment, and one of
them was homokaryotic for homologous integration (Fig. 3.8.2b). This *acaA-leuA* mutant was named MU367. Gene replacement in MU366 and MU367 were confirmed by PCR amplification with primers acaA7 (5’-CCACTAGAAGCCGCTTTAGGC-3’) and pyG-F2 (5’-GGCAAGTAACACCACATTCAGAGC-3’), which amplify a 1245 bp fragment only if the disruption fragment had been integrated at the *acaA* locus (Fig. 3.8.2c).

![Diagram](image)

**Fig. 3.8.1.** Schematic representation of the wild type *AcaA* locus (top) and after homologous recombination with the disruption fragment isolated from plasmid pMAT1096 (below). Dark gray box is the *AcaA* coding sequence and white boxes are upstream and downstream flanking regions. The positions of the primers, used for construction of the disruption fragment and to identify homologous integration events are indicated.
The expected sizes of the PCR fragments amplified from the wild type and \textit{acaA} loci are shown.

**A. Heterokaryotic transformants**

![Image](image1.png)

**B. Homokaryons**

![Image](image2.png)

**Fig.3.8.2.** PCR analysis of \textit{Ura}^+ transformants obtained by transformation of the MU223 (left) and MU402 (right) strains with the 5.6 kb \textit{SmaI-SacI} disruption fragment. (A). Amplification of the wild type \textit{acaA} allele produces a 4 kb fragment. Arrow indicates the size of the fragment amplified from the \textit{acaA} allele (5.6 kb). (B) Homokaryotic transformants marked by an asterisk were named MU366 (\textit{acaA}\textit{ergA}) and MU367 (\textit{acaA}\textit{leuA}), respectively. Confirmation of the disruption by PCR amplification using the
primer pairs acaA7-pyrG-F2. Arrow indicates the size of the fragment amplified from the
acaA' allele (1.25 kb).

3.9. Mating experiments

A successful mating is associated with the production of zygospores in Mucorales fungi. Hence the role of CrgA and AcaA associated to sex was analyzed by mating wild types and mutants in different combinations. Solid agar plates of CBS277.49 (-), its mutants MU367 (ΔacaA) and MU366 (ΔcrgAΔacaA) were independently co-inoculated with the wild type (+) strain CBS852.71. The co-cultivation was maintained in the dark at 23°C for up to 10 days. Zygospores were formed in wild type mating partners within 5 to 7 days post inoculation (Fig.3.9.1). Absence of zygospores in crosses with wild type (+) and MU367, MU366 or MU223, suggests that the carotenogenic repressor CrgA and putative apocarotenoid cleavage oxygenase acaA have crucial, yet independent mechanisms in regulating sexual cycle in M. circinelloides.

3.10. The carotenogenesis negative regulator CrgA, represses SexM and activates

_AcaA_ transcription

Since the ΔcrgA mutant is defective in sexual development, we investigated the crgA gene expression during sexual and asexual cycles. We checked the hypothesis that crgA regulates transcription of genes downstream carotenogenesis, by conducting the transcriptional analysis of two carotenoid cleaving oxygenases in the knockout mutant MU223(-) and in mated cultures of this mutant with a wild type (+) partner (Fig.3.9.1). Two-way ANOVA was performed for the independent variables time and genes by a balanced design through log transformation of relative fold change values (dependent variable). Normality test and equal variance tests were passed respectively at P =0.170 and P =0.810. A statistically significant
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difference exist in time (F= 26.476; P<0.001), genes (F= 88.754; P<0.001) and interaction between time and genes (F= 5.446; P=0.001). The temporal trend of AcaA transcripts in MU223 were similar but at a lower turnover rate compared to CBS277.49 (-) is a persuading link to consider AcaA as a CrgA target protein. There were no significant difference in CarS mRNA levels with developmental phases in MU223 (Fig. 3.10.1a) while CarRP had a reduction in transcript turn over compared to wild type (-) strain (Fig. 3.5.1c).

Fig.3.9.1. Mating experiments using wild type plus with wild type minus and mutant strains. (a,b) are the single and double knockout mutants of minus strains. No zygospores or sexual interactions took place in crosses between wild plus and mutants (c,d,e). The black spots are sexual zygospores formed during 5-7 days post inoculation when the wild type partners mate (f).
Fig. 3.10.1. Transcript analysis of all the known genes involved in sex and carotenogenesis independently in single knockout mutant of \textit{CrgA} (a) and on mating with wild plus (b). Small letters indicate significant differences between \textit{CarS}, \textit{AcaA} and \textit{CarRP} at a particular time point while capitals indicate significant differences of a single gene at different time points.
It is worthwhile to note that interaction of wild type CBS852.71 (+) with MU223 (-), instigated a 33 fold transcript upregulation for CarRP (Fig. 3.10.1b) which is 6 times more than the expression in wild type mated phase (Fig. 3.4.1c). Moreover, AcaA gene expression had a progressive increase up to 20 fold at 108 hpi (Fig. 3.10.1b), contrary to the steady mRNA level shown in the wild type mated (+/-) cultures (Fig. 3.4.1b). The SexM transcripts turned out to a maximum of 15 fold by 84 hpi in the absence of CrgA from (-) partner and SexP transcripts were constitutively maintained at an 8 fold upregulation (Fig. 3.10.1b) throughout the time points unlike in wild (+/-) phase (Fig. 3.5.3). Even though CrgA gene expression (Fig. 3.10.1b) had been contributed by the M. circinelloides (+) possessing the gene, lack of a functional protein in the (-) partner resulted in significant alterations in the expression pattern of genes involved in the sexual cycle, which might be a plausible reason for the lack of zygosporé formation on mating with mutants.

3.11. Does AcaA regulate the transcription of other genes?

To explore the regulatory function of AcaA the expression of all other genes involved in sexual cycle were analyzed independently, in knockout mutant and its cross with wild type (+) partner. As the trisporic acid biosynthetic pathway got blocked in MU367, the accumulation of the first cleavage product leads to gradual increase in CarS transcripts (Fig.3.11.1a). Contrarily, during sex (Fig.3.11.1b) the CarS transcripts maintained a steady state except 24th hour, which could be an attribute of the homeostasis maintained by the wild type (+) partner. On mating with wild type (+) the transcripts of SexM and SexP (Fig. 3.11.1b) maintained the same status quo as in wild type asexual phase (Fig. 3.4.3). To conclude, absence of AcaA had no effect upon sex genes while CarRP expression was lowered in the mutant as the trisporoid induced carotenogenesis was dysfunctional.
Fig. 3.11.1. The transcriptional analysis of genes involved in sex and carotenogenesis in *M. circinelloides* f. *lusitanicus* (a) ΔacaA mutant MU367 (-) and (b) while mating with wild type plus.
3.12. *Mucor mucedo* and sex hormones

*M. mucedo* belonging to the order Mucorales is a heterothallic zygomycetes fungus capable of producing copious amounts of β-carotene during sexual phase (Lampila et al., 1985, Vandenenden.H & Stegwee, 1971). The (+) (FSU621) and (-) (FSU620) strains at the mating zone develop specific slender aerial hyphae known as zygophores, within 3 days of inoculation on a solid agar surface (Fig.3.12.1a). They undergo a series of morphological changes where the zygophores fuse to form progametangia. During initiation of fusion the progametangial structures were translucent and gelatinous; gradually they bulge out and mature.

![Images of developmental stages](a) sexual and asexual phases (b) progametangia (c) gametangia (d) Zygospore

**Fig.3.12.1. Developmental stages during the life cycle of *M. mucedo*** (a). Sexual morphogenesis results in microscopic structures ranging from b-d at the mating zone of opposite mating partners.

The gametangial phase is associated to cross wall formation. Zygosporangium was designated as the transition phase from gametangium by the removal of fusion wall followed by plasmogamy. Here one of the suspensor remains translucent while the other develops slight
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yellow-orange colour. Wall of zygosporangium developed warty ornamentations and deposition of sporopollenin resulting in a double walled zygospore in 60-96 hours after incubation (Fig.3.12.1.b-d).

3.13. Raman Spectroscopy

"Investigation of macromolecules like nucleic acids, proteins or other metabolites during complex developmental phases of biological systems is tricky due to their heterogeneity and hence it needs spatial resolution to focus on subject of interest and chemical selectivity to probe particular biomolecules. Vibrational microscopy based on spontaneous Raman scattering permits direct chemical imaging of unstained samples"(Arcangeli, 2000). In this non-invasive method when a sample is irradiated with a laser light, an exchange of energy takes place between excited light and molecules in the sample that results in a measurable Stokes shift in the wavelength of incident laser light (Fig.4.5.1). "In Raman microspectroscopy where an optical microscope is coupled to a spectrometer such a biochemical fingerprint or Raman spectrum indicates the molecular vibrations of all chemical moieties in the interrogated region which could be of volume, even < 1µm³" (Swain & Stevens, 2007). But Raman imaging requires high power due to the small cross section of Raman scattering which in turn leads to a longer exposure time of cells and tissues that limits its application for studying dynamic living systems. Pure standards of β-carotene, trisporic acid C (TSAC), trisporic acid B (TSAB), D’orenone and methyl trisporate B (MTSPB) were subjected to Raman mapping (Fig.3.13.2).
Fig. 3.13.1. A simplified energy diagram of the shifts in wavelengths of incident radiation that provides chemical and structural information about the molecules under investigation.

A Raman shift of 1513, 1154 and 1006 cm\(^{-1}\) were respectively associated to C=C in phase stretching, C-C stretching and CH\(_3\) in plane rocking mode for \(\beta\)-carotene (Papaioannou et al., 2009, Brat et al., 2012, Withnall et al., 2003). The sex hormones, trisporic acids B and C had peaks at 1592 and 1595 cm\(^{-1}\) corresponding to the C=C functional group region, with shoulder peaks at 1621 and 1623 cm\(^{-1}\) (Fig. 3.13.2). The differences in the Raman shift were too weak to distinguish those compounds at a specific vibrational frequency. Early metabolite D’orenone involved in trisporic acid biosynthetic pathway of mucoralean fungi exhibited a distinct and sharp Raman shift at 1586 cm\(^{-1}\). But the spectra of methyl derivative of trisporic acid B were indistinguishable from the sex hormone.
Fig. 3.13.2. Raman spectra of the pure standard compounds ie., β-carotene, D’orenone, Trisporic acid C (TSAC) and Trisporic acid B (TSAB) dissolved in dichloromethane.

3.14. Coherent Anti-Stokes Raman microSpectroscopy

Coherent Anti-stokes Raman Scattering (CARS) microscopy allows vibrational imaging with high sensitivity, high speed and three dimensional spatial resolution (Cheng & Xie, 2004). CARS method targets a specific region of the Raman spectrum for the particular molecule. A molecule initially in its vibrational state excites resonantly its molecular bond,
when the incident frequency \( \omega_p - \omega_s \), between spatially and temporally overlapped pump and stokes laser pulses matches with its inherent vibrational frequency.

![Diagram of Raman Scattering and Coherent Anti-stokes Raman Scattering](newton.ex.ac.uk)

### Fig.3.14.1. Schematics indicating the principle behind the energy shifts leading to an anti-stokes scattering.

The interaction of excited molecule with incident energy from third photon known as probe results in an anti-stokes shift that occurs as the incident photon emit light at a higher energy (Cheng & Xie, 2004). As a result of emission in shorter wavelengths, the autofluorescence property inherent to the sample could be avoided by CARS rather than conventional Raman microscopy (Rodriguez et al., 2006). Intracellular monitoring of carotenoids in *B. trispora* had been reported by Raman spectroscopy (Papaioannou et al., 2009), but the question we faced was whether it would be possible to distinguish the highly abundant \( \beta \)-carotene from the sex hormone trisporic acid B or C that may be present in micromolar concentrations *in vivo*, in morphologically distinct sexual structures of *M. mucedo*? Due to their low abundance, bio transformations and feedback loop taking place *in vivo*, it’s diffic-
cult to isolate individual trisporoid compounds by solvent extraction followed by chromatographic analyses. Therefore, vibrational microscopy techniques like CARS, based on nonlinear Raman scattering with high sensitivity and spatial resolution was a feasible choice for the localization of sex hormones in live tissues.

Based on the Raman spectra we had chosen 1511-1526 cm\(^{-1}\) for β-carotene and 1592-1596 cm\(^{-1}\) for trisporic acids as the appropriate Raman shifts that distinguish two compounds in *M. mucido*. In the asexual hyphal structures β-carotene was present but not trisporic acids (Fig. 3.14.1). We observed the presence of trisporic acid even in the hyphae far away from the mating zone amidst of the abundant chromophore β-carotene in the sexual phase (Fig. 3.14.2). The sex hormones were abundantly present in progametangial structures. Trisporic acids being more water soluble compounds compared to nonpolar β-carotene might have been dispersed to the solid agar medium from the site of biogenesis. As the fusion proceeded, gametangial phase and zygosporangium had a distinct differential CARS signal observed for beta carotene at 1511-1513 cm\(^{-1}\) and trisporic acids at 1592-1596 cm\(^{-1}\). However mature zygospores bearing sporopollenin exhibited auto fluorescence or got burned up at the high intensity laser beam exposure which prevented us from receiving any CARS signals from sexual spores.
Fig. 3.14.1. CARS spectra observed for β-carotene (a) and trisporic acids (c) from the microscopic slide having asexual hyphal structures in M. mucedo. An enlarged view of the inset in (a) is depicted as (b). The white signals indicate presence of β-carotene in asexual phase while that of specific signals for trisporic acids were missing in (c).
Fig. 3.14.2. Progametangial (a) and gametangial (d) structures from *M. Mucedo* under a bright microscope field investigated for CARS analysis. The specific signals at 1522 cm\(^{-1}\) indicate β-carotene (b,e) and 1592 cm\(^{-1}\) that of trisporic acids (c,f) present in those structures.
3.15. GC-MS analysis

Gas chromatography coupled with mass spectrometry was adopted for the chemical analysis of *Mucor mucedo* sex hormones which are low molecular weight apocarotenoid metabolite compounds. Four replicates of solid agar plates having asexual and sexual phases after 72 hours and 96 hours of incubation were subjected to chemical extraction using chloroform: isopropanol (20:1) as solvent mixture (Schachtschabel & Boland, 2007). Contents in each plate were divided to three portions i.e. (+), (-) and (+/-) zone. As trisporic acids possess carbonyl group, derivatisation was done using diazomethane for the pH2 extracts. The methyl derivative of trisporic acid C had a molecular weight of 320 m/z which was observed only in the (+/-) zone of *M. mucedo* (+) and (-) co-inoculated plates (Fig. 3.15.1).

3.16. *TSP1* or 4 dihydromethyl trisporate dehydrogenase in *Phycomyces blakesleeanus*

Based on the results of amino acid sequence similarity and functional characterization of the protein, the *tsp1* gene in *Phycomyces* has the additional xylose reductase function (Kerstin et.al, unpublished results) as observed in *M. mucedo*. In order to check the xylose reductase function, the transcriptional dynamics of *tsp1* were assessed by real time quantitative PCR analysis of (+), (-) and (+/-) cultures using maltose (control) and xylose (treatment) as carbon sources. Quantification of gene expression was done based on 24<sup>th</sup> hpi as time zero. There was a slight up regulation in transcripts at 48hpi for (-) and (+/-) cultures on maltose medium while a constitutive down regulation was observed in xylose medium (Fig. 3.16.1). An interesting observation was the weaker expression of (-) in comparison to (+/-) when the carbon source was xylose. 3 way ANOVA was carried out using the log transformed relative fold change values and observed a statistically significant interaction among time, mating types and sugars. A significant interaction (P=0.004) exists among
time and mating types on treatment with xylose while it remains absent with maltose as sugar source (P=0.365). All pairwise multiple comparisons were carried out based on Bonferroni test.

![Gas chromatogram of the trisporic acids extracted from the sample (above) and that from the standard (below).](image-url)
Fig. 3.15.2. Mass spectrum for the derivatized trisporic acids obtained from sample and standard.
Fig. 3.16.1. The transcriptional dynamics of TSP1 in *P. blakesleeanus* cultivated using maltose and xylose as carbon sources. Small letters indicate the significant difference among (+), (-) and (+/-) at each time point.
CHAPTER 4. DISCUSSION

Molecular aspects of trisporoid signaling is yet unknown due to the lack of proteomics data downstream carotenoid production and the difficulties in gene transformations except in *Mucor circinelloides* CBS 277.49 (-). Here we explore the role of 4 different precursor metabolites involved in trisporic acid biogenesis as signal molecules influencing the transcript levels of structural genes in diverse genotypes. Besides, this study brings forth novel insights on the regulatory functions of CrgA and AcaA in context of fungal sexual communication. Many of the Mucorales are emerging opportunistic human pathogens and sex takes place as their survival strategy, hence it is important to understand the molecular background of trisporoid signaling as a key factor in their chemo ecological interactions.

4.1. How the gene expression of carotenoid metabolic structural genes varies with asexual and sexual phase in wild type strains of *B. trispora* and *M. circinelloides*. Do the early developmental phases influence gene transcription?

To understand the regulation of natural metabolic network, we focused upon the transcriptional dynamics of *CarRA/RP*, *TSP3/CarS*, *AcaA* and *TSP1* at sexual and asexual phases among (+) and (-) wild type mating types of two Mucorales members; having diverse physiology grown under same conditions in the lab. Expecting temporal transient gene expression in such a complex process, we adopted a 24 hr interval time series transcript analysis. A relative quantification of gene expression was previously reported for the carotenogenic genes *CarRA* and *CarB* in individual mating partners and mated cultures of *B. trispora* incubated for up to 3 days (Schmidt *et al.*, 2005, Kuzina *et al.*, 2008). In these studies, the reference genes were β-actin or the transcription elongation factor (*Tef1*) with a basal time point of 24 hours after inoculation for data analysis. The absence of significant temporal
variation even in a (+/-) culture that undergoes diverse physiological changes throughout its developmental phase made β-actin encoding cytoskeletal structural protein the optimal choice as internal standard for real-time PCR analysis (3.2.1). However, we observed the carotenoid production in *B. trispora* commences by 48 hours of spore inoculation as the biomass developed yellow pigmentation. Hence the kinetics of *CarRA* transcripts in mated partners, considering different growth phases (Fig.3.2.2) pinpointed the rationale of choosing early growth phase as basal time point in transcriptional data analysis. Therefore we focused upon an extended time series transcript analyses of the three functional genes known in *Blakeslea*, one involved in β-carotene production (*CarRA*) and two others in trisporic acid biogenesis (*TSP3, TSP1*) for testing the null hypothesis, trisporoids generated by mating partners do not differentially regulate gene transcription during asexual and sexual developmental phases (Fig 3.3.1). A statistically significant difference in *CarRA* gene up regulation was observed only at 48 hours among (-) and (+/-) cultures may be because of activation of molecular signaling cascades involved in early steps of TSA pathway in sexual phase. The constitutive gene expression beyond 72 hours in mated phase supports the ongoing trisporoid regulating feedback loop, while it is reasonable to have higher transcript levels for (-) that produces more β-carotene and negligible trisporic acids compared to (+) strain or (+/-) in *B. trispora*. The (+) strain known to produce 0.1% of trisporic acids compared to mated cultures (100%) in *B. trispora* had a progressive transcript turn over in *CarRA* on a temporal trend, supports the hypothesis that trisporoids synergistically enhances carotenogenesis at genetic level (Fig. 3.3.1a). In our time series experiments without any stimulation, *TSP3* transcripts (Fig. 3.3.1b) had a significant up regulation over the growth phases extending up to 144 hours or 6 days in (+) and (-). A striking fold change of 16,000
at 48 hours exclusively in (+/-) cultures and further decline to 4000 fold by 72 hours exemplifies the transcriptional bursts associated to dynamic biological networks controlled by molecular signals in eukaryotes (Raj & van Oudenaarden, 2008). A stable state of equilibrium might have acquired by the activity of functional proteins and metabolites involved in TSA pathway beyond 72 hours thereby maintaining a constitutive transcript level.

4-dihydromethyl trisporate dehydrogenase (TSP1) that produces methyl trisporate is the penultimate step involved in the putative trisporic acid biosynthetic pathway. TSP1 had been reported as a NADP dependent dehydrogenase localized in zygophores of (-) strains in heterothallic \textit{M. mucedo}. Meanwhile in homothallic \textit{Zygorhynchus molleri} the enzyme activity was observed in the copulating main branch by histochemical analysis (Werkman, 1976). Even though the enzyme was constitutively transcribed in (+) and (-) \textit{M. mucedo} strains, enzyme activity was observed only in trisporoid treated (-) strain. It is interesting to note that the trisporoid consists of trisporic acid fraction extracted from mated cultures of \textit{Blakeslea} (Schimek et al., 2005). A slight upregulation in transcript was observed only at an early timepoint of 24 hpi in both (+) and (+/-) cultures of \textit{Blakeslea} followed by a constitutive down regulation in all three mating types (Fig.3.3.1c). This is the first report on transcriptional dynamics of the \textit{TSP1} in \textit{Blakeslea trispora} based up on real time quantitative PCR assay and hence further investigations are essential to understand the enzyme function.

Sex is a highly dynamic developmental phase in these fungi where the “chemical dialect” among the complementary mating partners regulates a positive feedback metabolic loop. A ‘just in time” pattern of gene expression observed in \textit{TSP3} transcript levels in mated phase of \textit{Blakeslea} is a common trend among genes encoding metabolic and biosynthetic en-
zymes where the temporal activation of gene is a consequence of a cascade of impulse responses (Chechik et al., 2008, Ihmels et al., 2004). The transcript data on CarS, AcaA and CarRP, propose that during mating the apo/carotenoid cleaving enzymes maintain constitutive expression (until 72 hpi) unless until β-carotene synthesis accrue a threshold level within the system in M. circinelloides. A basal level of the putative carotenoid cleavage dioxygenase gene expression (Fig.3.4.1a, b) during sex among wild type mating partners may be an indication of faster biotransformation of the metabolites to produce a stable product or for maintaining homeostasis. The high transcript turnover of CarS in (+) with a temporal increase is an example for a long sustained response, a basic expression pattern in developmental processes. “A developmental time course is analogous to monitoring multiple cell types, some of which are more closely related than others and thus a partial overlap between consecutive time points is frequently observed” (Bar-Joseph et al., 2012).The striking contrast to the earlier reports on carotene super producers Blakeslea (Sahadevan et al., 2013) and Phycomyces (Medina et al., 2011) is that, in M. circinelloides during sexual phase both CarS and AcaA transcript abundance were much lower than asexual wild type strains. Unlike Blakeslea, exhibiting a high accumulation of β-carotene at sexual phase by trisporoid stimulation, M. circinelloides prefers light rather than sex for induced caroteneogenesis. M. mucedo belonging to the same genera showcases an intense yellow-orange pigmentation at the mating zone (Fig.3.12.1) where zygosporcs develop(Gooday & Carlile, 1997) but M. circinelloides do not offer such a visual treat. Moreover no reports are yet available about the identification or quantification of trisporic acids or its putative metabolites in latter. Therefore we reason, in the dark, mating partners of M. circinelloides pro-
duce an initial threshold level of β-carotene as a pre requisite for sex, but beyond that the mechanism of gene regulation varies in sexual communication and carotenogenesis.

On an ecological perspective, a long term exposure to light makes more sense while investigating the response of genes involved in a complex carotenoid metabolic network for understanding their transcriptional mechanism in soil borne fungi. Our data proposes _CarRP_ maintains its sensitivity to prolonged exposure of white light and even after a 24 hour light-dark cycle the transcripts were 18 fold at 96hpi (Fig.3.4.5), rather than dropping down to its basal expression level. It deserves mention that the earlier reports on photoinduction of _CarRP_ and _CarB_ transcripts were tested by northern blot analysis after 72hpi (Velayos et al., 2000a, Velayos et al., 2000b) while we quantified the data based on 12 hpi gene expression. However light play no role in the regulation of gene expression of putative carotenoid cleaving enzymes in _M. circinelloides_.

4.2. What are the apocarotenoid products formed by the activity of carotenoid/apocarotenoid cleaving enzymes in _B. trispora_ and _M. circinelloides f. lusitanicus_?

The high transcript abundance of carotenoid cleavage dioxygenase (CCD) in the sexual phase of _B. trispora_ (TSP3) vindicates its involvement in regulating the trisporic acid biogenesis. Phylogenetics (Fig.3.1.1) and amino acid sequence data (Fig. S2) suggest the presence of a single copy of CCD in _B. trispora_ and 4 copies in _M. circinelloides_ (CarS, AcaA, unk1 [189974], unk2 [114475]). _In vivo_ enzyme assays in heterologous β-carotene overproducing _E. coli_ co-expressed with the gene of interest (von Lintig & Vogt, 2000)is a promising technique to overcome the futility of _in vitro_ experiments using non polar substrates. The identification of C25 apocarotenoid, β-apo-12’-carotenal formed by the TSP3
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function analyzed via HPLC confirms that the early metabolites in putative trisporic acid pathway are conserved in β-carotene super-producers *P. blakesleeanus* (Medina et al., 2011) and *B. trispora* (Sahadevan et al., 2013). Cloning, heterologous expression and *in vivo* enzyme assay of *tsp3* homologue gene designated as *CarS* [146755] and *AcaA* [141273] in *M. circinelloides* did not yield any apocarotenoid product indicating β-carotene is not the ideal substrate for its activity. A computational prediction (Phyre²) for functional analysis of CarS postulates a cis carotenoid compound as the substrate (Fig.S3-4). Approximately about 30 CCOs (Kloer & Schulz, 2006) had been characterized as per the sequence databases and the proposed models have a >90% confidence interval only with 3 CCOS, ie., VP14, Synechocystis ACO and RPE65. β-carotene is not the ideal substrate for both VP14 (Messing *et al.*, 2010) and ACO (Scherzinger *et al.*, 2006). The cloning and *in vivo* co-expression experiments in *E.coli* using β-carotene overproducing vectors were futile supporting the prediction that β-carotene is not the suitable substrate for CarS or AcaA. The (+) specific trend of *CarS* transcripts (Fig.3.4.1a) need to be investigated but currently there is no successful transformation strategy known for *M. circinelloides*(+) to evaluate the gene function. *In vitro* enzyme assays using other carotenoids and even isocryptoxanthin (Gessler *et al.*, 2002, Schachtschabel *et al.*, 2008), speculated as an intermediate in trisporic acid biogenesis would be an alternative choice to for functional analysis. Moreover, cloning and heterologous expression of the unknown genes [189974 and 114475] would give more insights about the functions of carotenoid cleavage oxygenases.

A transient trend in *AcaA* expression even in mutants could be an outcome of its involvement in development and differentiation processes in those tested genotypes. The lack of
sexual zygospores during mating of ΔacaA with wild type partner could be the direct impact due to block in the biogenesis of trisporic acids. The temporal increase in m-RNA levels of the postulated carotenoid cleaving oxygenase, CarS in loss of function mutant ΔacaA (MU367) could be ascribed as the positive feedback induced by accumulation of β-12’-apocarotenal, the expected substrate for AcaA in *M. circinelloides*. Nevertheless, during mating of MU367 with wild type (+), an initial up regulation in CarS was replaced with a consistency in transcript turn over at later time points as the AcaA of (+) partner might have maintained the homoeostasis in the system. Lack of sexual zygospores on mating with wild (+) and MU367 enunciates the role of AcaA in regulating sexual communication.

4.3. How the *M. circinelloides* sex genes transcriptionally respond to mating under diverse genetic backgrounds?

“Mating occurs in complementary mating partners at close proximity” became an outdated concept as we have experimentally proven that the mere presence or gene up regulation of HMG domain transcription factor sex genes, SexP/SexM is not just enough for a successful sexual cycle. The higher m-RNA levels of sex genes during mating fit well with reports on northern blot experiments in *Phycomyces* (Idnurm et al., 2008). A “just in time pattern” (Bar-Joseph et al., 2012) of transient SexM turnover was observed by 96 hpi (Fig.3.4.3) suggesting the transcriptional activation as a consequence of signaling cascade. Among all those genetically diverse conditions, the SexM had a remarkably high transcript turn over (Fig.3.10.1b) during mating of MU223 (ΔcrgA) and CBS852.71(+). Surprisingly, the steady state up regulation of SexP transcripts (Fig.3.10.1b) was unique. In short, high transcript levels of HMG domain transcription factor sex genes need not necessarily be a characteristic of successful sexual cycle, at least in *M. circinelloides*. CrgA as an effector
positively regulate *AcaA* gene expression in wild type (-) (Fig.3.4.1b) while it is a negative regulator of transcription factor *SexM* (Fig.3.4.3) as per our transcriptional analysis data.

The gene expression of *SexP* was higher than *SexM* during mating of wild type (+) and (-) partners than asexual phases as per northern blot analysis in *P. blakesleeanus* (Idnurm et al., 2008). Similar trend was observed on a time series transcriptional analysis of sex gene expression by qPCR in *M. circinelloides*, for asexual and sexual phases (Fig.3.4.3). Sexual phase was futile on interaction of wild type (+) and Δ*acaA* mutant MU367, as trisporic acid biogenesis pathway was blocked, had a down regulation in *SexM* and low transcript turn over for *SexP*. Even though no zygospores were formed mating of wild type (+) and Δ*crgA* mutant MU223 had the maximum transcript turnover for *SexM* and *SexP* than wild type mating where sexual development was successful.

**4.4. Does the carotenogenesis repressor CrgA regulate sexual phase? Is there a variation in gene expression among different genera?**

CrgA possessing two RING finger zinc binding domains is present in almost all eukaryotes except *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. These domains mediate the linkage of ubiquitin residues to target proteins thereby altering their stability, localization or function and hence many cellular processes within the organism. In *M. circinelloides*, CrgA product targets Mcwc-1b, one of the three white collar photoreceptor genes, ubiquitinating and thereby hampering its function as a transcription factor activating the expression of carotenoid biosynthetic genes (Navarro *et al.*, 2013). No information is available about the regulatory function of CrgA in any of the Mucorales members either influencing sexual cycle or carotenoid cleavage oxygenases associated to sex hormone production. Hence our mating experiment that failed to develop sexual zygospores with wild type (+)
having CrgA and (-) MU223 (ΔcrgA mutant), is the first of its kind, unequivocally depicting the involvement of CrgA from (-) mating types in sexual communication. However, in the above mentioned mating, the gene expression of SexM was comparatively higher than asexual (-) partner or with other mating combinations. Reduction in transcript turnover of AcaA and CarRP in MU223 in comparison to wild type (-) suggests, CrgA probably targets those proteins too.

The discrepancies over lack of correlation in CarRP transcripts and carotene accumulation of MU223 had been reported earlier (Nicolas et al., 2008) citing similar outcomes in Neurospora crassa. A rational explanation for an impulse like pattern of CarRP expression, in a cross with wild (+) and MU223 (Fig.3.10.1b) could be the transcriptional activation induced by a specific signal molecule released from (+). Infact, our M. circinelloides data depicting futile sexual phases in mating experiments of wild (+) with (-) mutants, overaccumulating carotenoids (MU223, MU366) supports the findings on β-carotene overproducing and leaky carR mutants well known in Phycomyces(Ootaki et al., 1996). Hence there exists a non-linear relationship between zygospore development and content of β-carotene or its related metabolites, though the mechanisms are unknown. But the involvement of CrgA in development of sexual zygospores is a novel observation and a turning point for exploring its function in cell differentiation and interactions with genes involved in sexual cycle and development.

Sex in mucoralean fungi is a complex and fascinating phenomenon interlinked with secondary metabolism. Curiously, a 100 fold higher expression rate of CrgA in B. trispora(+/) phase having a higher rate of carotene production, compared to the asexual phase (Fig.3.4.4). This is also the first report unveiling the involvement of the carotenogenic re-
pressor ring finger protein CrgA and apocarotenoid cleavage oxygenase AcaA for the development of sexual zygospores. However, further studies on the regulatory mechanisms of CrgA which apparently activates AcaA transcription and represses SexM may bring forth the link between sex genes and a protein complex orienting trisporic acid signaling mediating sex in Mucorales.

4.5. Does the apocarotenoid impart a chemical dialect regulating the transcription of genes which varies with genes and genotypes?

Trisporins that activate zygophore development at millimolar concentrations (Ootaki et al., 1996) and methyl trisporates which enhances carotenogenesis even at micromolar units (Rao & Modi, 1977) are the exclusive morphogenetic factors known in Mucoralean sexual phase, while β-apo-13-carotenone (D’orenone) is an unexplored compound. At 50µM concentration, the latter induced transcript levels of CarRA and TSP3 in Blakeslea. Meanwhile, D’orenone or β-apo-13-carotenone did not stimulate the gene expression of carotenoid cleaving enzymes but positively induced CarRP transcripts in M. circinelloides(-).

The biological function of D’orenone is known as an apocarotenoid, inhibiting root elongation in Arabidopsis (Schlicht et al., 2008) and had been proposed as an intermediate in strigolactone biosynthetic pathway (Schwartz et al., 2004). But in rice seedlings supplementation of the metabolite neither inhibited tiller development nor it get bio transformed to strigolactone (Alder et al., 2012).

One of the late precursors in the pathway, trisporin C (TSPC), had a positive feedback up-regulation on CarRP and AcaA while the impact was comparatively weak on CarS transcripts. The concept of a “chemical dialect” among the mating partners in Mucorales varies with genotypes and genes, remains unabated as the trisporin C which had no influence on
gene transcription in either (+) or (-) *Blakeslea* is the most active compound in *M. circinelloides* (-). This may throw light on its differential effects and dose-dependency that varies with genus, species or strains as proposed in pheromonal-action-unitary theory (Sutter & Whitaker, 1981). It is interesting to note that the early trisporoid triggered *CarRA* transcripts while late trisporoid MTSPC influenced *TSP3*. On treatment with β-apo-12'-carotenal (C25), the putative apocarotenal product known among Mucorales, *AcaA* transcripts were 5 times more than untreated control which is a strong evidence that the substrate for *AcaA* activity could be apocarotenal while *CarS* had no change at all in its transcript levels.

In general, the higher transcript levels of mated culture without trisporoid stimulation for respective genes explain the synergistic effects of these metabolite cocktails naturally present in fungal partners. Trisporoids and apocarotenoids as signaling molecules determine the differential trends in the transcription of genes associated with the carotenoid metabolic network throughout the developmental phase in *Blakeslea* and *M. circinelloides*.

### 4.6. Is it possible to localize sex hormones of *M. mucedo* by the in vivo imaging method of Coherent Anti-stokes Raman micro Spectroscopy?

Based on Raman spectrum for trisporic acids (B, C), D’orenone and β-carotene we observed a clear difference in phase stretching of C=C conjugated double bonds between the substrate and sex hormones making it technically possible to distinguish β-carotene from the trisporic acids. But the difference in wavenumber among sex hormones and D’orenone was less than 10 cm⁻¹ that suggest it is impossible to get a spectral separation and hence identification of those distinct compounds in live tissues. The most powerful perspective of CARS method in biological systems is high spatial resolution, provided, the tuned vibra-
tional Raman resonances are sufficiently distinct and in high enough local concentration (Pezacki et al., 2011). This is exactly what we achieved with the CARS signal for both substrate and trisporic acid (either B or C) in the sexual progametangial structures.

β-carotene is a yellow pigmented highly carbon conjugated molecule with 11 C=C double bonds while trisporic acids possess only 3. Owing to their very low concentration and a positive feedback on carotenogenesis by trisporoids it was doubtful getting CARS signal from fungal hyphae. But we observed clear resonant signals from gametangial stages in *M. mucedo* for both β-carotene and trisporic acids as the comparisons were made with off time and non-resonant (different wavelength) signals. It is interesting to note that the translucent progametangial fusion phase of zygophores had no signal at all. The microscopic sexual structures had yellow pigmented compound developed only on one suspensor in gametangia and was maintained upto zygospore formation. Hence it is reasonable to propose the biogenesis of trisporic acids taking place in mature gametangial phase by the cleavage of β-carotene through a rapid sort of biotransformations.

4.7. Do the different sugar sources influence the transcription of *TSP1* belonging to aldo keto reductase superfamily in *P. blakesleeanus*?

Aldo keto reductases (AKR) are oxido reductases exhibiting broad substrate specificity widely distributed among prokaryotes and eukaryotes. Structural analysis of human aldose reductase revealed nonpolar aromatic compounds binding to the hydrophobic active sites are much better substrates than the polar monosaccharides (Czempinski et al., 1996, Wilson et al., 1993). The enzyme investigated within this study on a theoretical level showed all characteristics of an AKR belonging to the mannose and xylose family and it is now questionable whether the enzyme 4-dihydromethyltrisporate dehydrogenase is able to catalyze
xylose reduction as well (K. Hoffmann, unpublished results). The transcript turnover of TSP1 in both *Phycomyces* and *Blakeslea* grown in maltose as a sugar source was low. Trisporic acid production was facilitated in mated state and hence a higher expression level of TSP1 in (+/-) was observed even in presence of xylose (Fig.3.16.1b). A reasonable explanation is non polar 4 dihydro methyl trisporate which was abundantly produced during mating probably got bonded to the active site of TSP1 and might have further activated the gene transcription. It is important to note that no significant differences were observed either in development of sexual structures or zygospores in mating experiments of *Phycomyces* using maltose and xylose as carbon sources.

**4.8. Future perspectives on the chemical communication in mucoralean sex**

Fungal communication plays a vital role in growth, development, morphogenesis, mating, activation of virulence factors and pathogenesis. Does the sexual phase or the metabolite associate to the pathogenic phase in these fungi still remains as an open question?

A possible association of virulence and AcaA should be evaluated as the transient trend of gene expression with the development phases in highly virulent minus mating types (Li et al., 2011) was persistent, irrespective of the genetic variations and light stimuli. Moreover the phenotype of double mutant (∆crgA:∆acaA) resembles the single knockout mutant ∆crgA (Fig.5). This exemplifies an alleviating or “diminishing returns” gene interaction, as a mutation in one gene impairs the function of a whole pathway thereby masking the consequence of mutations in additional members of that pathway (Mani et al., 2008) and can be addressed by a systematic fitness and interaction studies. Molecular characterisation of the unknown genes mentioned in this study (Fig.2, Tab.1) need to be performed to identify their biological functions. The repertoire of single and double knockout mutants, amenable
genotype for genetic manipulation, potential as a human pathogen and genome data makes *M. circinelloides* the apt model to gain further insights on trisporoid signaling in Mucorales thereby exploring its broader impacts on host interactions. Besides, the possibility to identify metabolites like D’orenone *in situ* via Raman micro spectroscopy opens up a new track in plant physiology and biomedical studies.
CHAPTER 5. SUMMARY

A haploid asexual phase predominates life cycle in the saprotrophic soil borne fungi belonging to the order Mucorales, but adverse extrinsic factors along with darkness promote a “sexual communication”. During sex, a sequential cleavage of β-carotene produces trisporoid intermediates which undergo biotransformation among the plus and minus ‘complementary mating partners’ churning out to a cocktail of sex hormones known as trisporic acids. The latter stimulate a positive feedback regulation on carotenogenesis. But little is known about the physiochemical functions of the initial enzymes and products involved in the sexual phase among Mucorales. We hypothesise that most of the intermediate C18 trisporoid have a regulatory function upon the genes linked to sex pheromone production varying with their species, vegetative and sexual phases of development.

1. Transcript analysis of genes involved in hormone biogenesis

Real-time quantitative PCR kinetics conducted in Blakeslea trispora belonging to Choanephoraceae, displayed an exceptionally high transcriptional upregulation for carotenoid cleavage dioxygenase TSP3, during sexual phase. A reasonable explanation could be the higher amounts of protein facilitate the biogenesis of sex hormones that in turn switch on the sexual interactions regulating positive feedback metabolic loops and thereby enhancing faster substrate-product bioconversions. In the Mucoraceae member Mucor circinelloides f.lusitanicus, two putative carotenoid cleavage oxygenases (CarS and AcaA) were identified based on phylogeny and the conserved amino acid sequences among four other mucoralean species. Unlike B. trispora, during sex M. circinelloides f.
lusitanicus maintains a white phenotype that obviously supports the insignificant transcript up regulation for carotenoid cleaving enzymes. Meanwhile the transcript turnover was specific for CarS in (+) and with a consistent temporal trend for AcaA in (-) mating type. Even after an exposure to 12-24 hour dark-light cycles, neither CarS nor AcaA had any variation in its gene expression pattern, suggesting carotenoid cleaving enzymes in M. circinelloides (-) are not light dependent. Interestingly, TSP1 gene belonging to the aldo keto reductase superfamily, known in trisporic acid biosynthesis was down-regulated irrespective of asexual or sexual phase in both P. blakesleeanus and Blakeslea.

2. Heterologous expression of carotenoid cleaving enzymes in Escherichia coli

Cloning and heterologous co-expression of TSP3 with β-carotene overproducing plasmid in E.coli for in vivo enzyme assay followed by LC-MS analysis led to the identification of β-apo-12’-carotenal, as the first apocarotenoid in Blakeslea. Similar experiments conducted for the homologues in M. circinelloides were futile as CarS proteins were dysfunctional. The inefficiency of AcaA to break the C_{13-14} bond of β-carotene was expected, as its homologue in Phycomyces was exclusively specific for lycopene or apocarotenoid substrates. A computational prediction (Phyre²) for the functional analysis of CarS postulated a cis carotenoid compound as the ideal substrate.

3. Functional analysis of knock out mutants in association to sex genes and carotenogenesis repressor CrgA

A successful mating is associated with the production of zygospores in Mucorales fungi. Except wild type (-), the mutants derived from it, MU223 (ΔcrgA), MU367 (ΔacaA) and MU366 (ΔcrgA:ΔacaA) were futile in developing sexual zygospores while mating with
wild type (+). Hence CrgA and AcaA from (-) partner have crucial functions in trisporic acid biosynthesis and sexual morphogenesis. Besides, CrgA transcriptionally repressed the SexM and activated AcaA in *M. circinelloides* (-). A high transcript turnover was observed for SexP and SexM in mating experiments that failed in zygospore production. Therefore we proved that mere presence of specific sex genes is not enough for a successful sexual cycle and ‘complementarity’ criteria of the partners need to be redefined based on these new findings.

4. **Role of apocarotenoids as transcriptional activators**

Supplementation of C\textsubscript{18} trisporoids namely D’orenone, methyl trisporate C (MTSPC) and trisporin C (TSPC) increased CarRA and TSP\textsubscript{3} transcripts in (+) compared to (-) partners of *Blakeslea*. A phenomenal increase in AcaA transcripts after β-apo-12’-carotenal treatment probably denotes, the apocarotenoid as an efficient substrate for the enzyme activity. Or else the formation of apocarotenal product would be a rate limiting step in trisporic acid biosynthetic pathway. Trisporin C, which was inactive on *Blakeslea*, dominated β-apo-13-carotenone (D’orenone) enhancing CarRP and AcaA gene expression in *M. circinelloides*. We conclude that the apocarotenoids and trisporoids influence gene transcription and metabolite production depending upon the gene, corresponding genus and the developmental phase of the strain, representing a ‘chemical dialect’ during sexual communication.

5. **In situ localisation of hormones in sexual structures of *M. mucedo***

*M. mucedo* (Mucoraceae) was an ideal choice as they produce trisporic acids copiously and have a very distinct morphological differentiation from zygophore to zygosporangium. A Raman mapping of β-carotene and pure trisporic acids (B and C) enabled us to get the
specific vibrational Raman shift that distinguishes compounds. Coherent anti-Stokes Raman Spectroscopy (CARS) is a three dimensional non-invasive approach, and we successfully detected both sex hormone and its highly abundant precursor β-carotene in the pro-gametangial phase. Moreover GC-MS analyses of solid agar extracts clarified that detectable amounts of trisporic acid production is limited to mated cultures in Mucor mucedo. Application of CARS method seems promising to detect biologically unstable metabolites in situ, at the developmental phases of biological systems in a non-destructive manner.
ZUSAMMENFASSUNG


1. Transkriptanalyse von Gene der Hormonbiosynthese

Real-time quantitative PCR Kinetiken für Blakeslea trispora (Choanephoraceae) zeigen während der sexuellen Phase eine außergewöhnlich hohe transkriptionelle Hochregulation für die Carotenoid-spaltende Dioxygenase tsp3. Eine Erklärung hierfür wäre z.B. dass die erhöhte Proteinmenge die Biogenese der Sex-Hormone fördert, was wiederum positiv auf die metabolischen Loops der sexuellen Interaktion wirkt und dadurch die Geschwindigkeit der Substrat-Produkt Biokonversion erhöht.

Für Mucor circinelloides f. lusitanicus (Mucoraceae) konnten anhand von Phylogenie und aufgrund der hoch konservierten Aminosäuresequenz von vier weiteren Mucorales zwei putative Carotenoid-spaltende Oxygenasen (CarS und AcaA) identifiziert werden. Im

### 2. Heterologe Expression Carotenoid-spaltender Enzyme in *Escherichia coli*

Durch die Klonierung und heterologe Co-Expression von TSP3 und einem beta-Carotin überproduzierendem Plasmid in *E. coli* konnte anhand von *in vivo* Enzym-Assays und anschließender LC-MS Analyse das β-apo-12'-carotenal als erstes gebildetes Apocarotenoid in *B. trispora* identifiziert werden. Ähnliche Experimente zur Identifizierung der entsprechenden Homologen in *M. circinelloides* waren negativ, da alle putativen CarS Proteine nicht funktional waren. Die Ineffizienz von AcaA beta-Carotin am C_{13-14} zu spalten war erwartungsgemäß, da die entsprechenden Homologen in *Phycomyces* ausschließlich spezifisch für die Substrate Lycopin und Apocarotenoid sind. Eine computergestützte Vorhersage (Phyre^2^) zur funktionellen Analyse von CarS legt als das ideale Substrat eine cis-carotenoid-Verbindung nahe.
3. Funktionelle Analyse von knock out Mutanten von Sex-Genen und dem Carotenogenese Repressor crgA


4. Rolle der Apocarotenoide als transkriptionelle Activatoren

zeigte sich inaktiv in *B. trispora*, dominierte aber β-apo-13-carotenone (D’orenone) erhöhende *carRP* und *acaA* Expression in *M. circinelloides* (-). Man kann schlussfolgern, dass Apocarotenoide und Trisporoide die Transkription and Metabolitproduktion beeinflussen in Abhängigkeit vom jeweiligen Gen, der korrespondierenden Gattung und der Entwicklungsphase des Stammes und somit einen "chemischen Dialekt" während der sexuellen Kommunikation darstellen.

5. *In situ* Lokalisierung der Hormone in den sexuellen Strukturen von *Mucor mucedo*

6. REFERENCES


REFERENCES


7. APPENDIX

Fig. S1. Agarose gel electrophoresis for SexP and SexM amplicons from Mucor cir- cinelloides f. lusitanicus cDNA.

Lane1: DNA ladder (low mass range, fermentas), lane 2&3: cDNA from plus mating type amplified using sexP and sexM primers, lane 4&5: cDNA from minus mating type amplified using sexP and sexM

Fig.S2. Amino acid sequence alignments for CarS and AcA among five species in the order Mucorales

CarS

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Umbelopsis -----------------------------------------------MFRNTEIPQVFALPVN-GQL 20
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Mucor PSWLNGIMYRFPGKYNPSWLNGIMYRFPGKYN 120
Rhizopus PSWLNGIMYRFPGKYNPSWLNGIMYRFPGKYN 118
Umbelopsis PAWLNGTLFRGFKFNIKMKGDYSYHMKIFHDFGLPSVFAVNNQIYQNYRSNQL 80

Phycomyces FESSIADNGQKFGFHPGTMSVKSMTFVTSVQRLKDIFRFDSILLLRPLDTSASPQGVTA 169
Mucor LETKIKKNTSKGLLFFHIPETM-EPFWLYNFYIFNNLLPPHMDTGRIPSQAVGTV 179
Rhizopus LEKISSGADKGLVFFGHVPEVS-FLTWLIVRVLNLVRPPIILTLPGRSGVQTV 177
Umbelopsis VERQLA-DNDDYPLFFGHDPSATLSFIKLKYRKHRRSSIAN---ETRHPSSDAMGVTA 136
Phycomyces TPNFIPPPVKAADKNGESDRVLVAKTDANMLQQLNSDTELKRFNYGKSLLKGD 229
Mucor TPNFPLP-ARIKKAD---NENVLSDKANLQVQHAETLEPQINFNTSYDARIQGQL 234
Rhizopus TPNYTLF--ARVMENNELSLKSKSLVAKTDANLQVQKHAETLVEIFSYKNDPHLNGPF 234
Umbelopsis TPNFPLGNDHLADRKVQG-DOQLVAKTDANLQLLLVDHPSFVPPKIFNYYYIIIPEVQ7QG 194
Phycomyces SAAHQQYDPIKTETINFVDMF-ARLQVFSTPEGKITITLALFTHLDEKRTRVPVYI 288
Mucor SAAHQQYDPNTKEIFNFALTGPVPRVLVFSTSESGKATILADTFHDTFRT-DK-SPIQAPIY 292
Rhizopus SAAHQQYFDPNTEIFNFALTGPVPRVLVFSTSESGKATILADTFHDTFRT-DK-SPIQAPIY 293
Umbelopsis SAAHQQYDPIKTETINFVDMF-ARLQVFSTPEGKITITLALFTHLDEKRTRVPVYI 253
Phycomyces HAFNITKDYIILPEYSLAYTNMGVDFLVSGAVNTGMAWSNDRPTFHVISR-----HGKGL 344
Mucor HSLWLTENYVIPESPMVLLQKNGANMLKNGSFLILLTWKVDAPTYCHVIAIARRPAQEKEGL 352
Rhizopus HSFYLTQKVIPESPLVYGDQLNSLQLQAGVTSSMWIDQAPLHYVHR-----NEGGL 349
Umbelopsis HSFALTQNYUSWYFAGLQPGFILAEGG---IHFECAGPDFGIAHQLHTFQPKVRKFGTA 309
Phycomyces VASVFV-ETFSSFHVANADWVSQAGQRQVIMDICAENADIMCLHLTFAPVRQAEDS 403
Mucor VASIFPVGFFTFHVNGAFETKNEGEVMTLDAASSFCGDIMHQLHSGFTPQHRNGTAE 412
Rhizopus VASIFPA-PAFYTFHVANAEFVSLDQELLHHLDSSADSGDIIYQVQOFEGQTLQ-FDL 406
Umbelopsis VATIEA-DAFAPFHMNGCWEDEAEG-----IHFEAGPDFGIAHQLHTFQPKVRKFGTQ--- 363
Phycomyces HKKQLEDLSQSQQYMNIIPRQPSGFLRRYQI-----VLENHT distract ANVEFA 460
Mucor KVS---------QTTFNGISYPQQSQFGLVRYKLN---LDQST-TLSIDTLAKNAEF 460
Rhizopus SLK---------RTKFHGFPTFPAQQSVFGLHRHTL-----NQVRT-AVFAHTLAENIEFP 454
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Fig. S3. Phyre² homology predicted structures for CarS and AcaA from *M. circinelloides*. 
**Fig. S4. Template analysis for predicted CarS and AcaA structures**

<table>
<thead>
<tr>
<th>#</th>
<th>Template</th>
<th>Alignment Coverage</th>
<th>3D Model</th>
<th>Confidence</th>
<th>% I.d.</th>
<th>Template Information</th>
</tr>
</thead>
</table>
| 1  | c2bwc    | Alignment         | ![3D Model](image1) | 100.0      | 23    | PDB header: oxidoreductase  
Chain: C: PDB Molecule: apocarotenoid-cleaving oxygenase;  
PDB Title: crystal structure of apocarotenoid cleavage oxygenase from *Synechocystis* native enzyme |
| 2  | c3rpaA   | Alignment         | ![3D Model](image2) | 100.0      | 22    | PDB header: oxidoreductase  
Chain: A: PDB Molecule: 9-cis-apocarotenoid dioxygenase 1, chloroplastic;  
PDB Title: structure of vp1 in complex with oxygen |
| 3  | c3rfaA   | Alignment         | ![3D Model](image3) | 100.0      | 25    | PDB header: isomerase  
Chain: A: PDB Molecule: retinal pigment epithelium-specific 65 kDa protein;  
PDB Title: crystal structure of rpe65 at 2.14 Ångstrom resolution |
| 4  | c3qca2A  | Alignment         | ![3D Model](image4) | 54.4       | 21    | PDB header: hydrolase  
Chain: F: PDB Molecule: glycosyl hydrolase;  
PDB Title: crystal structure of a glycosyl hydrolase (bacova_03624) from *Bacteroides ovatus* at 2.39 Å resolution |

**Detailed template information**

<table>
<thead>
<tr>
<th>#</th>
<th>Template</th>
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<th>3D Model</th>
<th>Confidence</th>
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<th>Template Information</th>
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| 1  | c3rpaA   | Alignment         | ![3D Model](image5) | 100.0      | 21    | PDB header: oxidoreductase  
Chain: A: PDB Molecule: 9-cis-apocarotenoid dioxygenase 1, chloroplastic;  
PDB Title: structure of vp14 in complex with oxygen |
| 2  | c2bwc    | Alignment         | ![3D Model](image6) | 100.0      | 23    | PDB header: oxidoreductase  
Chain: C: PDB Molecule: apocarotenoid-cleaving oxygenase;  
PDB Title: crystal structure of apocarotenoid cleavage oxygenase from *Synechocystis* native enzyme |
| 3  | c3rfaA   | Alignment         | ![3D Model](image7) | 100.0      | 22    | PDB header: isomerase  
Chain: A: PDB Molecule: retinal pigment epithelium-specific 65 kDa protein;  
PDB Title: crystal structure of rpe65 at 2.14 Ångstrom resolution |
| 4  | d1qqa2   | Alignment         | ![3D Model](image8) | 34.2       | 15    | Folded-bladed beta-propeller  
Superfamily: C-terminal (heme d1) domain of cytochrome cd1-nitrite reductase  
Family: C-terminal (heme d1) domain of cytochrome cd1-nitrite reductase |
Fig. S5. LC-MS spectrum of β-carotene and β-apo-12’ carotenal based on *in vivo* enzyme assay. TSP3 cleaves the substrate at C_{11’}-C_{12’} position leading to the formation of a C_{25} apocarotenal and confirmed results using reference standards.
8. ACKNOWLEDGEMENTS

The time I spent over in Jena, professionally as a graduate student and personally as a young foreigner, will remain forever as one of the most memorable phases of life. An entirely different experience to interact with people from diverse cultures, regions, cuisine, opinions and those travels to exotic places are worthy for a lifetime; it was a great chance to explore myself critically and in depth. I am always grateful to all the members of Bioorganic Chemistry department for maintaining a contagious friendly ambience that made life easy and energetic.

First of all, I express my sincere gratitude to Prof. Wilhelm Boland, for a studentship in zygomycetes project, inspiring suggestions, freedom and always being supportive bestowing trust in me. I am thankful to Doreen for her patience and help in the initial days in analytical chemistry, gas chromatography, setting up experiments for collecting VOCs and her sincere advises on research. I owe thanks to Dr. Kerstin Hoffmann for being so proactive and helpful in analyzing sequence data, phylogeny and for the excellent teamwork. It was very interesting the appointments with you Dr. Kerstin Voigt and am thankful for the patience and wise advices about research that I gained from you. I would like to thank you Prof. Erika Kothe for your talks as well as persona influenced me to dream about a career in academia.

Thank you Anja David for being always helpful and offering a solution with a smile to every problem that I approached you. I am indebted to Dr. Kerstin Ploss for her guidance and time in helping me to run HPLC measurements. I appreciate the time and technical help offered by Dr. Maritta Kunert and Dr. Michael Reichelt in developing a better method.
for chromatographic analyses. It was always a pleasant experience with you Grit Winnefeld and I thank you for perfectly organizing my appointments like that of foreigner’s office or facilitating all amenities needed for conference trips. I acknowledge my gratitude to Dr. Axel Mithöfer and Dr. Antje Burse for being so friendly and approachable. May I thank you Dr. Christian Kost for the positives vibes you spread through smiles and the time you spared to listen patiently about my worries on the project. I would like to express gratitude to Dr. Jan Kellmann for his wise suggestions in making deals with stuffs that I could not comprehend and sharing his experiences on maintaining balance at work and life. Dr. Karin Groten, you were always motivating with your bountiful optimism and thank you for your kind support in finding a solution for every problem that I sought your help.

I mesmerize those amazing time we shared together inside lab, around Jena, Weimar and at Leipzig with you Guanjun Li and am so happy and thankful for being a great and trustworthy friend. I highly appreciate the funny moments and timely help offered by Guillermo, re-purifying the chemical compounds thereby saving me from further depression. I do acknowledge the camaraderie of Amra, Erika, Huijuan, Glen, Abith, Mina, Jens, Gerhard, Frenzie, Sandra, Nadja, Rene, Sindy, Karla, Sabrina and Wenhua. I thank Daniela for her help during IMPRS parenting and Radhika Venkatesan for her graceful motivation and whole hearted support in helping me adapt to the new environment. I am grateful to Peter, Dr. Shabab and Dr. Jyothi for their umpteen and wise tips that facilitated my endeavours in protein chemistry and molecular biology. Holger deserves special mention being both irritating to the core and at the same time very helpful in the extremely needy hours in lab life like offering JM 109 strains.
The administrative staffs in our institute are unique for being so cordial, responsible and always having a pleasing demeanor and thank you Christine Rams, Jens, Eleonore, Antje Buchwald and Evelyn. I highly appreciate the service provided by Martin, Hendrik, Marcel, Matthias and Dieter from the IT desk, Linda Maack from the library section. I would like to thank Samay for patiently teaching me about SPSS. I am thankful to Soumya, Elke-Martina and Riya for their time and assistance in doing microscopy at Prof. Kothe’s lab. My sincere gratitude to Ina Weissflog from IPHT for nicely coordinating the experimental set up and conducting Raman spectroscopic analysis as a collaboration partner.

I am so lucky to have such a foster family in Jena with Jacqueline, Herald and Arnold, who took pain to care about my welfare in every possible way. I learnt about German lifestyle and culture in those three years sharing your home. Besides, it was always wonderful the moments I spent with you my dear Indian friends and Jena Malayalees, who add not only rainbow colors but also four seasons to my world. Let me thank you Dr. G. Prasad for being more than a mentor and always energizing me with your positive attitude to life. I owe you for the freedom to share my joys and disclose sorrows without a second thought and grateful to the trust in me. I am thankful to you Vaibhav for the love, trust and being a reliable companion through thick and thin.

Thanking you my dear parents for sharing my dreams and guiding me throughout the rough path with your emotional support and unconditional love.
9. Eigenständigkeitserklärung


Yamuna Sahadevan
10. CURRICULUM VITAE

Personal data

Name : Yamuna Sahadevan
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Sex : Female
Nationality : Indian
Marital status : Single
Email : iyamuna@gmail.com

Scientific career

Since 01/2009 PhD student at the Department of Bioorganic Chemistry, Max
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06/2006- 09/2008 Master’s thesis in Agricultural Microbiology titled ‘Studies on
Developing antioxidant compounds from pigmented microorganisms’

guided by Prof. K. Chendrayan, Dept. of Agricultural Microbiology,

Tamil Nadu Agricultural University, Coimbatore, India

04/2001-12/2005 Bachelor’s in Agricultural Sciences at College of Agriculture, Kerala
Agricultural University, Thiruvananthapuram, India

Scientific publications

trisporoids differentially regulate β-carotene production and gene transcript levels in the
mucoralean fungi Blakeslea trispora and Mucor mucedo. Appl. Environ. Microbiol . (Dec
2013)


**Oral presentations**

1. Sahadevan. Y and Boland. W: Apocarotenoids as signaling molecules in mucoralean fungi (*Invited talk; Carotenoids, Gordon-Kenan Research Seminar; Emerging roles of carotenoids in living organisms, Ventura, California, USA, January 2013*)

2. Sahadevan. Y and Boland. W: The chemistry of sex in zygomycetes (*9th International Max-Planck Research School Symposium, MPI for Chemical Ecology, Dornburg, Germany, February 2010*)


**Poster presentations**


**Membership in scientific societies**

Member of International Carotenoid Society

**Fellowships and awards**

- Max Planck Gesellschaft (MPG) funding for pursuing PhD at Max Planck Institute for Chemical Ecology, Jena, Germany
- Indian Council of Agricultural Research (ICAR) Junior Research Fellowship for Master’s Degree programme in Agricultural Microbiology.
- State Government of Kerala merit placement in professional Bachelor’s Degree in Agricultural Sciences.