Molecular aspects of sex and trisporoid signaling in mucoralean fungi

Dissertation

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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

Wm^{-2}- 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) (Ituriaga *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 multi-cellular 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 20th 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 *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. *Aspergillus* responds to environmental cues influencing secondary metabolism by signal transmission through Cys_2 His_2 zinc-finger transcription factors that mediate carbon (CreA) (Dowzer & Kelly, 1989), nitrogen (Wong *et al.*, 2009) and pH (PacC) signaling (Tilburn *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 *et al.*, 2011). In bread mold fungus *Rhizopus* (zygomycetes) or ascomycete *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.

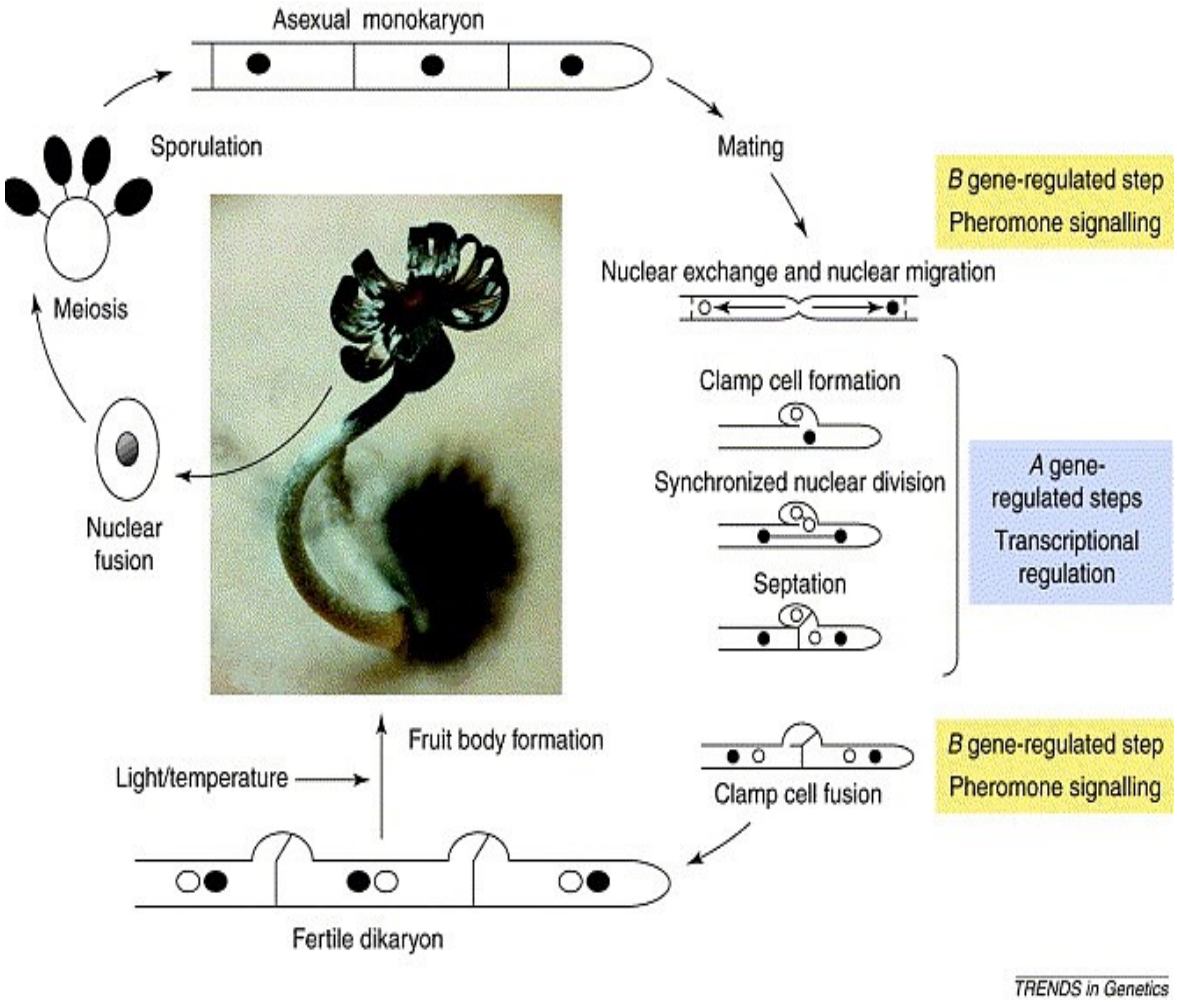

Fig.1.3.3.1.Mating in inkcap mushroom *Coprinus cinereus* orchestrated by MAT loci

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 a1 and α1in 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.

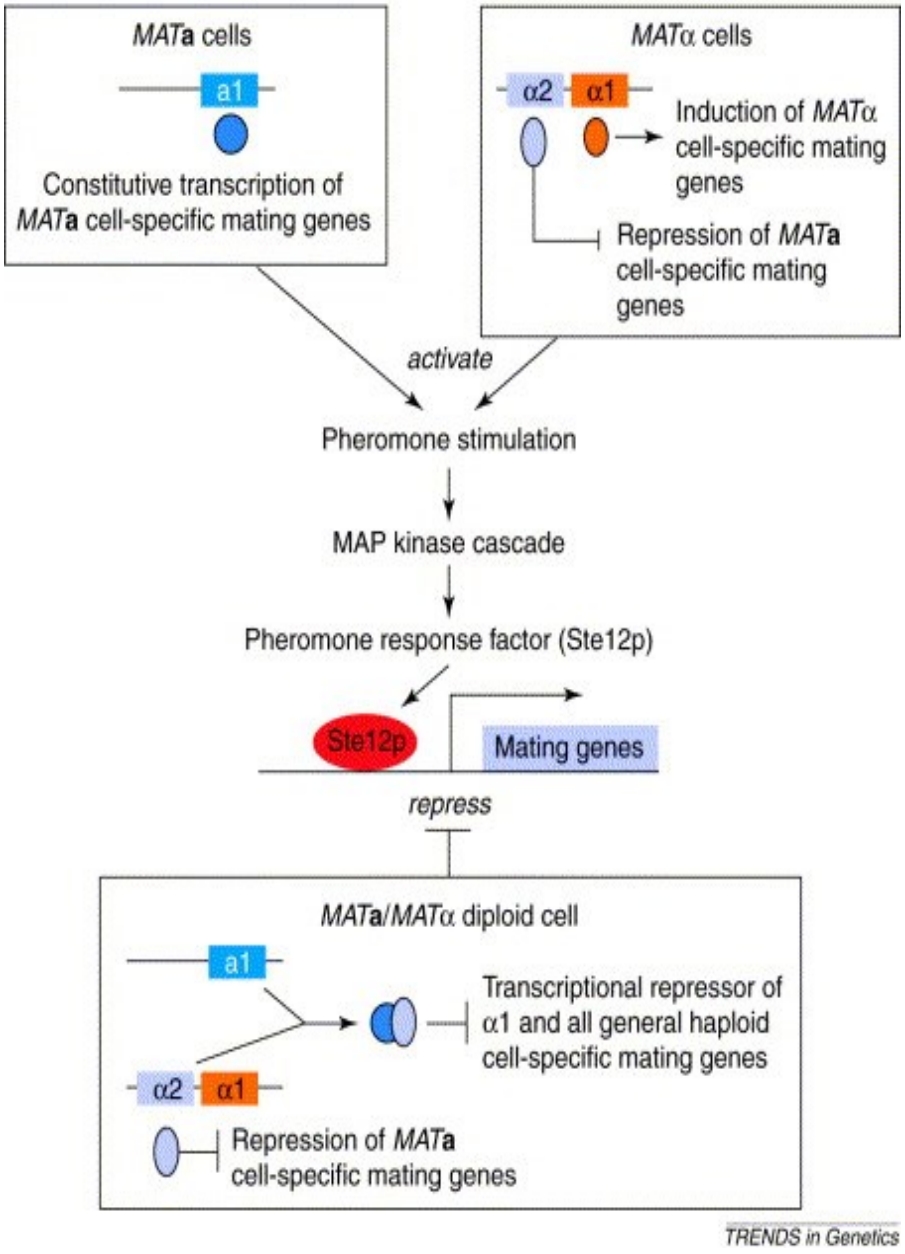

Fig.1.3.3.2.Mating in budding yeast, *Saccharomyces cerevisiae*.Binding of pheromones to the Ste receptors activates a heterodimer G-protein which in turn initiates phosphorylation through mitogen activated phospho kinase cascade signaling.

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 zygomycota is replaced with an uncertain systematic position (*insertae cedis*) having the monophyletic subphylum Mucoromycotina, Kickxellomycotina, Zoopagomycotina and Entomophthoromycotina (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 consists of approximately 1% of the true fungi. Mucorales are fast growing soil saprobes which compriseabout 205 species (Kirk *et al.*, 2008). They feed on dead and decaying organic 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, germinating 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).

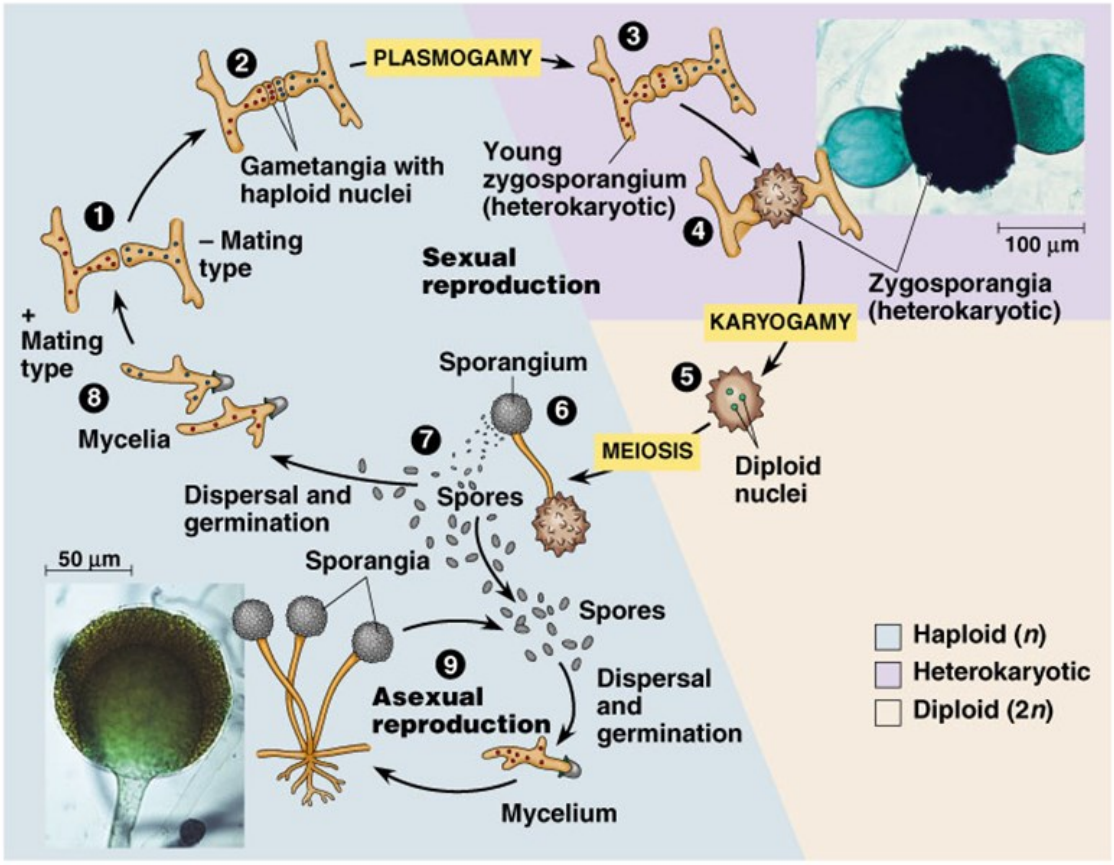

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Fig.1.4.2.Asexual and sexual phases in life cycle of a typical heterothallic mucoralean fungus.

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 & Vandenen.H, 1973). Δ*sexM* 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_{18} $H_{26}O_4$, 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_{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_{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_{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_{13}-C_{14} bond of β-apo-12'-carotenal to produce the first C_{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_{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).

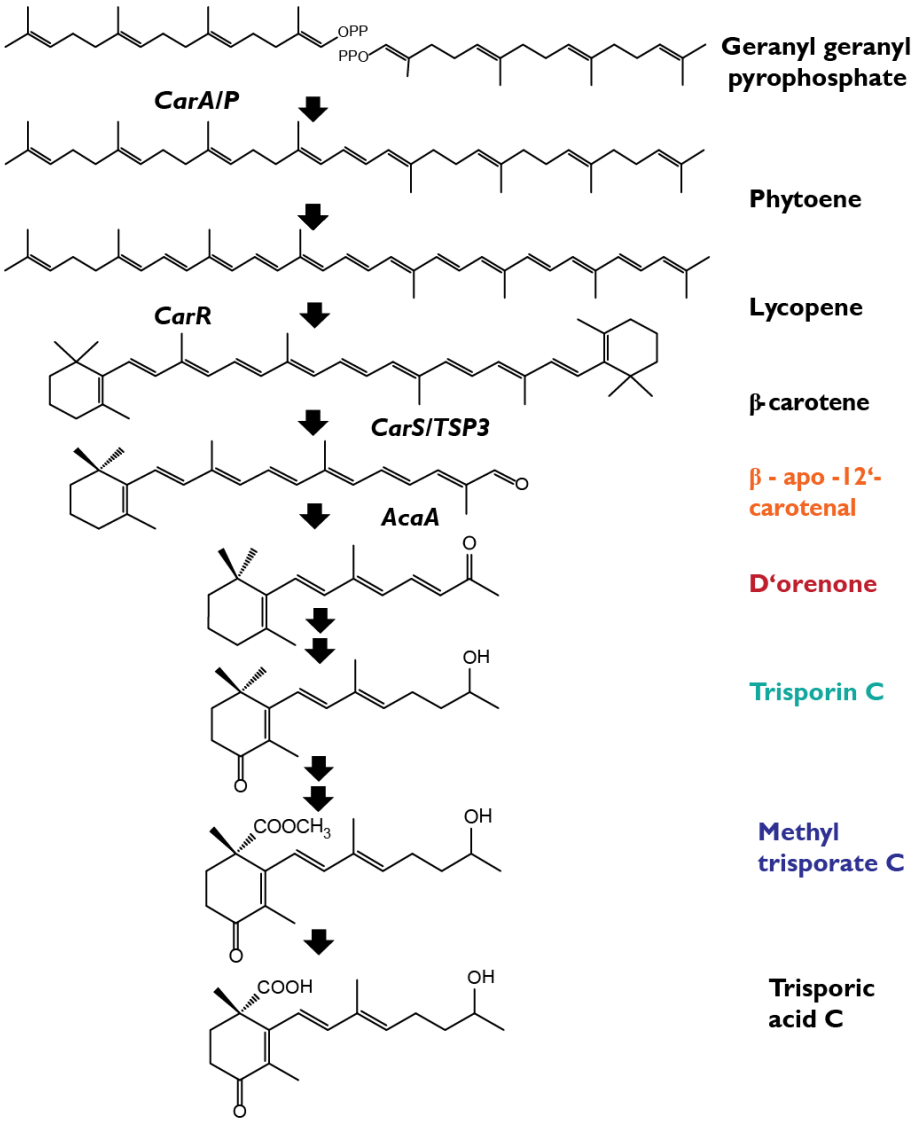


Fig.1.4.3. Schematic diagram of the putative biosynthetic pathway and genes encoding proteins involved in β-carotene metabolism. The enzymes depicted on left side of arrows lead to β-carotene biosynthesis while those on right are involved in trisporic acid biogenesis. CarRA is a bifunctional enzyme having 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 C_{18} 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 *crgA*Δ *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.

I	10g	Potassium nitrate (KNO_3)
	5g	Potassium hydrogen phosphate (KH_2PO_4)
	2.5g	Magnesium sulphate hepta hydrate ($MgSO_4.7H_2O$)
	1.0g	Yeast extract
	200ml	Distilled water
II	13.0g	Agar
	700ml	Distilled water
III	20g	Maltose
	100ml	Distilled water

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%.

Primer	Gene amplified	Tm (°C)	Sequence (5'-3')	Amplicon (bp)	Accession No.
Bt ***Car-RA***	Phytoene synthase&lycopene cyclase	65.5 63.5	for: CCGTGCACGTGAGATGGGGC rev: GCTACGGGCGTTGCCTTGCT	151	AY176663.1
Bt ***TSP3***	Carotenoid cleavage dioxygenase	62.1 59.8	for:TCGGAACAAACGCTGCTTTGC rev:AGCTTCTCCACGCTTGACACCT	125	AM409182.1
Bt ***Act***	β-actin	63.7 64.6	for: TCCGTGCCCCTGAAGCTCTCT rev:CGCTTCATGATGGAGTTGTAGGTG	91	AJ287143.1
Bt ***Gpd***	Glyceraldehyde-3-phosphate dehydrogenase	62.0 61.0	for:GGGGTGCAACGTACGATGAAATCA rev:AGCGAGATACCTGCGGAGGCA	157	AJ278318.1
Bt ***Pyrg***	Orotidine-5'-monophosphate decarboxylase	61.0 60.1	for:TGCTCACACAGTTCCTGGAG rev: GGCGAGCCATATTGAACAGAT	152	AY262090.1
Bt ***Tef***	Translation elongation factor 1-α	63.1 60.5	for: CTCCCCGGTGACAACCTCCCCGGTGACAACGTCG rev: GAACCGGCTTCCTTGGCG	110	AF157235.1
Bt ***TSP1***	4-dihydromethyl trisporate dehydrogenase	61.8 61.8	for:TGGTCAAGTGGGTCAAGGACC rev:GCACAACGTATCTGCCGGTCA	165	FSU331 JX470964
Bt ***CrgA***	Negative regulator of carotenogenesis	55.0 55.5	for:ATGCTTGAACGACAACAGATCTTG rev: CTGGCCATCATACTAGCGGGT	160	AJ585199
Mcl ***CarS***	Carotenoid cleavage dioxygenase	59.4 58.0	for: CATCAGCCCATCTGGCGTCAT rev:ATTGGCAGTATGGAAGGAGAAAGCA	261	146755|e_gw1.06.133.1
Mcl ***AcaA***	Apocarotenoid cleavage oxygenase	58.0 54.8	for:AGACGTGATGGCCGTCCTGAT rev:AGTTGACCTTGAATGCGAGCA	208	141273|e_gw1.03.260.1

Mcl ***CarRP***	Phytoene synthase & lycopene cyclase	63.7 59.8	for:AACGACCAACTGCCTGCCTCG rev:AATCCCACTTGTACCCGTCGA	156	AJ250827.1
Mcl ***SexP***	Transcription factor (plus) sex gene	56.9 57.4	for:ATGTGGAAGTCCGTTGGAAAGTCT rev:TACACCAATCGAGATAGTTTTAAAAAT	160	FJ009107
Mcl ***SexM***	Transcription factor (minus) sex gene	57.4 60.6	for:ATTACAAACGACACCTGCACCAGA rev:CAAGTCATCGAAATGTTGAACATGCTG	113	FJ009106
Mcl ***CrgA***	Negative regulator of carotenogenesis	56.9 55.6	for:ATGGCATCCAGCAGAAGACGA rev:AGCATGTGTTCTTGCTCTCTGTCA	250	AJ250998.1
Mcl ***Act***	β-actin	56.5 55.7	for:TCCTGATGGGCAAGTCATTACTGT rev:CGGAAACTAAATGAACAACCAACC	114	AJ287173.1
Pb ***TSP1***	4-dihydromethyl trisporate dehydro-genase	55.5 54.5	for:AGCAGACTCGCCTCGTTGAGT rev:TGTTGGAGCATGAGGTTATCAAGT	140	NRRL1555
Pb ***Act***	β-actin	60.7 59.4	for:ATGTCGCTCTCGACTTTGAGCAGG rev:GGCTGGAAGAGAGCTTCGGGA	118	AJ287184.1
Bt ***TSP3***full	Carotenoid cleav-age dioxygenase	57.6 57.1	for:ATGAAATTGCTTGAAGGTGAAGAG rev:TCAATTAACAGCTATGCCTCTTC	1986	AM409182.1
Mcl ***CarS*** full	Carotenoid cleav-age dioxygenase	57.8 54.0	for :ATGATCACTCCCGCTGAAGCC rev: TTAATTGACGG-CAATGCCTCT	1875	146755|e_gw1.06.133.1
Mcl ***AcaA***full	Apocarotenoid cleavage oxygen-ase	55.7 54.6	for:ATGATTGTAGGATTGCTTACAT rev: TCACAATGACATGACAGT	1024	141273|e_gw1.03.260.1

Tab.2.3.1. List of primers used for both qPCR and standard PCR reactions

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 RN*ase* 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 DN*ase* treatment (Turbo DNA-free™ kit, Ambion, AM 1907, Carlsbad, USA) as prescribed by the manufacturer.

2.4.3. RNA clean up

DN*ase* treated RNA was subjected to clean up using RNeasy-MinElute Cleanup kit (Qiagen, Hilden, Germany) for improving the purity of the DN*ase* 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^{TM} 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 DN*ase*/RN*ase*free distilled water (Gibco, Karlsruhe, Germany) upto 13 µl in a DN*ase*/RN*ase* 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 ˉ80°C until use. All the reactions were carried out quickly using DNA*ase*/RNA*ase* 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.

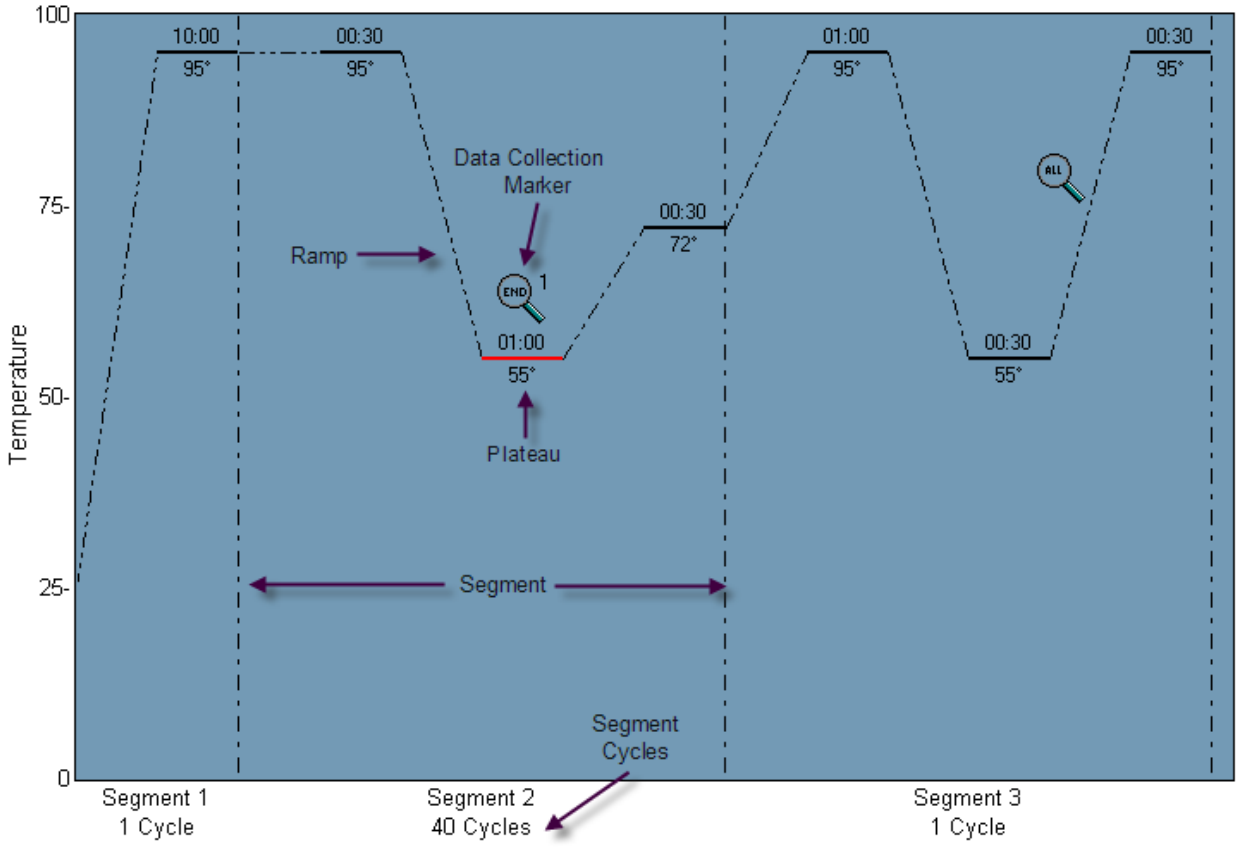


Fig.2.5.2. The thermal profile set up for qPCR as per Stratagene. Segment 2 had been modified to 35 cycles and plateau temperature optimized for 60°C to avoid unspecific binding.

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, 0.375µl 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 compoundβ-carotene was purchased from Sigma-Aldrich (Seelze, Germany) and β-apo-12'-carotenal from Carotenature (Lupsingen, Switzerland). Both standards and samples were treated with 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'-ATTATTCCCGGGCATCCGTACTCACTGTCTGGCACC-3' and acaA2 (5'-CAGCACGAGCTCCAAGACTACATAGTGTAAGTTTGTAC-3'), which contain *Sma*I

and *Sac*I sites, respectively (underlined). The 3990 bp amplified fragment was digested with *Sma*I and *Sac*I 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 oligo-nucleotides acaA3 (5'-AAGCCGGGATTCGGTAACGAGATCAATGTAAGCAATCC-3') and acaA4 (5'-TGGTCAGGATTCCGCCGTTACCTTCCATGGCTC-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 *BamH*I 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 ω_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 signalswere 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 *Phycomyces* 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.

protein ID	gene	function	reference
***Phycomyces blakesleeanus* NRRL1555 v2.0**			
183749|estExt_Genemark1.C_330115	*carS*	Carotene cleavage dioxygenase	Medina et al. 2011; Tagua et al. 2012
143867|Genemark1.6058_g	*acaA*	apocarotenoid cleavage oxygenase	Medina et al. 2011
143846|Genemark1.6037_g	*acaA2*	putative apocarotenoid cleavage oxygenase	Medina et al. 2011
76627|Phybl1.estExt_fgeneshPB_pg.C_30900	unkn1	unknown	Medina et al. 2011
184656|estExt_fgenesh1_pg.C_20034	unkn2	unknown	Medina et al. 2011
***Mucor circinelloides* CBS277.49 v2.0**			
146755|e_gw1.06.133.1	*carS*	carotene cleavage dioxygenase	this study
141273|e_gw1.03.260.1	*acaA*	apocarotenoid cleavage oxygenase	this study
189974|estExt_fgenesh1_pg.C_050311	unkn1	unknown	this study
114475|Genemark1.10238_g	unkn2	unknown	this study
***Rhizopus delemar* 99-880**			
RO3G_03330	*carS*	carotene cleavage dioxygenase	this study
RO3G_01507	*acaA*	apocarotenoid cleavage oxygenase	this study
RO3G_11848	unkn1	unknown	this study
RO3G_16933	unkn2	unknown	this study
***Umbelopsis ramanniana* AG # v1.0**			
170528|e_gw1.8.23.1	*carS*	carotene cleavage dioxygenase	this study
170664|e_gw1.8.75.1	*acaA*	apocarotenoid cleavage oxygenase	this study
295994|estExt_fgenesh1_pg.C_10029	unkn1	unknown	this study
219685|fgenesh1_kg.2_#_1609_#_combest_scaffold_2_40762	unkn2	unknown	this study
***Blakeslea trispora* CBS131.59**			
AM409182	*tsp3*	carotene cleavage dioxygenase	Burmester et al. 2007

Tab.3.1.1.Details of apo/carotenoid cleavage dioxygenases identified in the genome of five species in the order Mucorales.

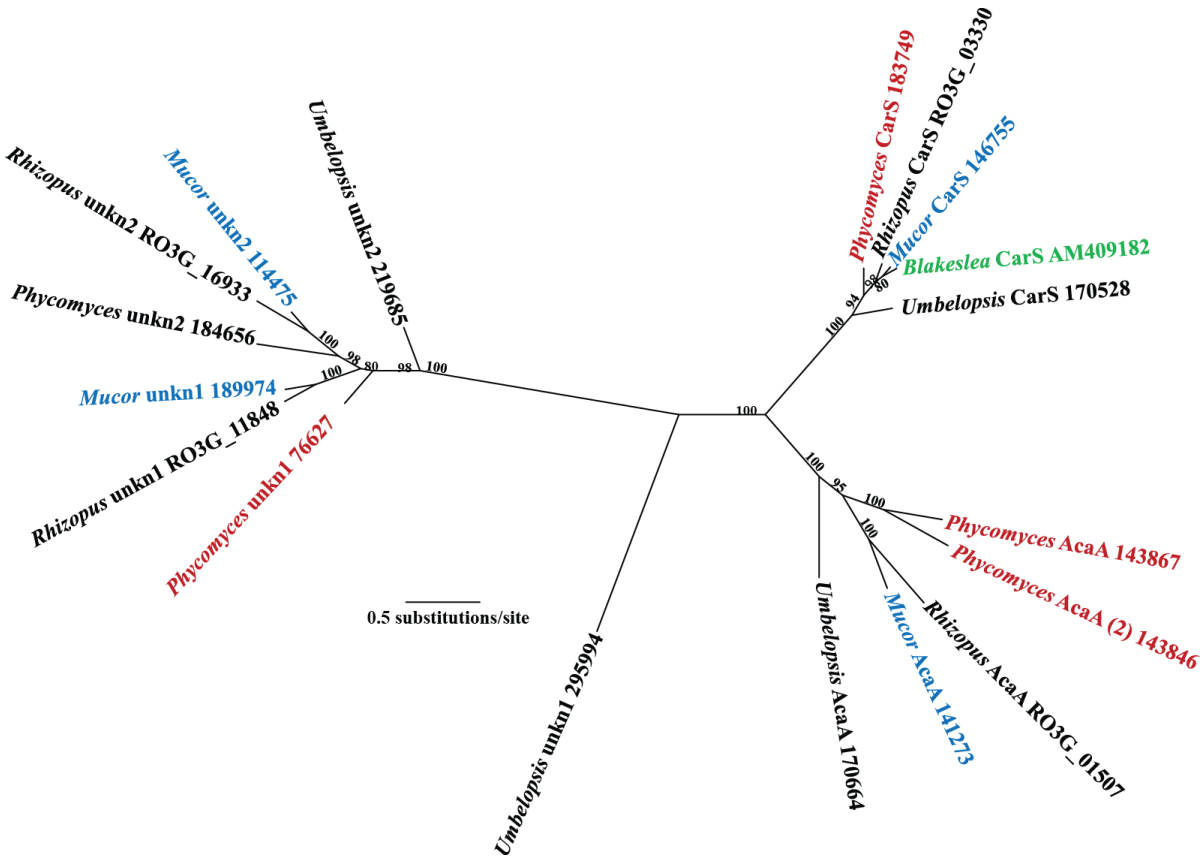


Fig.3.1.1. Phylogenetic tree of the apo/carotenoid cleaving enzymes designated as CarS (carotenoid cleavage dioxygenase) and AcaA (apocarotenoid cleavage oxygenase).

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 **M**inimum **I**nformation for Publication of **Q**uantitative Real time PCR **E**xperiments (Bustin *et al.*, 2009). Actin showed higher expression levels, lower Cq values and the least temporal variation over the growth period.

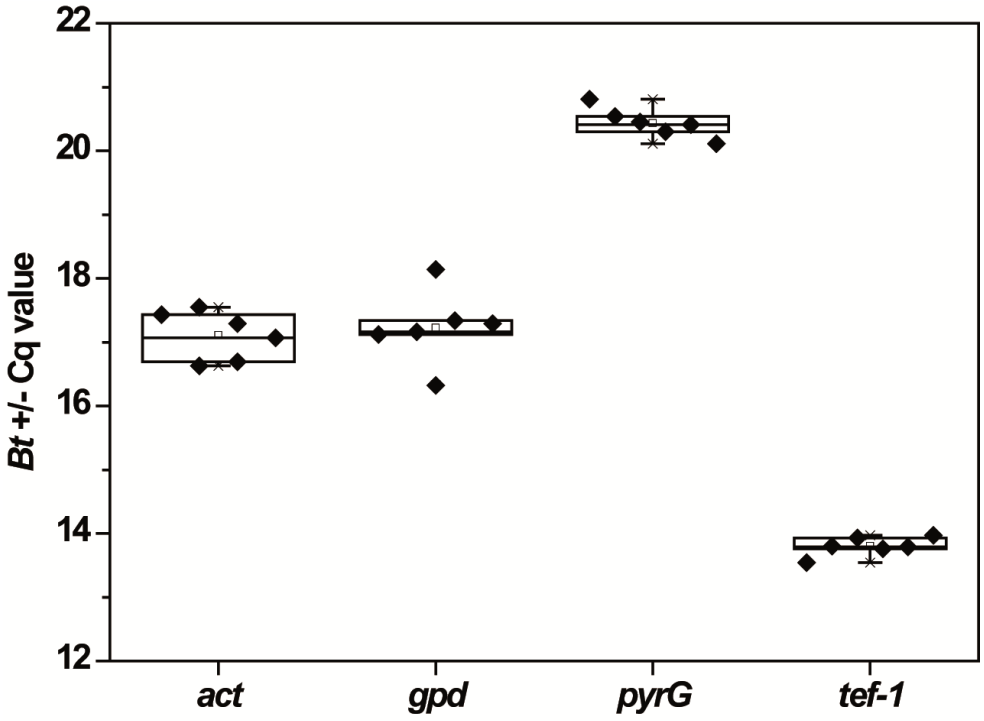


Fig.3.2.1.Real time PCR cycle threshold values (Ct/Cq) of 4 different internal standards actin (*Act)*,glyceraldehyde phosphate dehydrogenase (*Gpd*), orotidine monophosphate decarboxylase (*PyrG*) and translation elongation factor (*Tef-1*) plotted for *B. trispora* cross culture over six time points 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).

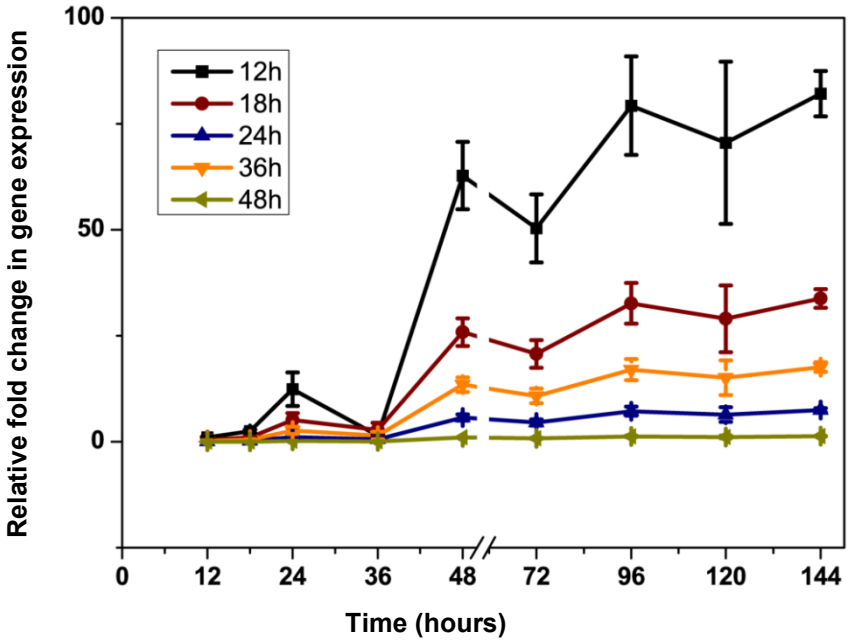


Fig.3.2.2. Relative gene expression of *CarRA* in mated culture of *Blakeslea trispora* (*Bt*+/-) on different time point chosen for data analysis. Data analyses were performed with samples collected at 12, 18, 24, 36 and 48 hours of growth as zero hour or basal expression level as per the $2^{-\Delta Ct}$ 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 $2^{-\Delta Ct}$ 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).

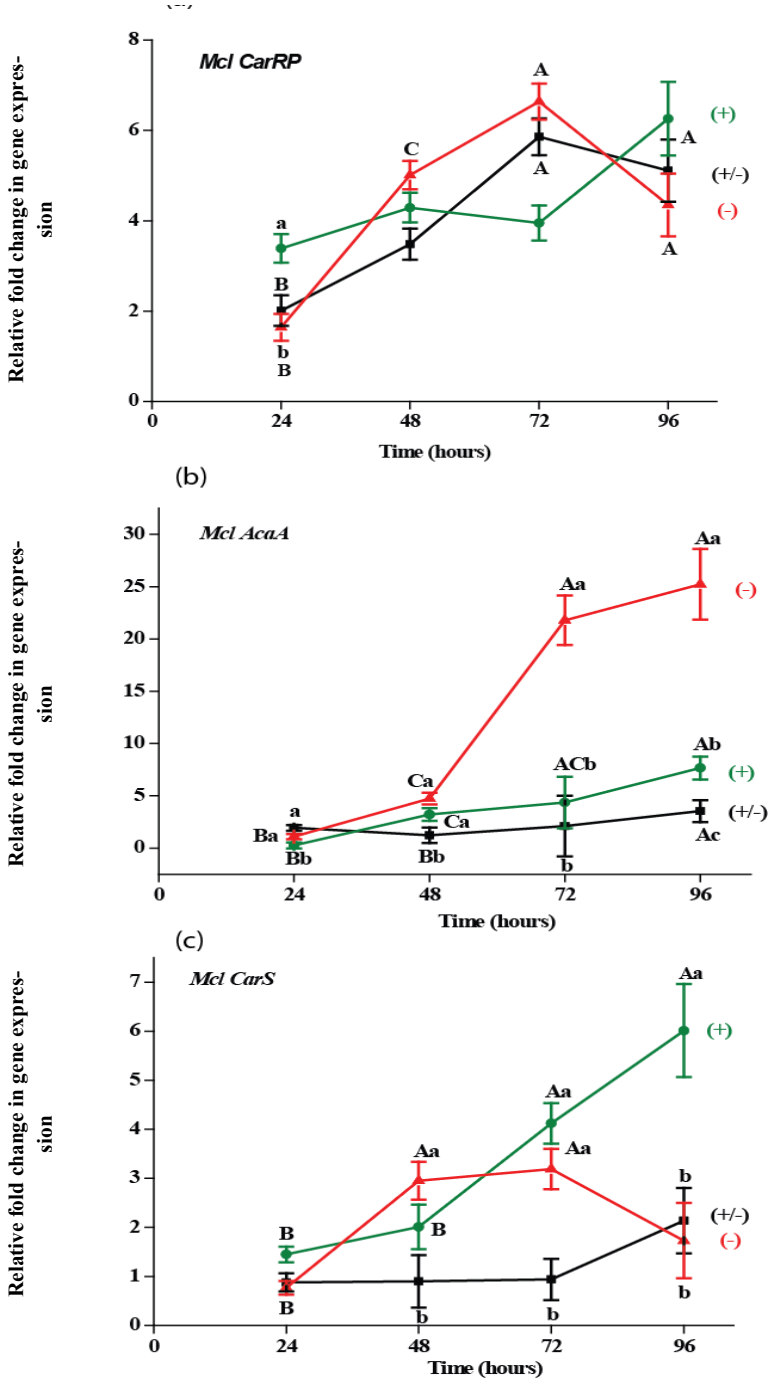


Fig.3.3.1.Real time qRT-PCR analyses on dynamics of *CarRA* (a), *TSP3* (b) and *TSP1* (c) in *Blakeslea trispora* (+), (-) and (+/-). The small letters indicate significant difference based on Tukey's test (post hoc) at each time point among (+), (-) and (+/-) mating types and capital letters between 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 ingene 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 of3.5 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.

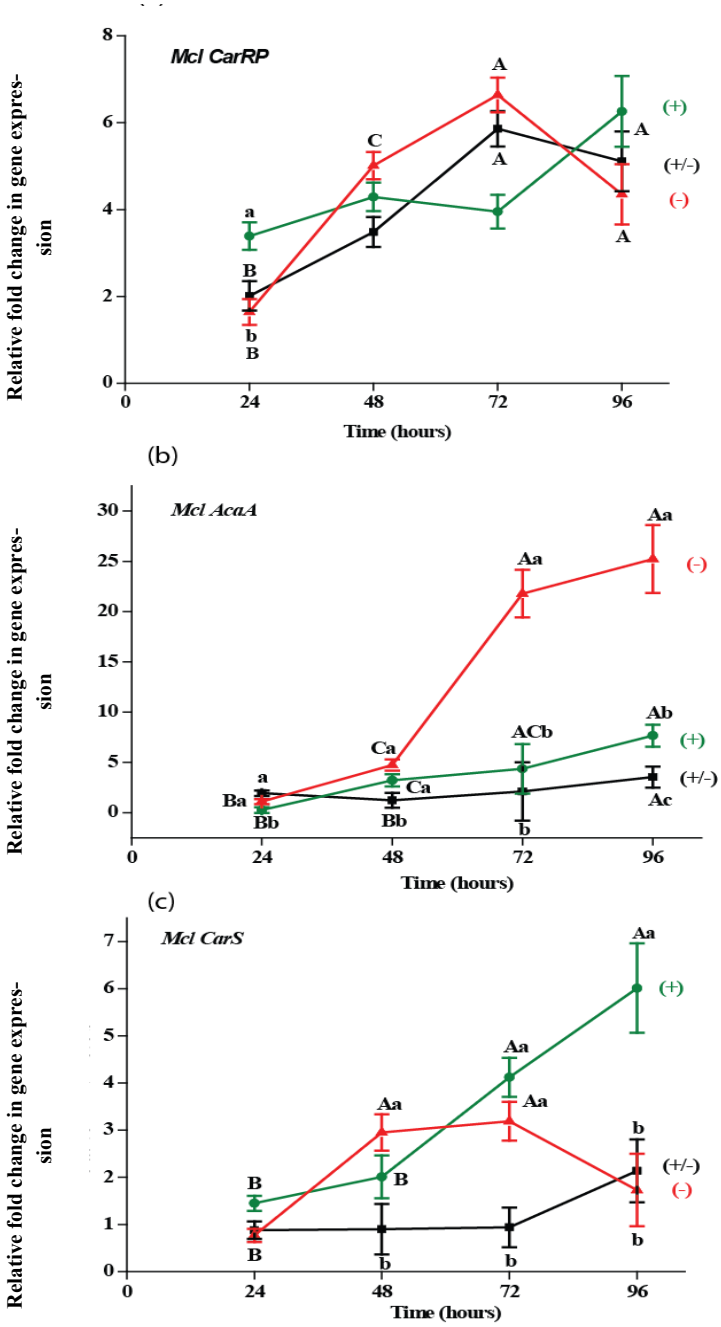


Fig.3.4.1. Differential gene expression in the plus, minus and mating phase of *M. circinelloides* grown in dark normalized with actin as internal standard. Graphs are plotted on the mean value of 3 biological replicates with error bars indicating standard error

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.

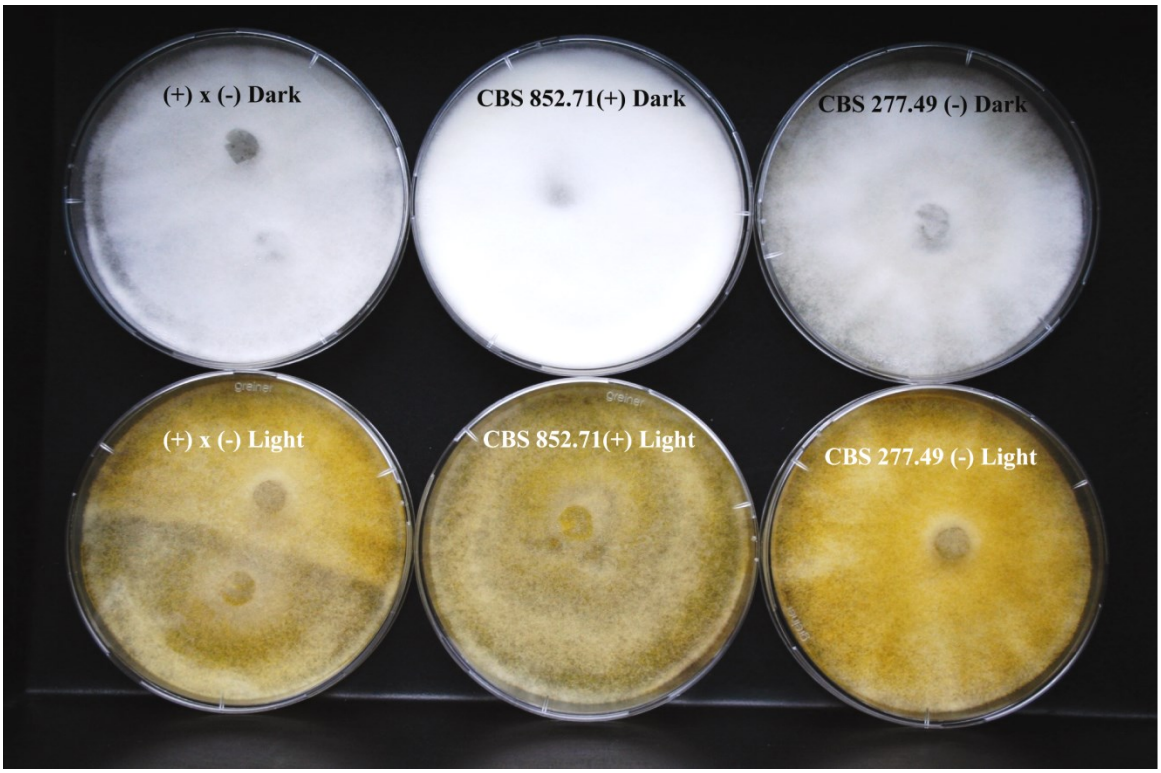


Fig.3.4.2. *Mucor circinelloides* f. *lusitanicus* exposed to continuous dark and light conditions in solid induction medium agar plates.

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-

lation 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).

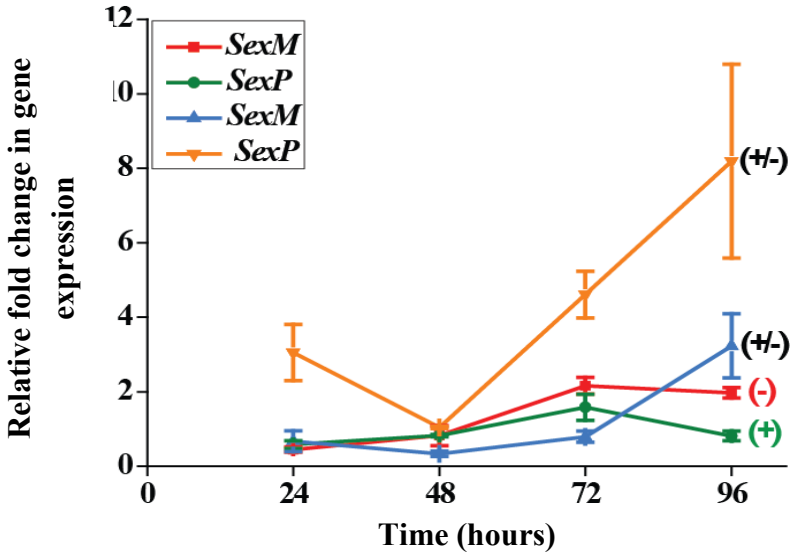
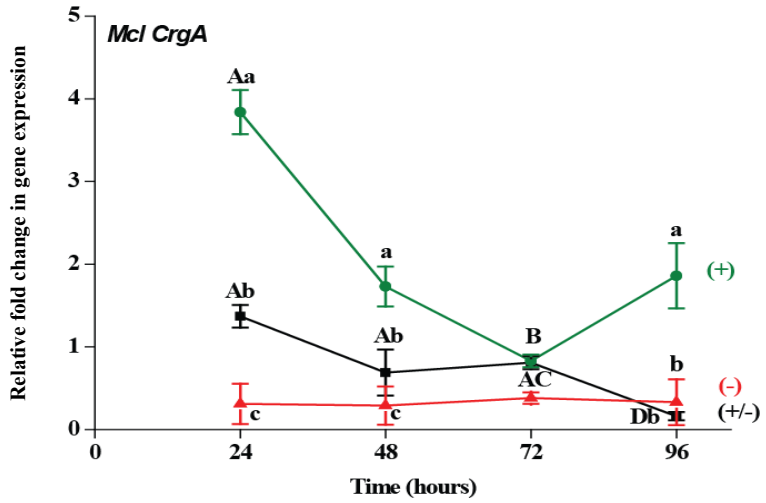


Fig.3.4.3. Transcriptional dynamics of HMG domain mating type specific transcription factor sex genes *SexM* and *SexP* at asexual and sexual phases in *M. circinelloides*. The graphs are based on data from three independent replicates and error bar indicates the standard error mean.

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.

a.



b.

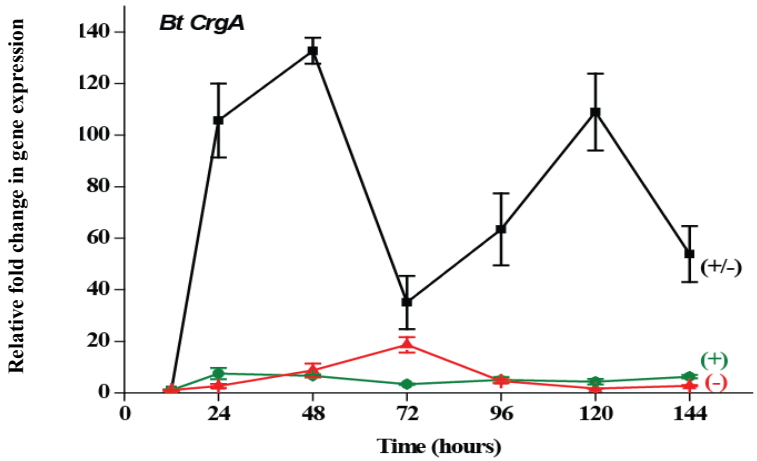


Fig.3.4.4.Therelative fold changes of *CrgA* gene expression in *M. circinelloides* and *B. trispora*. The notation +/- indicates sexual phase where the spore inoculum from both mating partners were inoculated to liquid broth in equal ratio.

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).

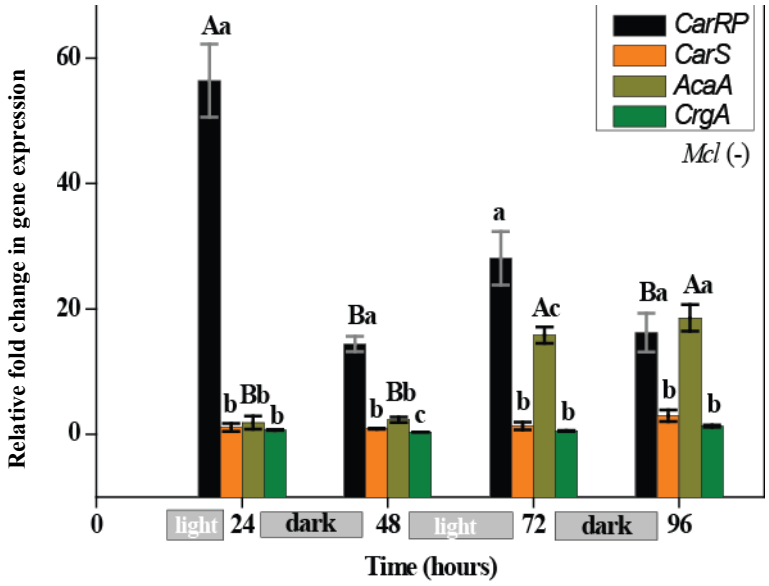


Fig.3.4.5.Transcript levels of genes involved in carotenoid metabolism in CBS 277.49 (-) exposed to a dark and light regime during growth. Small letters indicate significant differences at P<0.001 among *CarRP*, *CarS*, *AcaA* and *CrgA* at a particular time point and capital letters indicate significant 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.

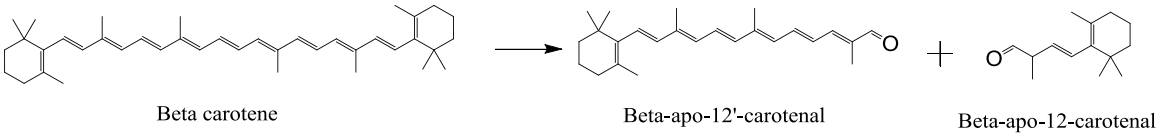
3.5. Prediction of CarS and AcaA function by template based homology modeling

The protein fold recognition server Phyre^2 (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 (λ_{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).



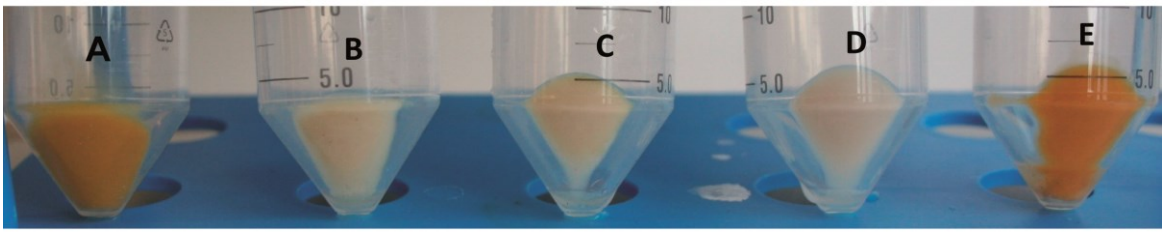


Fig.3.6.1.β-carotene got bleached by carotenoid cleavage dioxygenase (TSP3) activity. *Escherichia coli* cells harbouring both pβ-carotene plasmid and *TSP3* gene inserts at 0.08% **(B)** and 0.2% **(C)** arabinose grown at 28°C for 24 hours were centrifuged and pellets were decolorized by the enzyme 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 β-carotene cleavage and hence no apocarotenoid products were detected in chromatographic analysis. The inability of AcaA to cleave β-carotene provides strong evidence that like its characterized homolog in *Phycomyces*, the appropriate substrate would be any other carotenoid like lycopene or apocarotenoids.

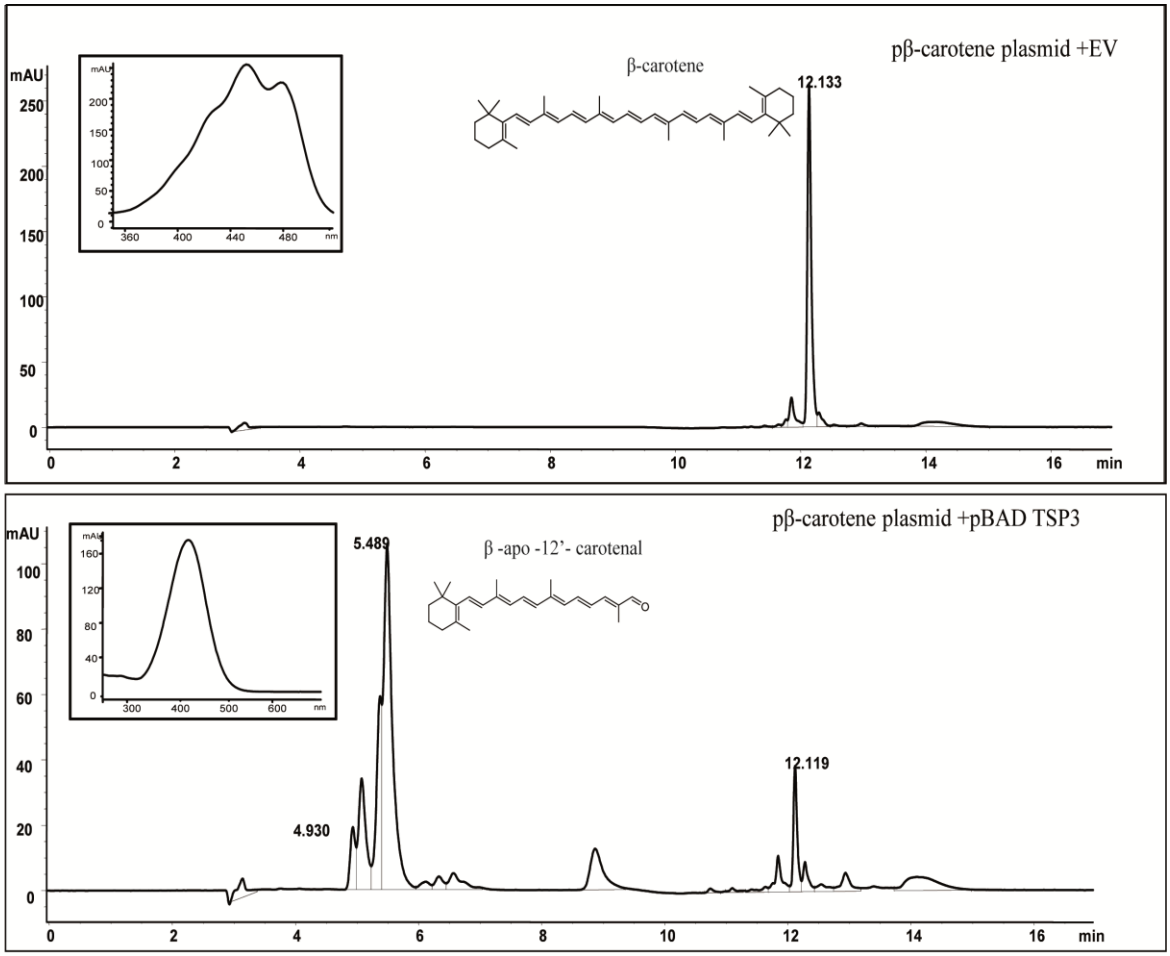


Fig.3.6.2. HPLC chromatogram of *in vivo* enzyme assay with TSP3 co-expressed along β-carotene producing plasmid after 24 hours and EV- empty pBAD vector. Insets show the Ultraviolet-Visible spectra of β-carotene substrate and β-apo-12'-carotenal.

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 ($C_{19}H_{28}O$; 320Da), D'orenone ($C_{18}H_{26}O$; 258Da) and trisporin C/TSPC ($C_{18}H_{28}O$; 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-

ments 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).

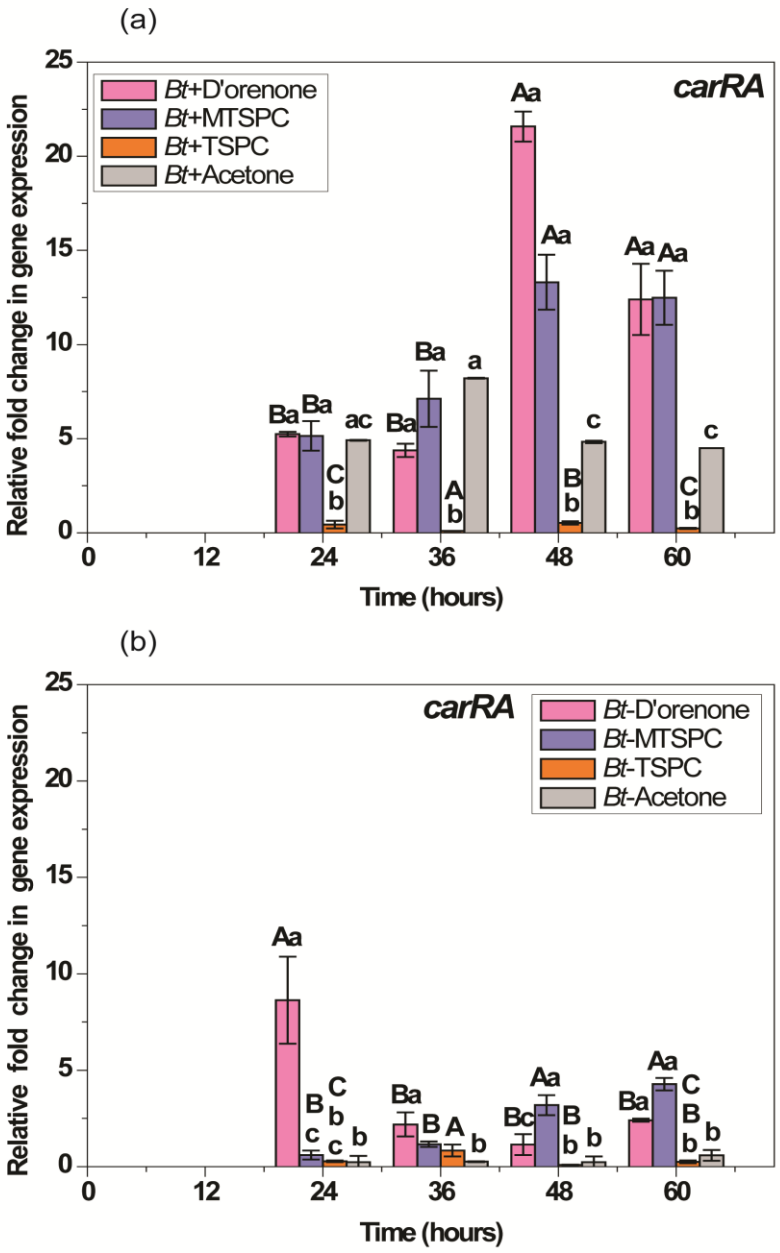


Fig.3.7.1. Dose-response transcriptional data of *B. trispora* plus (a) and minus (b) partners on treatment with early and late trisporoids. Letters indicate the statistically significant differences among the treatments namely, D'orenone, MTSPC and TSPC along with control solvent acetone at each time point (small) and among different time points for each treatment (capital).

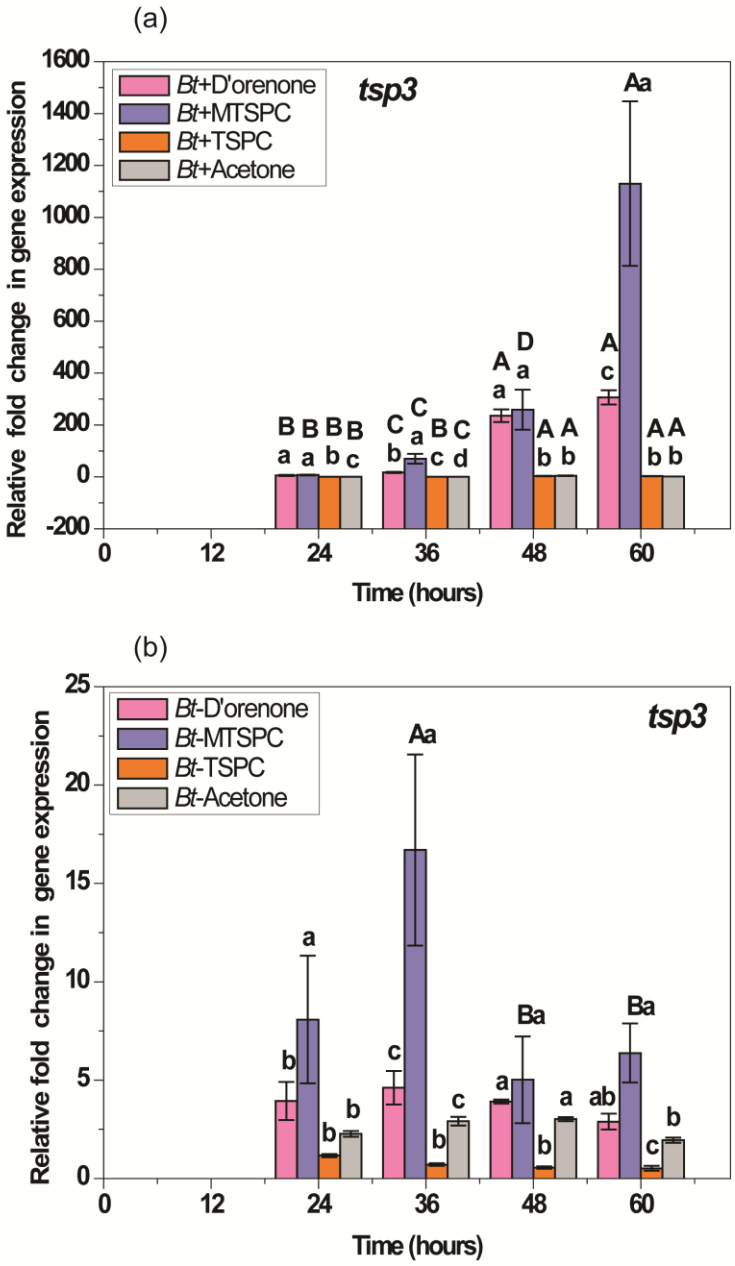


Fig.3.7.2. Transcriptional dynamics of carotenoid cleaving dioxygenase (*TSP3*) in *B. trispora* plus (a) and minus (b) mating types after treatment with trisporoids at 12 hours post inoculation. The difference in scale of graphics should be noted. Notations using letters indicate statistically significant 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.

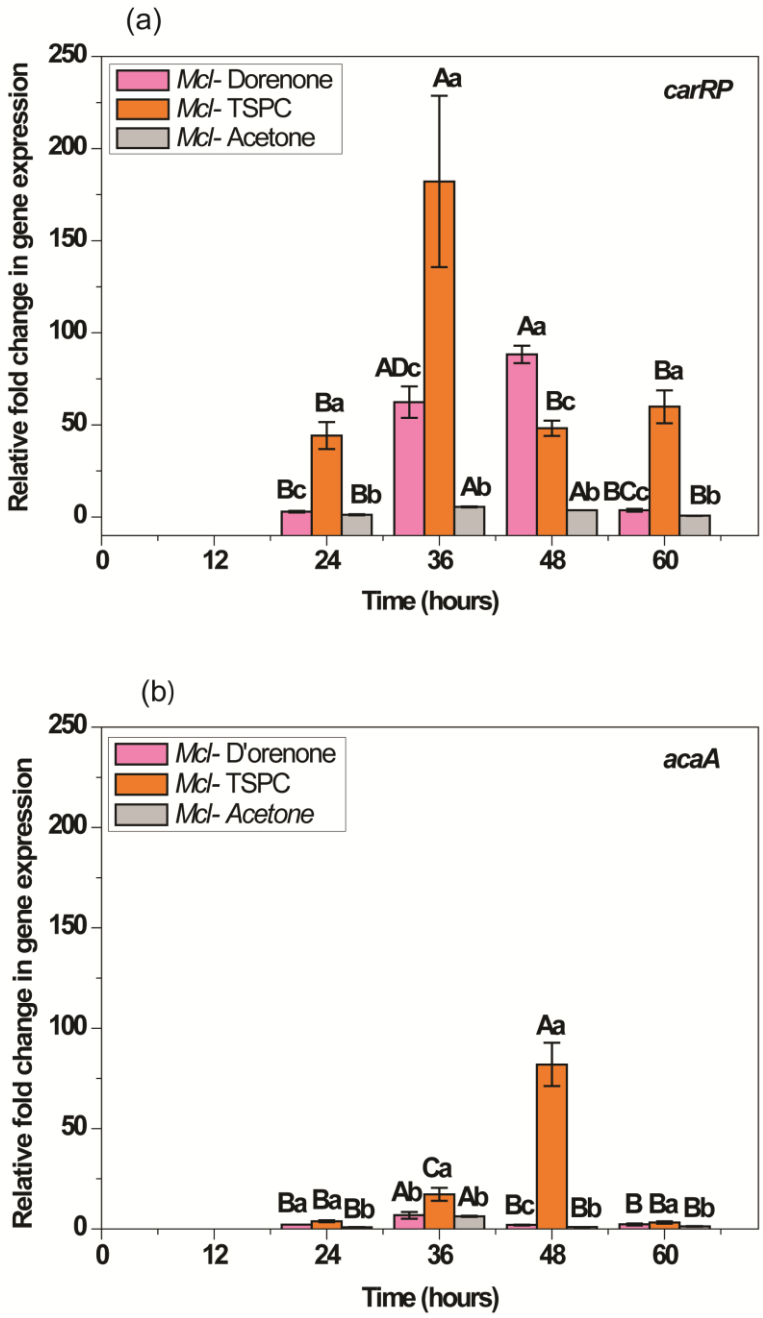


Fig.3.7.3.Transcriptional dynamics of *CarRP* and *AcaA* in minus mating type of *M. circinelloides* after treatment with two trisporoids namely D'orenone and trisporin C. Acetone is the solvent control. The letters indicate significant statistical differences as described previously.

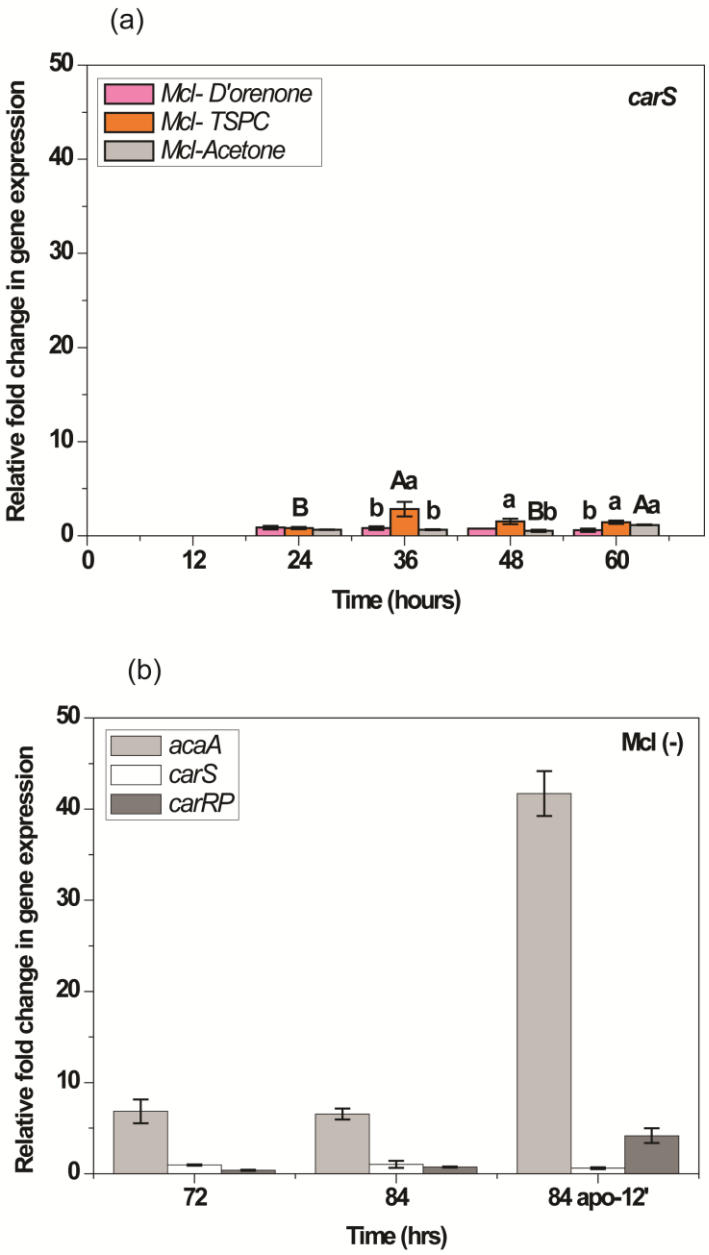


Fig.3.7.4. Transcriptional dynamics of carotenoid metabolic genes in (-) *M. circinelloides* treated with different apocarotenoids. (a)*CarS* transcript after being treated with D'orenone, TSPC and acetone at 12 hpi; (b)*AcaA*, *CarS* and *CarRP* gene expression after β-apo-12'-carotenal treatment at 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 *Sma*I-*Sac*I 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).

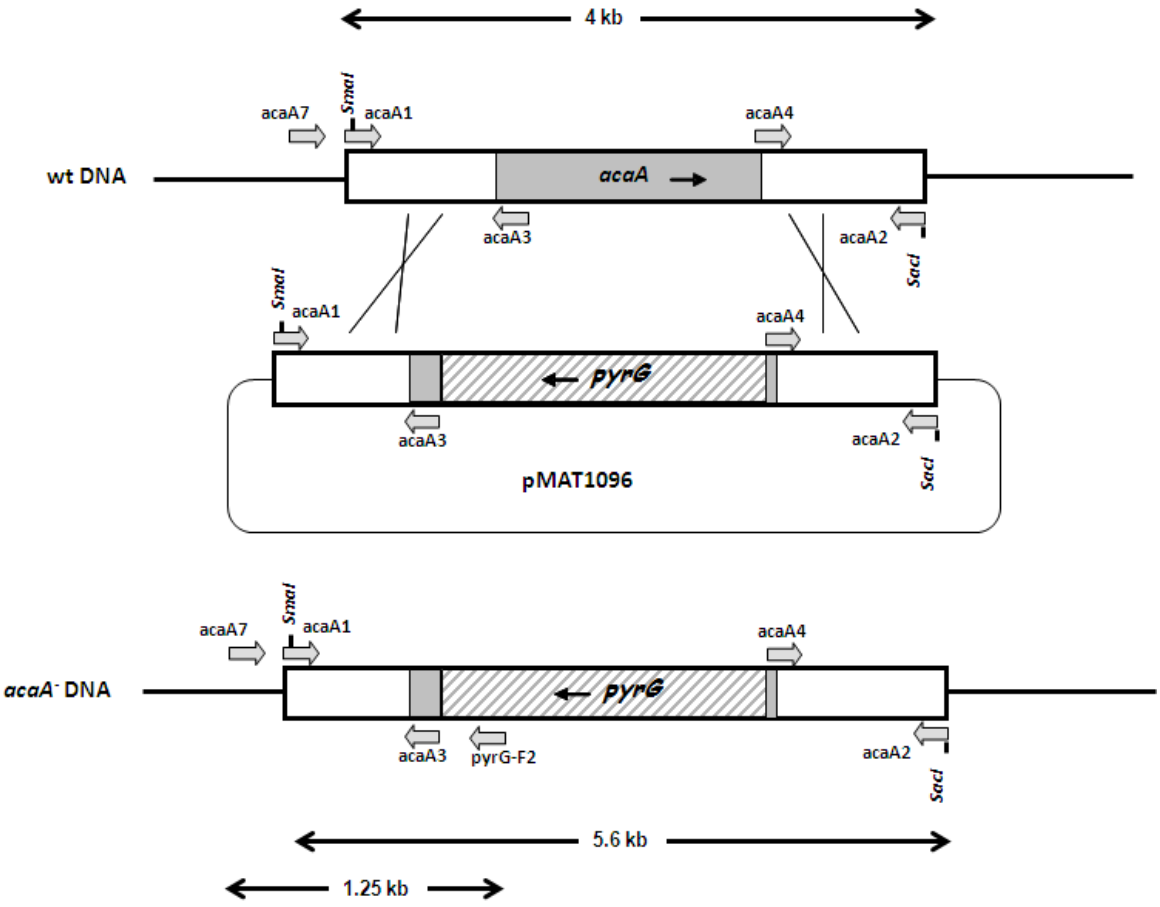
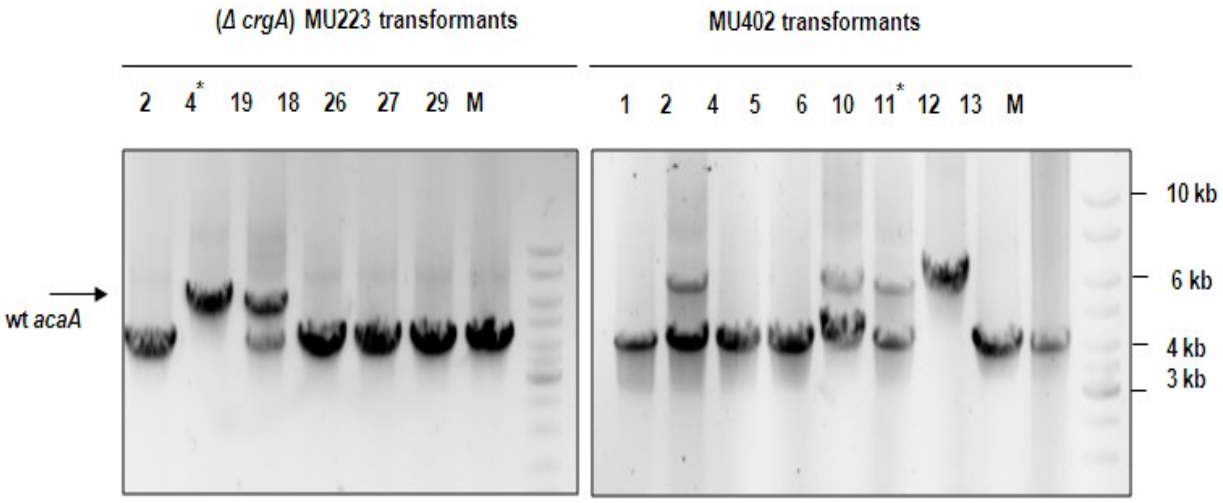


Fig.3.8.1. Schematic representation of the wild type *AcaA* locus (top) and after homologous recombination with the disruption fragment isolated from plasmid pMAT1096 (below). Dark gray box is the *AcaA* coding sequence and white boxes are upstream and downstream flanking regions. The positions of the 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 *acaA*$^{-}$ loci are shown.

A. Heterokaryotic transformants



B. Homokaryons

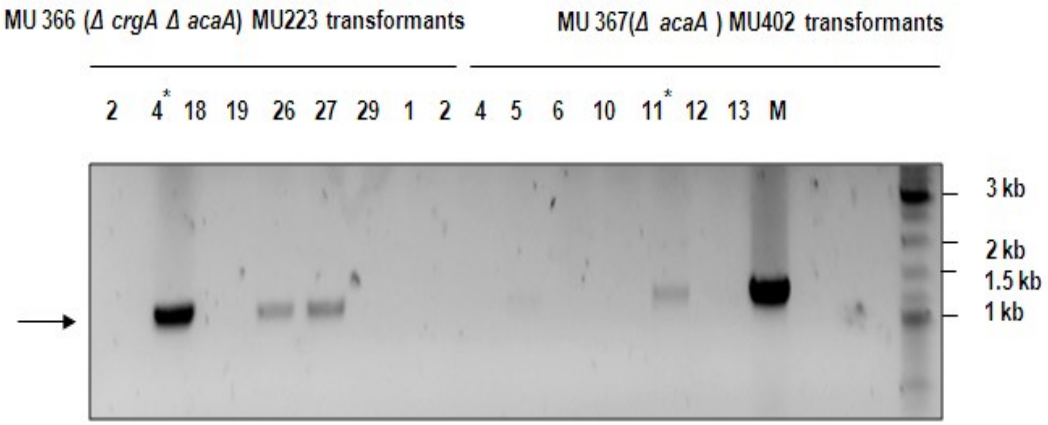


Fig.3.8.2. PCR analysis of Ura^{+} transformants obtained by transformation of the MU223 (left) and MU402 (right) strains with the 5.6 kb *Sma*I-*Sac*I disruption fragment. (A). Amplification of the wild type *acaA* allele produces a 4 kb fragment. Arrow indicates the size of the fragment amplified from the *acaA*$^{-}$ allele (5.6 kb). (B) Homokaryotic transformants marked by an asterisk were named MU366 (*acaA*$^{-}$*crgA*$^{-}$) and MU367 (*acaA*$^{-}$*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 (Δ*acaA*) and MU366 (Δ*crgA*Δ*acaA*) were independently co-inoculated with the wild type (+) strain CBS852.71. The co-cultivation was maintained in the dark at 23°C for upto 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 Δ*crgA* 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).Twoway 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

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).

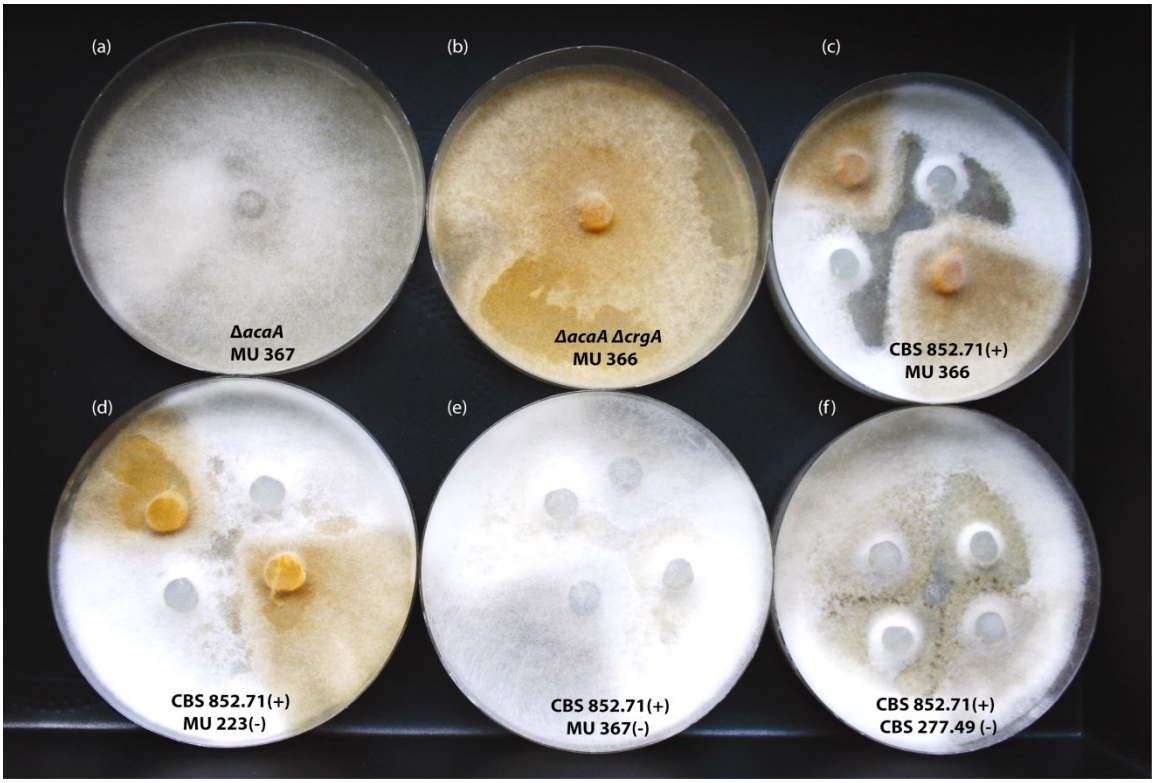


Fig.3.9.1.Mating experiments using wild type plus with wild type minus and mutant strains. (a,b) are the single and double knockout mutants of minus strains. No zygospores or sexual interactions took place in crosses between wild plus and mutants (c,d,e).The black spots are sexual zygospores formed during 5-7 days post inoculation when the wild type partners mate (f).

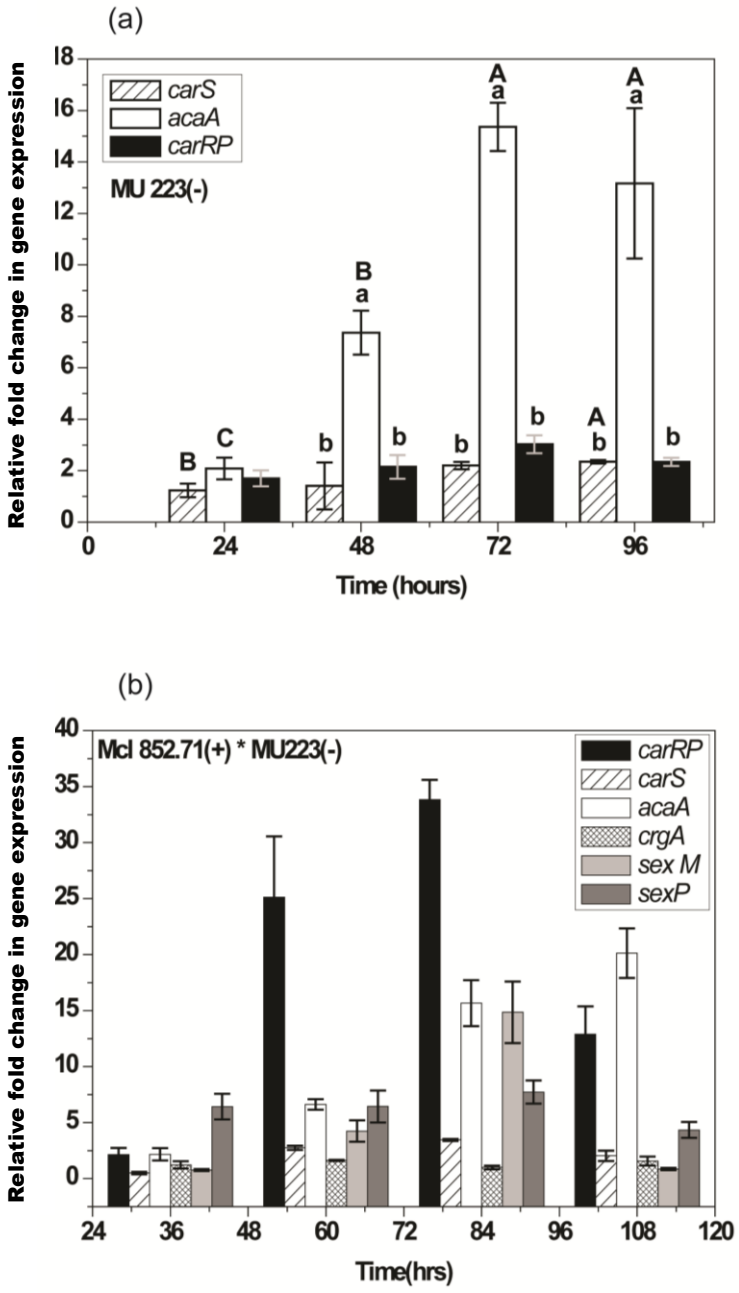


Fig.3.10.1. Trancript analysis of all the known genes involved in sex and carotenogenesis independently in single knockout mutant of *CrgA* (a) and on mating with wild plus (b).Small letters indicate significant differences between *CarS*, *AcaA* and *CarRP* at a particular time point while capitals 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. 3.4.1.b).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 zygospore 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 carotenogeneis was dysfunctional.

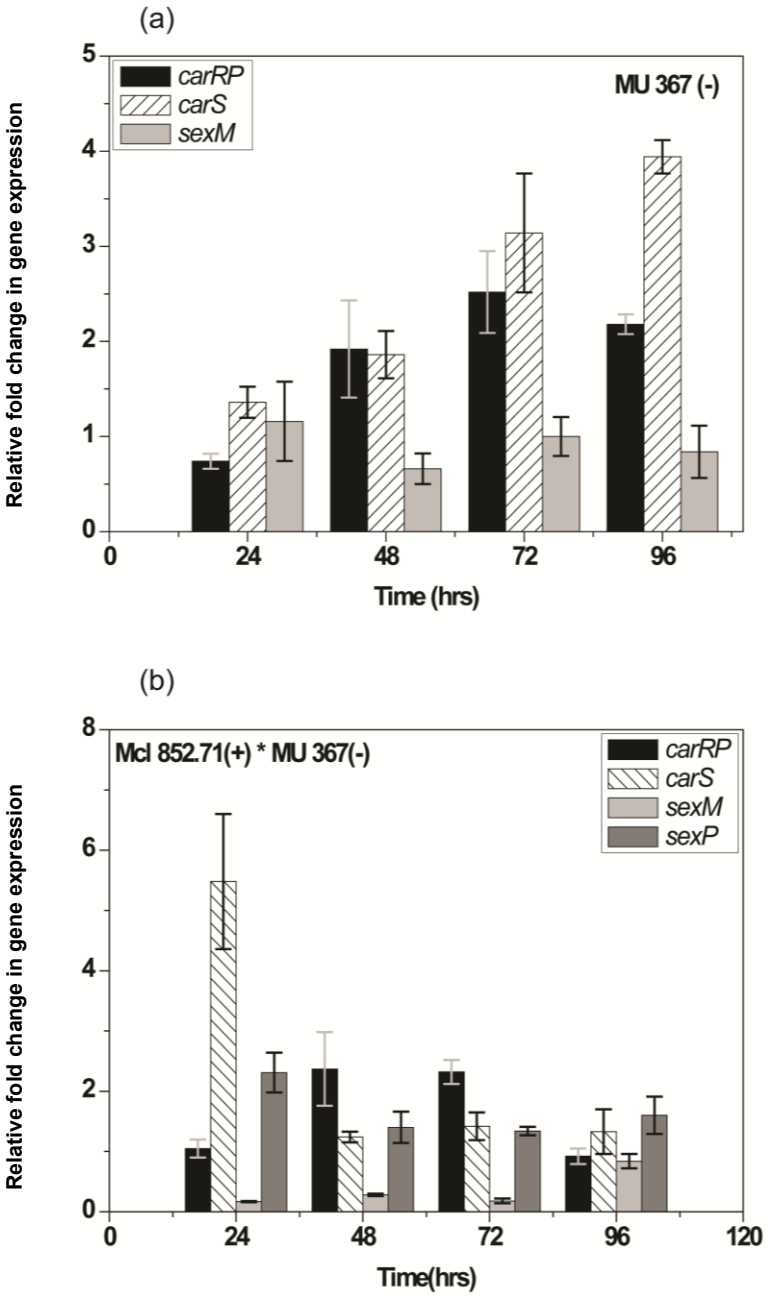


Fig.3.11.1. The transcriptional analysis of genes involved in sex and carotenogenesis in *M. circinelloides* f. *lusitanicus*(a) Δ*acaA* mutant MU367 (-)and (b)while mating with wild type plus.

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, Vandenen.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.

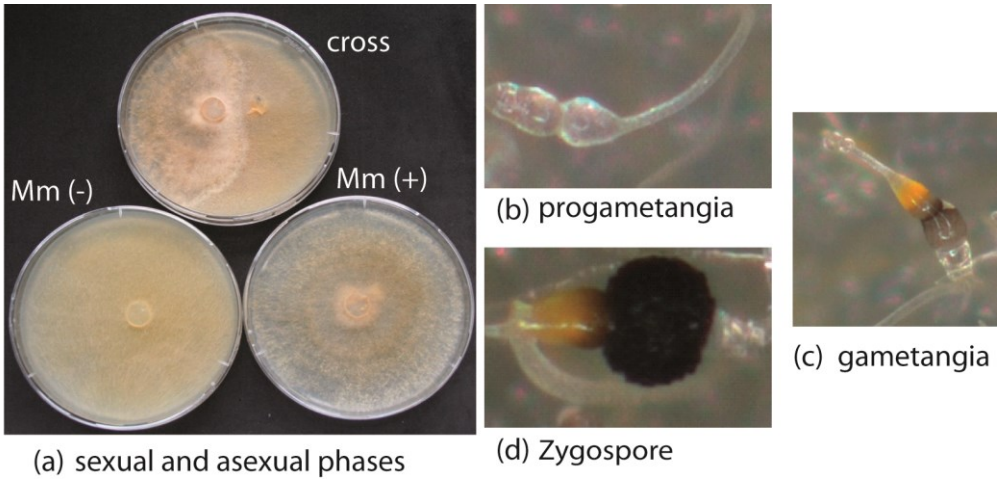


Fig.3.12.1.Developmental stages during the life cycle of *M. mucedo* (a). Sexual morphogenesis results in microscopic structures ranging from b-d at the mating zone of opposite mating partners.

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

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 < $1\mu m^3$" (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).

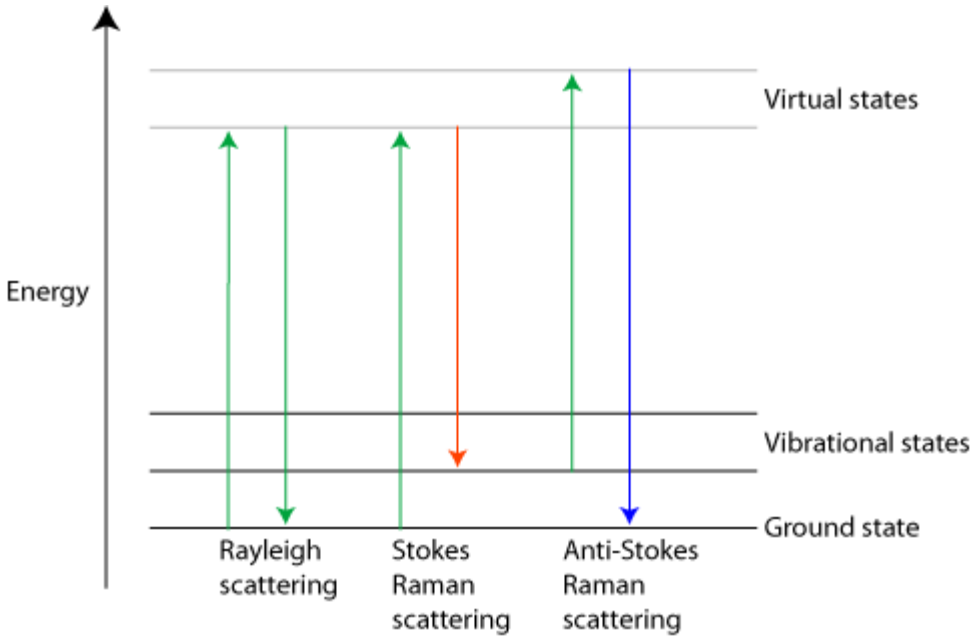


Fig.3.13.1.A simplified energy diagram of the shifts in wavelengths of incident radiation that provides chemical and structural information about the molecules under investigation

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 β- 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.

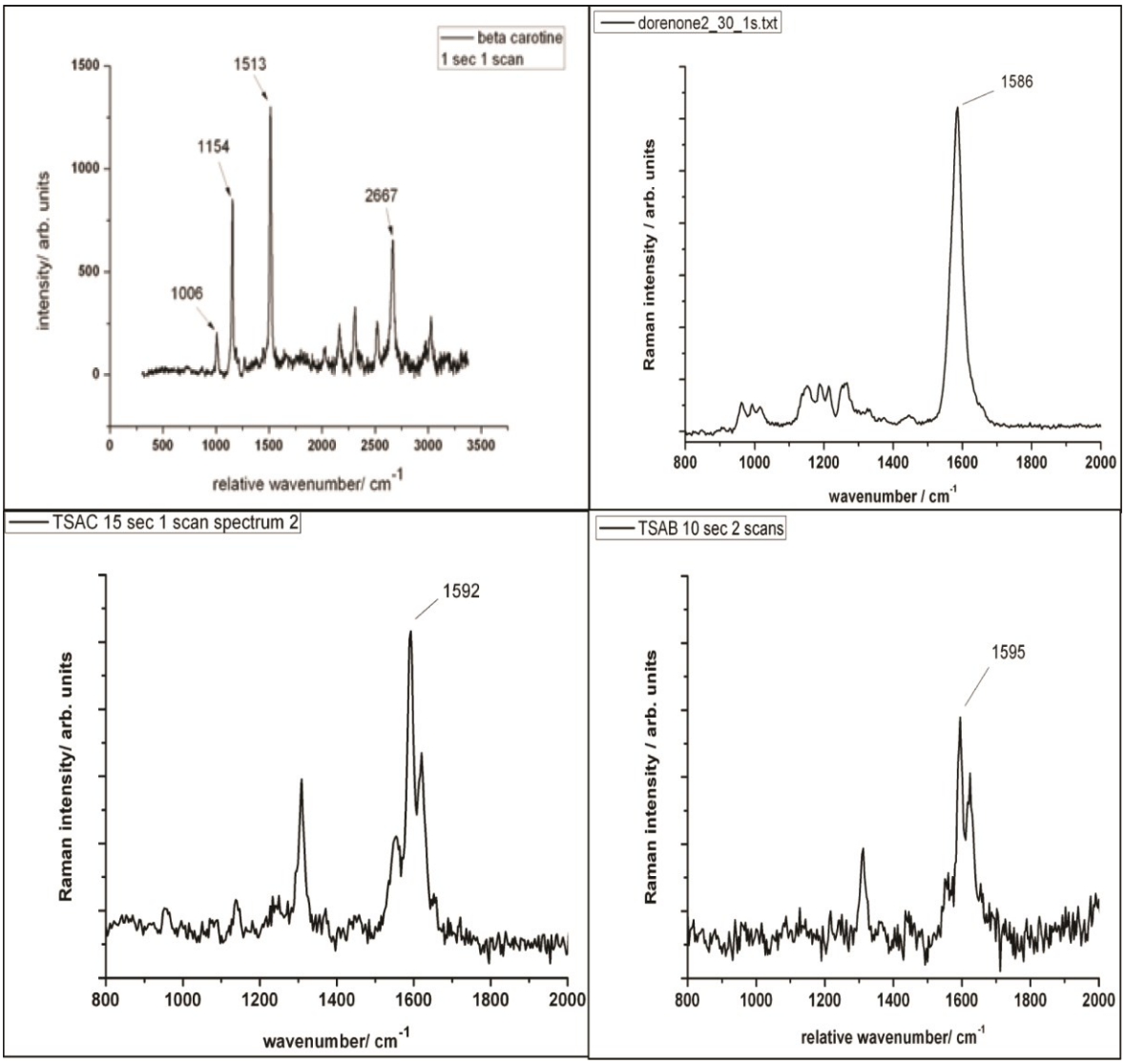


Fig.3.13.2. Raman spectra of the pure standard compounds ie., β-carotene, D'orenone, Trisporic acid C (TSAC) and Trisporic acid B (TSAB) dissolved in dichloromethane.

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 ωp- ωs, between spatially and temporally overlapped pump and stokes laser pulses matches with its inherent vibrational frequency.

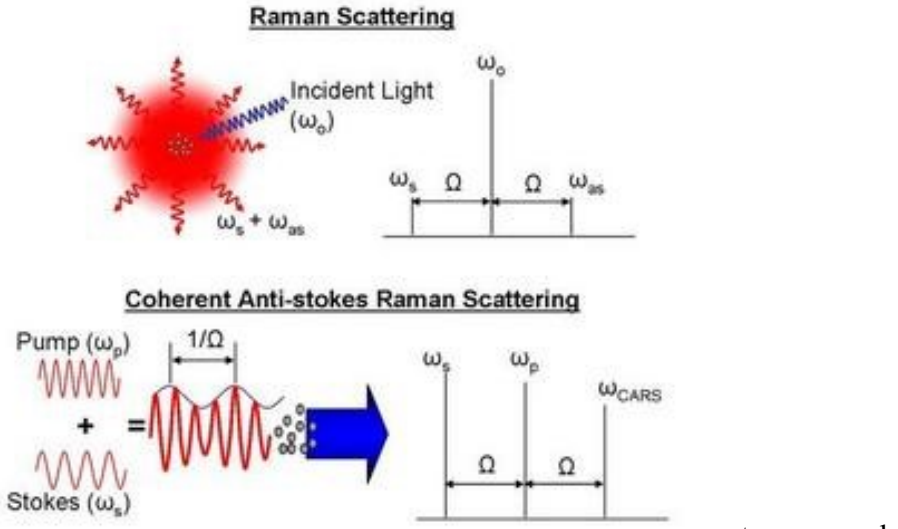


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Fig.3.14.1. Schematics indicating the principle behind the energy shifts leading to an anti-stokes scattering.

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 β-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 diffi-

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 1511-1526cm^{-1} for β-carotene and 1592-1596 cm^{-1} for trisporic acids as the appropriate Raman shifts that distinguish two compounds in *M. mucedo*. 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.

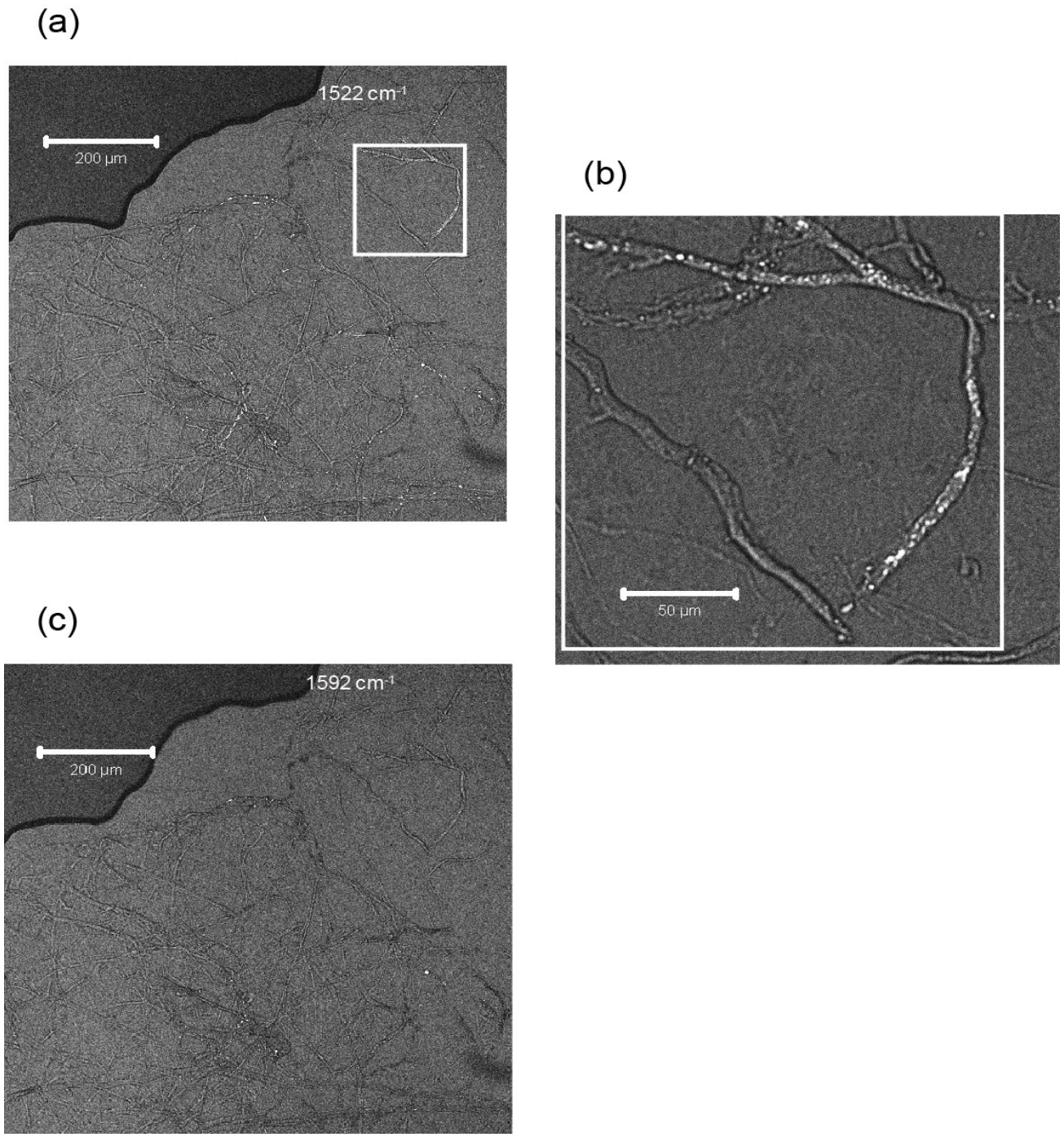

Fig.3.14.1.CARS spectra observed for β-carotene (a) and trisporic acids (c) from the microscopic slide having asexual hyphal structures in *M. mucedo*. An enlarged view of the inset in (a) is depicted as (b).The white signals indicate presence of β-carotene in asexual phase while that of specific signals for trisporic acids were missing in (c).

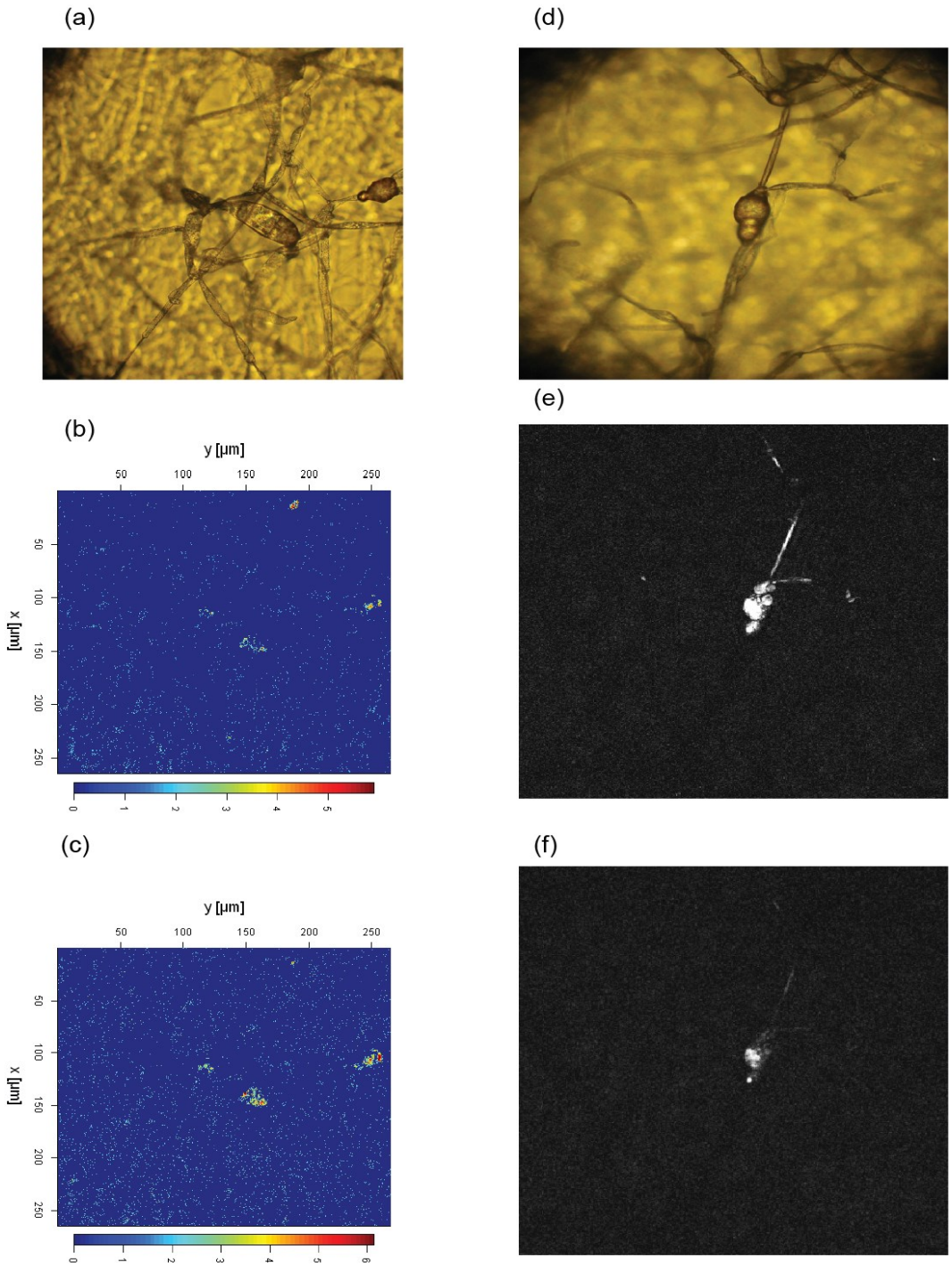

Fig.3.14.2.Progametangial (a) and gametangial (d) structures from *M. Mucedo* under a bright microscope field investigated for CARS analysis. The specific signals at 1522 cm^{-1}indicate β-carotene (b,e) and 1592 cm^{-1} that of trisporic acids (c,f) present in those structures.

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 ie.(+), (-) 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^{th} hpi as time zero. There was a slight up regulation intranscripts 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.

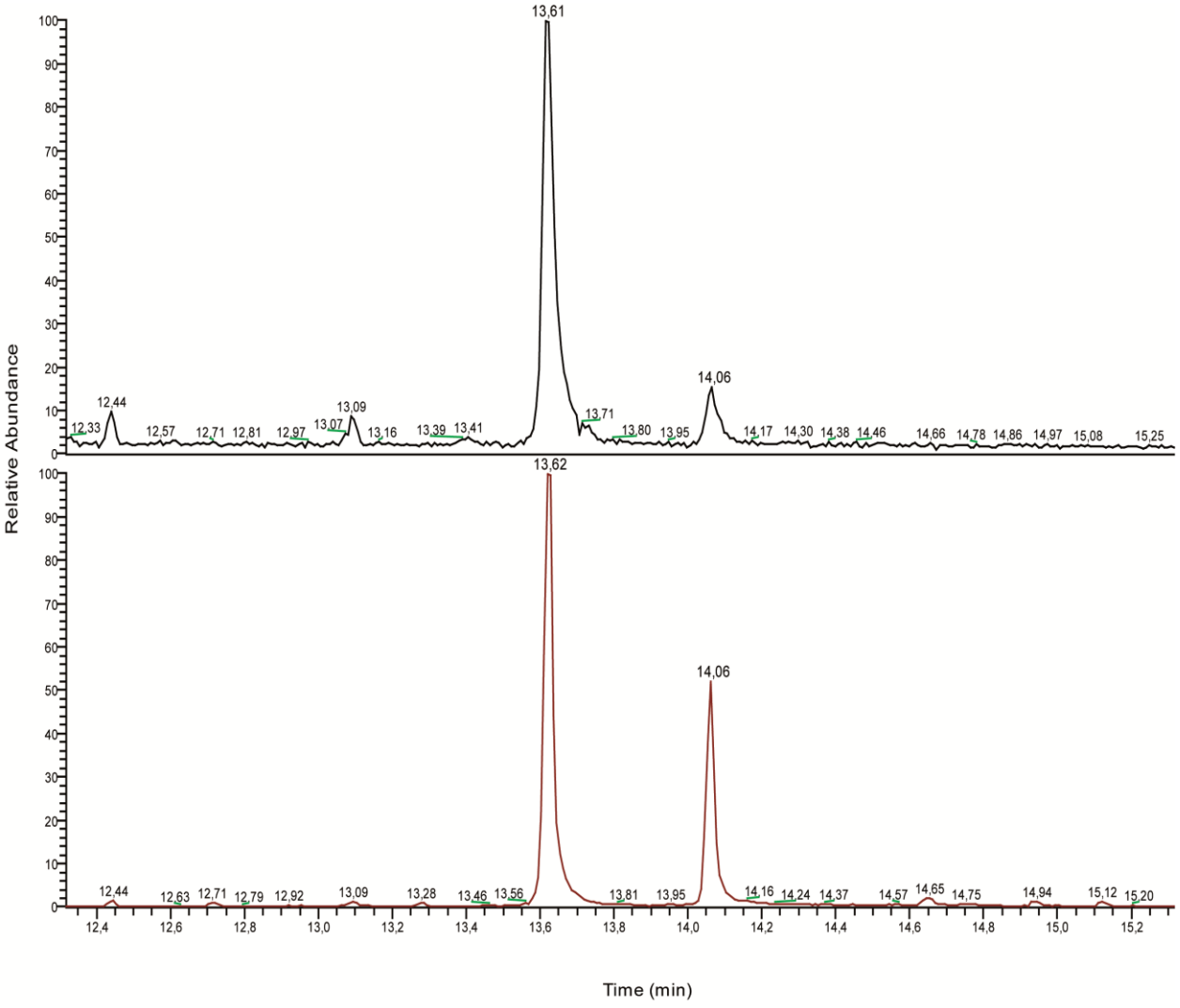

Fig.3.15.1.Gas chromatogram of the trisporic acids extracted from the sample (above) and that from the standard (below).

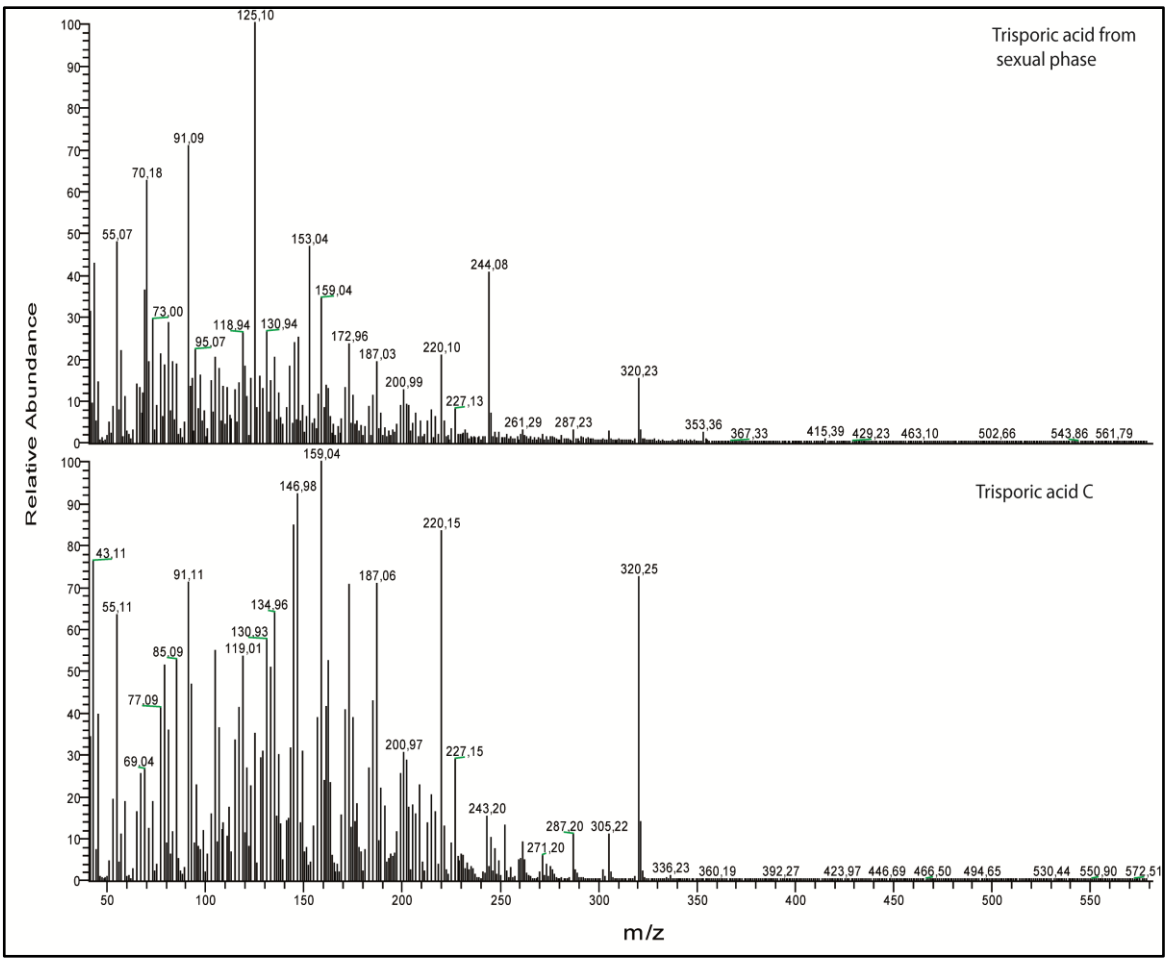

Fig.3.15.2. Mass spectrum for the derivatized trisporic acids obtained from sample and standard

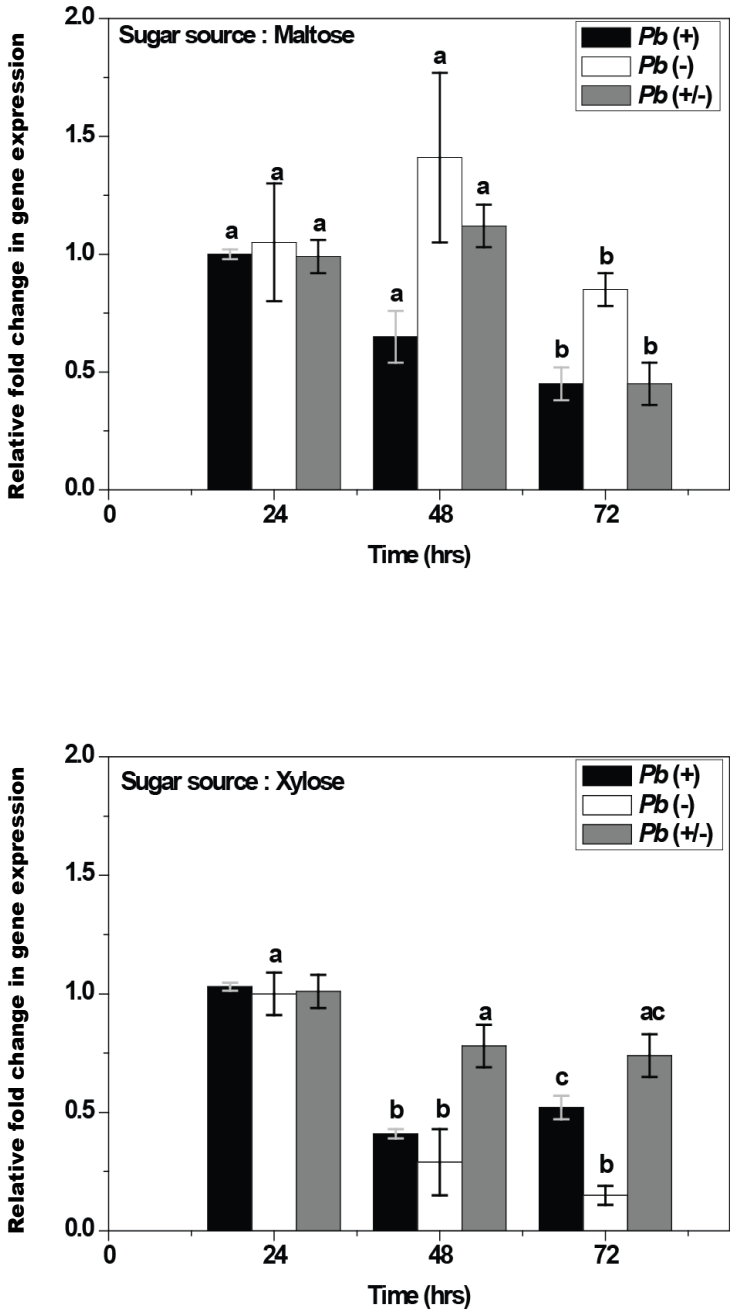

Fig.3.16.1. The transcriptional dynamics of *TSP1*in *P. blakesleeanus* cultivated using maltose and xylose as carbon sources. Small letters indicate the significant difference among (+), (-) and (+/-) at each time point.

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 *M. mucedo*. Meanwhile in homothallic *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 (-) *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 *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 *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 *TSP1* in *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 *TSP3* transcript levels in mated phase of *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 carotenogenesis. *M. mucedo* belonging to the same genera showcases an intense yellow-orange pigmentation at the mating zone (Fig.3.12.1) where zygospores 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*, unkn1 [189974], unkn 2 [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 C_{25} apocarotenoid, β-apo-12'-carotenal formed by the TSP3

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 (Phyre^2) 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

exual zygospores during mating of Δ*acaA* 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 Δ*acaA* (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 (Δ*crgA*) 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

postively 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 (Δ*crgA* 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 upregulation 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 (C_{25}), 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 distinguishβ-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 (Δ*crgA*:Δ*acaA*) resembles the single knockout mutant Δ*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 C_{18} 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 $C_{13\text{-}14}$ bond ofβ-carotene was expected, as its homologue in *Phycomyces* was exclusively specific for lycopene or apocarotenoid substrates. A computational prediction (Phyre^2) 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 caroteno-genesis repressor CrgA

A successful mating is associated with the production of zygospores in Mucorales fungi. Except wild type (-), the mutants derived from it, MU223 (Δ*crgA*), MU367 (Δ*acaA*) and MU366 (Δ*crgA*:Δ*acaA*) 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_{18} trisporoids namely D'orenone, methyl trisporate C (MTSPC) and trisporin C (TSPC) increased *CarRA* and *TSP3* 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

Pilze der Ordnung Mucorales sind bodenbewohnende Saprophyten, mit einem überwiegend haploiden, asexuellen Lebenszyklus. Äußere Einflüsse und Dunkelheit begünstigen eine „sexuelle Kommunikation“. Während der sexuellen Interaktion werden durch sequenzielle Spaltung von beta-Carotin Trisporoide gebildet, welche durch die komplementären Plus- und Minus-Kreuzungstypen zu einem vielfältigen Cocktail von Sexualhormonen, bekannt als Trisporsäuren, umgewandelt werden. Diese stimulieren wiederum positiv die Carotinogenese. Allerdings ist bisher wenig über die physiochemische Funktion der Enzyme und deren Produkte in der frühen sexuellen Phase der Mucorales bekannt. Es wird vermutet, dass die meisten C_{18} Trisporoid-Intermediate regulatorisch auf Gene der Sex-Pheromonsynthese wirken und innerhalb von Arten, sowie zwischen der vegetativen und sexuellen Phase der Entwicklung variieren.

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

Gegensatz zu *B. trispora* behält *Mucor circinelloides* f. *lusitanicus* während der sexuellen Interaktion seinen weißlichen Phenotyp, was ganz offensichtlich die nicht-signifikante Hochregulierung der Carotenoid-spaltenden Oxygenasen bestätigt. Der Transkript-turnover für *carS* ist hingegen spezifisch im Plus-Kreuzungstyp, sowie für *acaA* deutlich Zeit-abhängig im Minus-Kreuzungstyp. Selbst nach einem 12-24 stündigem Nacht-Tag Zyklus zeigen weder *carS* noch *acaA* irgendwelche Änderungen in ihren Expressionsmustern, was nahelegt, dass die Carotenoid-spaltenden Oxygenasen in *M. circinelloides* (-)Licht-unabhängig sind. Interessanterweise wird in *Blakeslea* und in *Phycomyces* (Phycomycetaceae) das Gen *tsp1* aus dem Trisporsäure-Biosyntheseweg unabhängig von der sexuellen oder asexuellen Phase runter-reguliert.

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*

Innerhalb der Mucorales ist eine erfolgreiche Kreuzung durch die Bildung reifer Zygosporen gekennzeichnet. Entgegen dem Wildtyp-Isolat des Minus-Kreuzungstyps von *M. circinelloides* f. *lusitanicus* sind dessen Mutanten MU223 (Δ*crgA*), MU367 (Δ*acaA*) und MU366 (Δ*crgA*:Δ*acaA*) nicht in der Lage mit einem komplementären Plus-Kreuzungstyp Zygosporen zu bilden. Folglich haben CrgA und AcaA im Minus-Kreuzungstyp eine entscheidende Funktion in der Trisporsäure-Biosynthese und der sexuellen Morphogenese. Zudem unterdrückt CrgA in *M. circinelloides* (-) transkriptionell das Gen *sexM* und aktiviert *acaA*. Ein hohes Transkript-turnover konnte hingegen für *sexP* und *sexM* in Kreuzungsexperimenten ohne Zygosporen-Bildung beobachtet werden. Daraus ergibt sich, dass die reine Präsenz spezifischer Sex-Gene nicht ausreichend ist für einen erfolgreichen sexuellen Zyklus, und dass die sogenannten Kriterien der „Komplementarität“ der Kreuzungspartner anhand der hier gezeigten Ergebnisse neu definiert werden müssen.

4. Rolle der Apocarotenoide als transkriptionelle Aktivatoren

Die Supplementierung der C_{18} Trisporoide D’orenone, Methyltrisporat C und Trisporin C führt zu einem Anstieg von *carRA* und *tsp3* im Plus-Kreuzungstyp von *Blakeslea* verglichen mit dem Minus-Kreuzungstyp. Ein gewaltiger Anstieg von *acaA* Transkripten nach der Behandlung mitβ-apo-12’-carotenal kennzeichnet möglicherweise die Apocarotenoide als effiziente Substrate. Andernfalls wäre die Bildung des Apocarotenals ein Geschwindigkeitslimitierender Schritt im Trisporsäure Biosyntheseweg. Trisporin C

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*

M. mucedo (Mucoraceae) ist die ideale Wahl aufgrund der reichlich produzierten Trisporsäuren und seiner ausgeprägten morphologischen Differenzierung von Zygophoren hin zu Zygosporen. Die Vermessung von beta-Carotin und reinen Trisporsäuren (B und C) mittels Raman lieferte uns die spezifischen Schwingungsänderungen zur Unterscheidung von Verbindungen. Koherente anti-Stokes Raman Spektroskopie (CARS) ist ein dreidimensionaler nicht-invasiver Ansatz mit welchem wir erfolgreich sowohl die Sex-Hormone als auch ihren reichlich vorhandenen Vorläufer, das beta-Carotin, in der Pro-Gametangien Phase nachweisen konnten. Ferner zeigten Extrakte von Agar-Platten in der GC-MS Analyse das detektierbare Mengen an Trisporsäuren auf gekreuzte Kulturen von *M. mucedo* gegrenzt sind. Die Anwendung von CARS ist vielversprechend um biologisch instabile Metabolite *in situ* in unterschiedlichen Entwicklungsphasen nachzuweisen ohne das biologische System zu zerstören.

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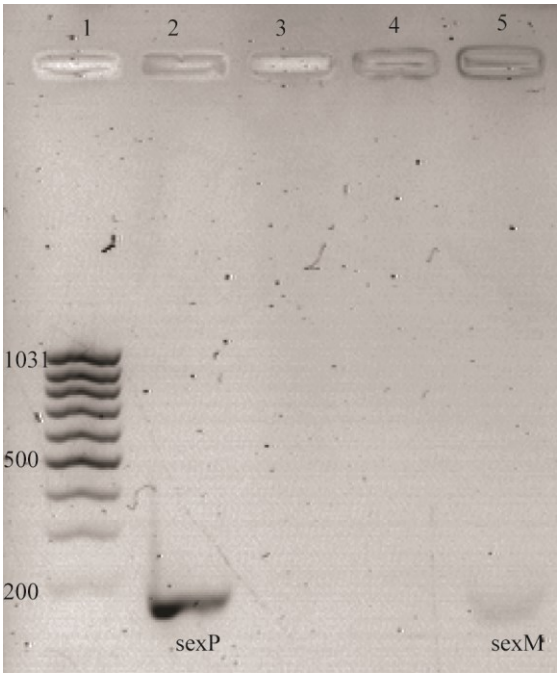
7. APPENDIX

Fig. S1. Agarose gel electrophoresis for *SexP* and *SexM* amplicons from*Mucor circinelloides 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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Phycomyces  ----------------------------------MLTPAAENPLREQGLPAPSPTGYNNV  26
Mucor       ----------------------------------MITPAEANPN--INEAAPSPVGFKNV  24
Blakeslea   MKLLEGEENKIYKSKQSFIFVVLYLFLFCSLVKKMLTPACENPNAKVGEAAPSPVGFKNI  60
Rhizopus    ----------------------------------MITLAENNPNREKNEAAPSPVGFKNI  26
Umbelopsis  ----------------------------------MKSHS--------EDITESPLGLKNT  18
Phycomyces  PAFNKPVELTIEGTIPEWVNGVMYRA---GSGRYNLLLENGDTFHIGHPFDGLAMLHRFE  83
```

```
Mucor       PSVESPVELKLEGQIPSWVHGVMYRS---GSGRYNVLLDNGDTFHIGHPFDGLAMLHRFE  81
Blakeslea   PATESPKTIMLDGYIPEWIEGVMYRS---GSGRYNVLLDNGDTFHIGHPFDGLAMLHRFE  117
Rhizopus    PAFEKPIELEIDGKIPSWINGVMYRS---GAGRYNILLENGDTFHVGHPFDGLAMLHRFE  83
Umbelopsis  PENATPIELEVKGTIPSWLCGVMYRAGFQGAGTFNIPLQDGSTFHIKHPFDGLAMLHRFE  78
Phycomyces  LSGETQTVQYSSRHTSHGVERRIREKDPTLLTFGPDPCKTIFGRIQSVYHHISKFGANAQ  143
Mucor       INGETQTVKYNSRHTSHGVERRIGQRDPTLLTFGPDPCKTIFGRIQSVYHHISKFGANAA  141
Blakeslea   IDGKTQTIKYNSRHTSHGVERRIGERDPTLLTFGPDPCKTIFGRMQTVYHHISKFGTNAA  177
Rhizopus    ISGEDQSIRYNSRHTSHGVERRIGKRDPTLLTFGPDPCKTIFGRIQSIYHHISKLGSNAA  143
Umbelopsis  INGVQNSVSYCSRHTSNGVKRRIMEKDPTLLTFGPDPCKTIFGRMQSVFHHISRMKKNAD  138
Phycomyces  IQEGDPEFDMVNVTITPNFPLGERLEAETGVKRGDALVVKRDANTLQLVDNKTLKPIKMF  203
Mucor       LQENDAEFDMVNVTVTPNFPLGADLEQETGVKRGEALVVKRDANTLQVVDPESLKPLKMF  201
Blakeslea   LQEHDPEFDMVNVTVTPNFPLGEALEKETGVKRGEAVVVKRDADTLQLVDSESLKPLKMF  237
Rhizopus    LQENDPEFEMVNVTVTPNFPLGEKLEEETGVKRGEAVVVKRDANTLQVVDKDTLKPIKMF  203
Umbelopsis  DMEEDPEFDMVNVTITPNFPIGKTLEEKTGVLPGEAVVVKRDANTLQLVDSKTLEPLKMF  198
Phycomyces  TYGHVNDKLQGQLCASHHQYDEETDEYVNFTVRLGPIPSFQSYTLGPYLPTPPGSKEKMP  263
Mucor       TYAHVSKKLQGQLCASHHQYDEETDEYINFMVKLGPFPTFQTFSLGPYLPSPPGEKEALP  261
Blakeslea   TYNHVSKKLQGQLCASHHQYDEETDEYVNFMVKLGPFPTFQTFTLGPYLPHPE-QKEQVP  296
Rhizopus    TYGHVSEQLNGQLCASHHQYDEETDEYVNFMVKLGPIPSFQTFSLGPYLPLPADEKENMS  263
Umbelopsis  TYGHVDKSIKGQLCASHHQYDEETDEFVNFMVTLGPFPSFQSFTIGPYLPKMSIGTQEVM  258
Phycomyces  APQVRLHEPIYRHLGAWRTLEPLKPAYIHSFSMTKNYIIVPNFPYYYSFGGMSALYYSCA  323
Mucor       APEPRLHQPIWRHLGAWKTMEALKPSYIHSFSMTKNYIIIPNFPYYYSFGGLSAIYYSSA  321
Blakeslea   APEARLHQPIWRHLGAWKTMEALKPSYIHSFSMTKNYIVIPNFPYYYSFGGLSAIYYSSA  356
Rhizopus    KPDVRLHQPIWRHLGAWKTLEALKPSYIHSFSMTKNYIIIPNFPYYYSFGGLSAIYYSCA  323
Umbelopsis  A---VLHEPVWRHLGAWKTMEPLKPSYIHSFSMTKRYIIIPNFPYYYSFGGLSALYYSNA  315

Phycomyces  YQTFYWDETRPTLFHVVDRNTGRHVATYDADPCFSFHSANAWDEEVDLPGGGKERVIYMD  383
Mucor       YQTFYWDETRHTLFHVIDRHTGHHVATYEADPCFSFHTANAWDEDVELPGGRKERVIYMD  381
Blakeslea   YQTFYWDETRHTLFHVIDRHTGHHVATYEADPCFSFHTANAWDEEVELPGGGKERVIFMD  416
Rhizopus    YQTFYWDETRHTLFHVIDRHTGYHVATYEADPCFSFHTANAWDEDVELPGGRKERVIFMD  383
Umbelopsis  YQTFHWEENRHTLFHVIDRNSGRHIATYEAEPCFAFHTANAWDEDIPTFDGKTETVIYFD  375
Phycomyces  YCVYENTDIVDASFDLGKTPTG-FDASKVEPARFKIKRHTDDKKDNSISPSQLRRYRLGN  442
Mucor       YCMYENTDIVDASFELGKTPTGKLDLDLVQPARYVFDKNTAHKEEHQIAPSQFRRYRLGQ  441
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Blakeslea   YCMYENTDIVDASFELGKTPTGKLDLDKVQPARFRFDKLTDHKTEHQIAPSQLRRYRLGR  476
Rhizopus    YCTYENTDIIDASFELGKSPSGKLDLDLVQPA--CFIKNTDGKKEHKISPSQFRRYRLGQ  441
Umbelopsis  MSVYENTDIVDASFEFGK-GVDFFDAEKVKPAPFVIKKKAFGKANNKIAPSQVRRYRLSN  434
Phycomyces  VPVSSNAPETSRWSPKGIT-GLLSGIFDFNKRRVASYTVLGSDIELPRFNSNFNLRKYRY  501
Mucor       VPVAK--PGADAWTPSWSN--AFSKFTQFNKRRVASYTVIGHDLEMPRFNPRYSMKPYRF  497
Blakeslea   VPVAK--PDADKWSPSWSDSFNFGKWTQFNKRRVASYTVIGHDIEMPRFNPRYNLKPYRY  534
Rhizopus    VPVSQ--PGAETWSSSWSN-LNISSFTKYNKRRVASYTVIGYDIEMPRFNPKHNLRKYRY  498
Umbelopsis  VPVVN--------ESNRVNTGLFGISTDFNSRKVASYEVLGYDLELPRFNQKYALKPYRY  486
Phycomyces  VWGVCESKHAPSYASG-AVVNGLIKLDLDKPTLCKNTEEGSSAKIWDEPGCSCSEPIFVA  560
Mucor       CWGVCESRHAPSYASG-SVVNGLIKLDLNNVYLGPNTDKESSAKIWDEPSCSCAEPIFIP  556
Blakeslea   CWGVCESQHAPSYASG-AVVNGLIKLDLNHPYLGPNTSEESSAKIWDQPGCSCAEPIFIP  593
Rhizopus    CWGLCESRFAPPYASGGAIVNGLIKLDLDQPYLGPNTDEASPAKIWDYPGCSCAEPIFVP  558
Umbelopsis  LWGVCQSKHAPSYASG-AVVNGIIKLDLNNP-----KAQSSTALYWDEPGCSCAEPIFVP  540
Phycomyces  HPEQRAEDDGVLISTVNTTTPDGKESCFLLIVDAATMVEVGRTTLGAFTAMTIHGSFVDT  620
Mucor       HPDAQDEDEGVVMSIVNTTLEDGSESCFLLILDACSFTEIARTTIGAFNAVTLHGSFVDK  616
Blakeslea   NPDALEEDDGVVMSIVNAVLEDGSESCFLLILDAATMKELARATLGNFNAVTLHGSFVDK  653
Rhizopus    SPEGINEDDGVIMSIQQV----------------------------------------  576
Umbelopsis  SDHGKAEDDGVVISVVNASLPEG-DSCFLLILNAATLEELARTTMGQWHTMTIHGSFVDQ  599
Phycomyces  NGKGVAVN  628
Mucor       HGRGIAVN  624
Blakeslea   HGRGIAVN  661
Rhizopus    --------  576
Umbelopsis  AGRGIAVN  607
```

Phycomyces blakesleeanus_183749 [estExt_Genemark1.C_330115]	%ID(AA) CarS	0,715	0,688	0,666	0,627
Mucor circinelloides_146755 [e_gw1.06.133.1]	0,715	%ID(AA)	0,809	0,732	0,621
Blakeslea trispora AM409182	0,688	0,809	%ID(AA)	0,686	0,596
Rhizopus delemar [RO3G_03330.3]	0,666	0,732	0,686	%ID(AA)	0,565
Umbelopsis ramanni-ana_170528 [e_gw1.8.23.1]	0,627	0,621	0,596	0,565	%ID(AA)

AcaA

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Phycomyces -------MVWVNPFGLVSRFTTYFN---GQDLLAAAQTTVFRNTPEKRTPEWFPVK-GRV  49
Mucor      MIVGLLTLISLPVSGFVLFLYRNYYNDVYRRFQVILKSHKLSNTPEVQDPIVLQVQSGQI  60
Rhizopus   --MLLVASIVLGPLGYVFYLLYSNYNHVYKKIQSMQKPVMLGNTPECQDRITLEKVSGQL  58
Umbelopsis ---------------------------------------MFRNTTEIPQPVALPVN-GQL  20
Phycomyces PDWFNGIMYRVGPGKYNIEQANGTTFAINHAFDGMPFMHRFEISSERQAVRYNSRNISEE  109
Mucor      PSWLNGIMYRIGPGRFNIQQDDGSTFNIRHAFDGLPFMHRFEVNGSTQTLKYNSRMLAKS  120
Rhizopus   PSWLNGVMYRVGPGVFNIKQKNGITYTIRHAFDGLPMVHRFQLNGSTQTVTYNSRHTAKS  118
Umbelopsis PAWLNGTLFRIGPGKFNIKRKDGSYMHIRHAFDGLPYLHRFVFSAVNNQIQYNSRNLSQE  80
Phycomyces FESSIAKDNGQGKIFFGHQPTVTSVKQRLKDIYLRFDSMLLSRRPLDETSPSSQPVGVTA  169
Mucor      LETKIKNKTSKGLIFFGHIPEMT-FPEWLYNFYIRFNNLILFPKSRRDGRPDSQAVGVTV  179
Rhizopus   LEKEISSGADKGLVFFGHVPEVS-FLTWLINWIVRLNNLVLRPKPIHLTKPDGRSVGVTV  177
Umbelopsis VERQLA-DNDDYPLFFGHSDPATSLFIKLYRAYKRLRSSSIAN---ETRHPSDAMVGVTA  136
Phycomyces TPNFPIPPVYKAADKNNGESDRVLVAKTDANMLQQLNSDTLEPKRLFNYGNFEKKLKGDL  229
Mucor      TPNFPLP---ARIKKAD--NENVLVSKTDANVLQKIHAETLEPQNIFNYTSYDARIQGQL  234
Rhizopus   TPNYTLP---ARVSMNNDLSKKSLVAKTDANVLQKLHAETLVPEIIFSYKNYDPHLNGPF  234
Umbelopsis TPNFPLGNDHLADVRKVG--DQVLVAKTDANLLQLIDVDSLVPKKIFNYTTIIPEVQGQL  194
Phycomyces SAAHHQYDPITKETINFVLDMFP-ARLQVFSSTPEGKITILADFTHRLDEKRTRVQPVYI  288
Mucor      SAAHHQYDPNTKEIFNFALTTGPVPRLVVFSTSESGKATILADFTHRT-DK-SPIQAPYI  292
Rhizopus   SAAHHQFDPETNEIFNFSLHLFPKSKMTVFKTSKEGQTTLLAEITHRKGDH-SEFRASYI  293
Umbelopsis SAAHHQTCPYTKELFNIVLSFGRSTTIKVFSINPEGKGTLYGDIVKRLHDN-VPIKSSYI  253
Phycomyces HAFNITKDYIILPEYSLAYTNMGVDFLVSGAVNTGMAWSNDRPTFFHVISR----HGKGL  344
Mucor      HSLWLTENYVIIPESPMVLKDRGANMLMNGSFLSSISWVKDAPTYCHVIARRPAQEKEGL  352
Rhizopus   HSFYLTKNYVIIPESPLVYGDQGLNSLLQGAVLSSMHWIDQAPLYFHVIHR----NEGGL  349
Umbelopsis HSFALTQNYIVIPEYPLYFAGGGLPILLEGTIEAAFAWDSNAKTYFHVISR----EKKKH  309
Phycomyces VASVPV-ETFFSFHVANAWDSVDAQGRQVIDMDICAFENADIMCQLHTFAKPVRQAEYDS  403
Mucor      VASIPVNPGFFTFHVGNAFETKNESGETVLTLDAASFCDGDIMHQLHSFGTPHRKGFTAE  412
Rhizopus   VASIPA-PAFYTFHVANAFEEVSLDGELLLHLDSSAFSDGDIIYQVQNFGGTFLQ--DDL  406
Umbelopsis VATIEA-DPAFAFHMGNCWEDAEG-----IHFECGAFPDGDIAHQLHTFGKPVRKTQLSS  363
Phycomyces HVKKQLELSKQSQQYNGMNIPPLRQPSFGDLRRYQI---VLENGTGEATYRTIASNVEFA  460
Mucor      KVS--------QTTFNGISYPPRQQSGFGDLVRYKLN---LDQST-TLSIDTLAKNAEFP  460
Rhizopus   SLK--------RTKFHGFTFPPAQQVSFGHLTRHTLN---LNQRT-AVFAHTLAENIEFP  454
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Umbelopsis QDNHHGDN--KQKKSKGITVNPPRQSSFGDLRRYTISYDAIQRGAGQATYKEIAPNVEFP  421

Phycomyces RYSQDYAMRK-HKFVYGCQLISVTAKSNERYDLVKVNLDDGS-VIRYSQEGCACSEPIFA  518

Mucor      RFNQDYALKEESRFVYGCQLQGFTEKRDETGGLIKVDLTGQGEPLTFVEEGFNCSEPIFV  520

Rhizopus   RFHQELIGKP-YKFVYGCRVL--DDKR--STGLAKVDVSNQS-VIQHQESGYAYSEPIFV  508

Umbelopsis RFNAKMTGKP-SRYVWACKLHPATMDKNETVSVVKVDTSTGK-TVQYYRPGYYCSEPIFI  479

Phycomyces PRPGGTEEDDGVLMSLVNKLDKEDPSKDYCFLLLLDAKTMQEVATCQVGQFTATTFHGSF  578

Mucor      PKPAGTKEDDGVLLSLVNNF-------DCCYFIVFDASNLRELARIKIGQFIAVTFHGSY  573

Rhizopus   PHPQAKSEDDGVLLSLANHT-------ECCYLVILNAMDMKELARFKIGQFTAITFHGSF  561

Umbelopsis PKPGAENEDDGIVVSLANDFHESDESLDLCYVVILDGETLEELGKAQIGDMTPVTFHGSF  539

Phycomyces VDHHFENVSIN  589

Mucor      VDHEFKSININ  584

Rhizopus   VDYEFKSINLN  572

Umbelopsis VDKDFQDVSVN  550
```
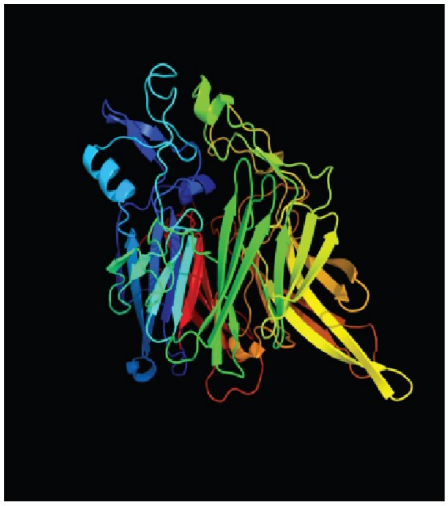
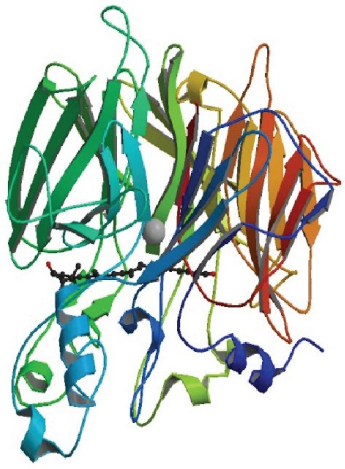

Phycomyces blakesleeanus_143867 [Genemark1.6058_g]	%ID(AA) AcaA	0,415		0,386	0,394
Mucor circinel-loides_141273 [e_gw1.03.260.1]	0,415	%ID(AA)		0,522	0,372
Blakeslea trispora					
Rhizopus delemar [RO3G_01506]	0,386	0,522		%ID(AA)	0,347
Umbelopsis ramanni-ana_170664 []e_gw1.8.75.1	0,394	0,372		0,565	%ID(AA)

Fig. S3. Phyre[2] homology predicted structures for CarS and AcaA from *M. circinelloides*.

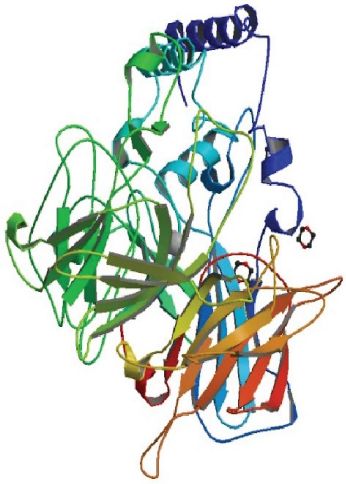
CarS



AcaA



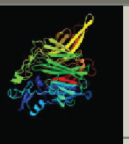
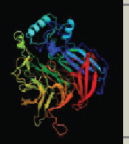
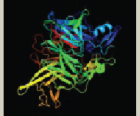
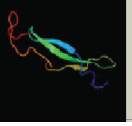
Apocarotenoid cleavage oxygenase from *Synechocystis*



9-cis epoxycarotenoid dioxygenase 1 or maize viviparous VP 14

Fig. S4. Template analysis for predicted CarS and AcaA structures

Detailed template information

#	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
1	c2biwC_	Alignment		100.0	23	**PDB header:**oxidoreductase **Chain:** C: **PDB Molecule:**apocarotenoid-cleaving oxygenase; **PDBTitle:** crystal structure of apocarotenoid cleavage oxygenase from2 synechocystis, native enzyme
2	c3npeA_	Alignment		100.0	22	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**9-cis-epoxycarotenoid dioxygenase 1, chloroplastic; **PDBTitle:** structure of vp14 in complex with oxygen
3	c3fsnA_	Alignment		100.0	25	**PDB header:**isomerase **Chain:** A: **PDB Molecule:**retinal pigment epithelium-specific 65 kda protein; **PDBTitle:** crystal structure of rpe65 at 2.14 angstrom resolution
4	c3qc2A_	Alignment		54.4	21	**PDB header:**hydrolase **Chain:** A: **PDB Molecule:**glycosyl hydrolase; **PDBTitle:** crystal structure of a glycosyl hydrolase (bacova_03624) from2 bacteroides ovatus at 2.30 a resolution

Detailed template information

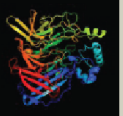
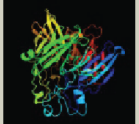
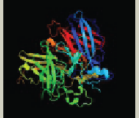
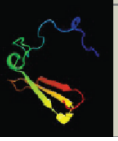
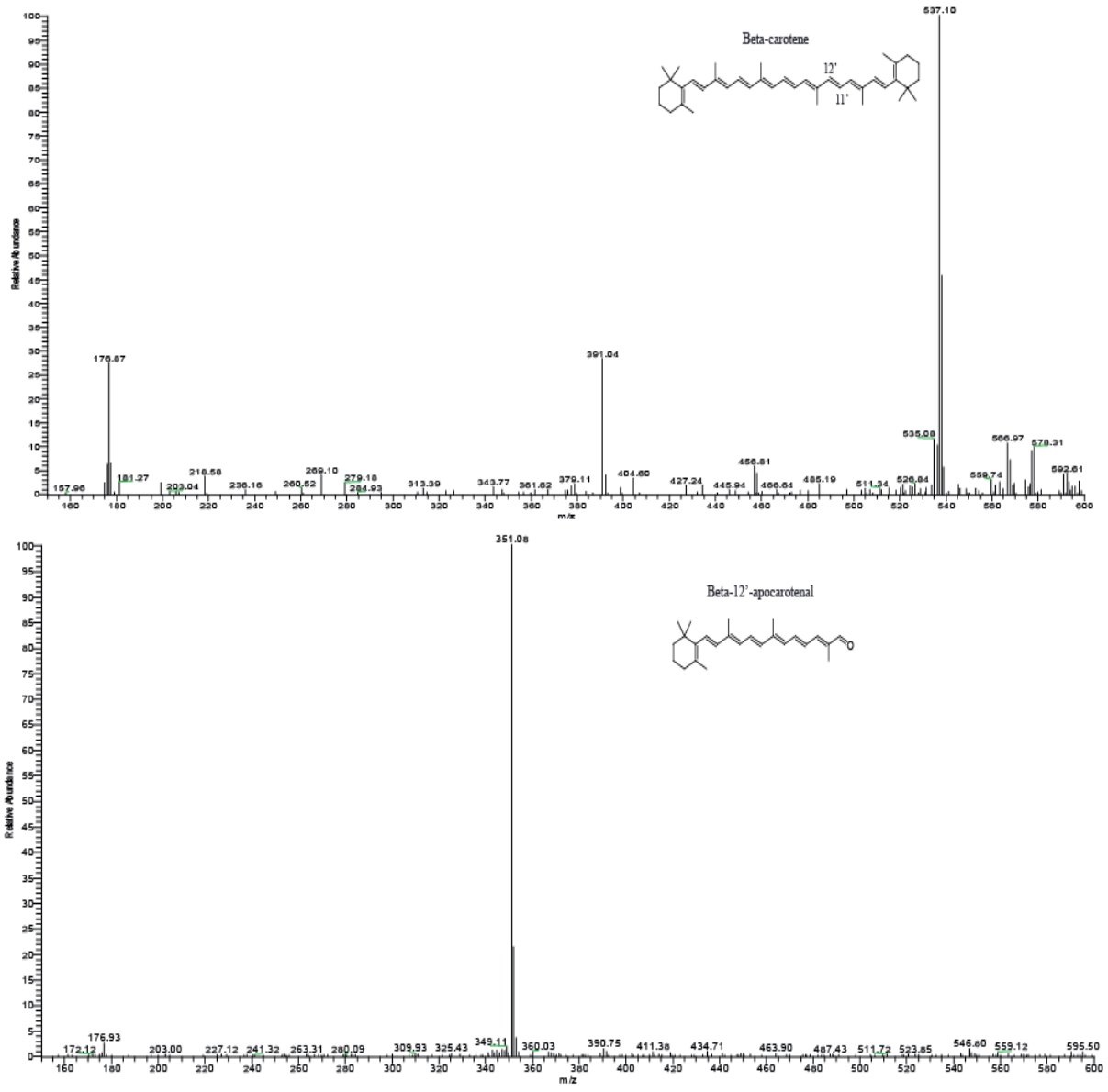
#	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
1	c3npeA_	Alignment		100.0	21	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**9-cis-epoxycarotenoid dioxygenase 1, chloroplastic; **PDBTitle:** structure of vp14 in complex with oxygen
2	c2biwC_	Alignment		100.0	23	**PDB header:**oxidoreductase **Chain:** C: **PDB Molecule:**apocarotenoid-cleaving oxygenase; **PDBTitle:** crystal structure of apocarotenoid cleavage oxygenase from2 synechocystis, native enzyme
3	c3fsnA_	Alignment		100.0	22	**PDB header:**isomerase **Chain:** A: **PDB Molecule:**retinal pigment epithelium-specific 65 kda protein; **PDBTitle:** crystal structure of rpe65 at 2.14 angstrom resolution
4	d1qksa2	Alignment		34.2	15	**Fold:**8-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.

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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

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Yamuna Sahadevan
Jena, den 15. Nov, 2013

10. CURRICULUM VITAE

Personal data

Name : **Yamuna Sahadevan**

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Scientific career

Since 01/2009 PhD student at the Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena,Germany

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

1. Sahadevan.Y, Richter. M, Hoffmann.K, Voigt.K, Boland.W (2013).Early and late trisporoids differentially regulate β-carotene production and gene transcript levels in the mucoralean fungi *Blakeslea trispora* and *Mucor mucedo*. ***Appl. Environ. Microbiol*** .(Dec 2013)

2. Sahadevan.Y, Hoffmann. K ,Navarro. E, Voigt.K, Ruiz-Vázquez. R, Boland.W. The RING finger protein CrgA and putative apocarotenoid cleavage oxygenase AcaA of the minus mating type regulate sexual zygospore production in an opportunistic human pathogen *Mucor circinelloides* f. *lusitanicus.* (In preparation)

3.Sahadevan.Y, Weissflog.I, Dietzek.B and Boland.W. *In situ* localisation of β-carotene derived fungal sex hormones in *Mucor mucedo* by Coherent Antistokes Raman microspectroscopy (In preparation)

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)

3.Sahadevan. Y and Boland. W: Unraveling the unknown volatiles directing sexual interaction in zygomycetes fungi (5th Plant Science Student Conference, Halle, Germany, June 2009)

Poster presentations

1.Sahadevan. Y, Hoffmann. K, Weissflog. I, Voigt. K, Dietzek. B and Boland. W: Apocarotenoid signals regulate sex in mucoralean fungi (11th International Fungal Biology Conference, September 2013, Karlsruhe, Germany.)

2. Sahadevan. Y and Boland. W: Kinetics and differential expression profiling of genes involved in β-carotene metabolism and apocarotenoid feedback in mucoralean fungi (Gordon Research Conference on Carotenoids, January 2013, Ventura, California, USA)

3. Sahadevan. Y and Boland. W: How sex influences carotene metabolism in zygomycetes fungi?(11th European Conference on Fungal Genetics, May 2012,Marburg,Germany)

4. Sahadevan. Y, Boland. W, Hoffmann. K, Voigt. K. Developing an optimal qRT – PCR Strategy for fungal transcriptomics on carotenoid metabolism.(5th International qPCR Symposium,Industrial Exhibition and Application Workshop, May 2011, Munich, Germany)

5. Sahadevan. Y, Weissflog. I, Dietzek. B and Boland. W: Performance and localization of Fungal sex hormones.(10th International Max Planck Research School Symposium, MPI for Chemical Ecology, February 2011, Dornburg, Germany)

6. Sahadevan. Yand Boland. W.Signaling molecules associated with Zygomycetes fungi (8th International Max Planck Research School Symposium, MPI for Chemical Ecology, March2009, Dornburg, Germany)

7. Sahadevan. Y: GeoChip-A novel microarray technology (International Symposium on Structural Bioinformatics, Bharathiar University, January 2008, Coimbatore, India)

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.