Down regulation of gene expression in *Mucor mucedo* and *Mucor circinelloides* by transformation with antisense morpholino oligonucleotides

Dissertation

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by

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Gutachter:

1. …….....
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I dedicate this work to My Heavenly Father
and
My Beloved Family Members

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Abbreviations

Amp	=	Ampicillin
ARS	=	Autonomously Replicating Sequence
BCA	=	Bicinchoninic acid
BLAST	=	Basic Local Alignment Search Tool
DAPI	=	4',6-Diamidino-2-Phenylindole
DSB	=	Double strand breaks
DTT	=	Dithiothreitol
EDTA	=	Ethylene diamine tetraacetic acid
EGFP	=	Enhanced Green Fluorescence Protein
FAD	=	Flavin Adenine Dinucleotide
FSU	=	Friedrich Schiller University
GFP	=	Green fluorescent protein
GLA	=	Gamma linolenic acid
HR	=	Homologous Recombination
IPTG	=	Isopropyl β-D-1-thiogalactopyranoside
Kb	=	Kilobase
LB	=	Luria Britani
mbar	=	Millibar
mins	=	Minutes
miRNA	=	micro RNA
MO	=	Morpholino
NADP	=	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	=	National Center for Biotechnology Information
NHEJ	=	Non-Homologous End Joining

OD	=	Optical density
PCR	=	Polymerase Chain Reaction
PEG	=	Polyethylene glycol
RISC	=	RNA-induced silencing complex
RNAi	=	RNA interference
RNase	=	Ribonuclease
rpm	=	Revolutions per minute
S-DNA	=	Phosphorothioate linked DNA
SDS	=	Sodium dodecyl sulfate
SiM	=	Silent mutated
siRNA	=	small interfering RNA
SSC	=	Saline-sodium citrate
SUP	=	Supplemented minimal medium
TA	=	Trisporic acid
TAE	=	Tris Acetate EDTA
TBE	=	Tris Borate EDTA
TDH	=	4-dihydromethyl-trisporate dehydrogenase
TNDH	=	dihydrotrisporin-dehydrogenase
Tris HCl	=	Tris hydrochloride
u	=	Units
UTR	=	Untranslated region
VDJ	=	Variable diverse joining
VIS	=	Visible
Xgal	=	5-Bromo-4-chloro-3-indoly- galactopyranoside

Summary

The regulation of gene expression by means of antisense oligo-morpholino nucleotides (MO) allows many new experimental approaches. The morpholino antisense technology has been used for the first time in zygomycetes succeeding in the down-regulation of the expression of the *crgA*-gene from *Mucor mucedo*, and of the *ku70* gene from *M. circinelloides*.

Stable genetic manipulations in zygomycetes are very rare events. In almost all attempts at transformation, the transformed vectors are autonomously replicated within the transformants. Ectopic as well as homologous integration are only very rarely observed and hundreds of transformants need to be characterized in order to find such integrative transformants.

To prove the efficiency of the method, two different MO were created: The CrgA-MO is derived from the sequence of the newly established gene fragment of the *crgA* gene in *M. mucedo*. The Ku70-MO was designed based on the genome sequence from *M. circinelloides*. Both nucleotides have a length of 25 bp. The following genes were the target of the MO antisense regulation:

1. The crgA protein is a negative regulator of carotene synthesis. A decrease in its expression should lead to an increased carotene production. The increase or decrease of carotene is easily determined by photometric measurements after extraction of the cells. The CrgA-MO was transported into *M. mucedo* protoplasts by electroporation. After treatment with the CrgA-MO, an increase in the cellular carotene content was found. This is the first successful transformation experiment reported for *M. mucedo*. The method for protoplasting *M. mucedo* was also established.

2. Expression of *ku70* in *M. circinelloides* was also modulated, using a Ku70-MO. In this experiment, *M. circinelloides* was transformed simultaneously with the Ku70-MO and a mutated version of the pEUKA400 vector. The marker gene *Leu2* for leucine biosynthesis contains a silent mutation, result of a single nucleotide-exchange, leading to the formation of a new *Xba*I restriction enzyme recognition site. This allows for easier screening for transformants with homologous integration of the transformed gene and differentiates them from revertants, which

might also occur. Southern blot-analysis of the transformants revealed stable integrative transformants and only a few containing the autonomously replicating vector.

These results show, that MO are useful tools in the inhibition of gene expression. In future, this technique might help with the analysis of selected genes and in the modulation of gene expression in zygomycete fungi.

Zusammenfassung

Die Regulierung der Genexpression unter Verwendung von Antisense-Oligo-Morpholino-Nukleotiden (MO) ermöglicht viele neue Anwendungen. Diese Technik mit MO Nukleotiden wurde zum erstem Mal in Zygomyceten eingesetzt und es gelang die Genexpression von *crgA* aus *Mucor mucedo* und die *ku70*-Genexpressions aus *Mucor circinelloides* herunter zu regulieren.

Stabile genetische Manipulationen in Zygomyceten sind sehr seltene Ereignisse. In der überwiegenden Mehrzahl der Fälle werden die transformierten Vektoren in den Transformanden autonom repliziert. Sowohl ektopische als auch homologe Integration von Vektoren sind äußerst selten zu finden und es ist die Charakterisierung von vielen 100 Transformanden notwendig, um diese Integranten zu finden.

Um die Funktionaltät der Methode zeigen zu können, wurden zwei verschiedene MOs entwickelt: das CrgA-MO wurde abgeleitet von der Sequenz des neu isolierten Genfragments für *crgA* aus *Mucor mucedo*. Das Ku70-MO wurde aus der Genomsequenz von *Mucor circinelloides* abgeleitet. Beide Nukleotide habe eine Länge von 25 bp. Die Expression der folgenden Gene soll durch die MOs gesteuert werden.

1. Das CrgA-Protein ist ein negativer Regulator der Carotinsynthese und ein Abfall der Genexpression sollte zu einer erhöhten Carotin-Synthese führen. Die Zu- oder Abnahme von Carotin kann nach Extraktion aus den Zellen einfach photometrisch bestimmt werden. Das CrgA-MO wurde mit Hilfe von Elektroporation in Protoplasten von *M. mucedo* eingeschleust. Es konnte die Zunahme von Carotin nach Behandlung mit CrgA-MO nachgewiesen werden. Dies ist das erste Transformations-Experiment in *M. mucedo*. Die Methode zur Protoplastierung von *M. mucedo* wurde ebenfalls etabliert.

2. Die *ku70*-Genexpression in *Mucor circinelloides* wurde ebenfalls durch ein Ku70-MO moduliert. Dabei wurde *Mucor circinelloides* gleichzeitig mit dem Ku70-MO und einer mutierten Version des pEUKA400 Vektors transformiert. Das Marker-Gen *LeuA* für die Leucin-Biosynthese enthält eine stille Mutation durch Austausch eines einzelnen Nukleotids, die zu einer neuen *Xba*I Restriktions-

Erkennungsstelle führt. Dies ermöglicht ein einfacheres Screening von Transformanten, die das Gen homolog integriert haben und unterscheidet diese von möglichen Revertanten. Die Southern-Blot-Analyse der Transformanden zeigte stabile integrative Transformanten und nur wenige, die den Vektor autonom replizieren.

Diese Ergebnisse zeigen, dass MOs die Genexpression blockieren können. In Zukunft könnte diese Technik nützlich sein für die Untersuchung von ausgewählten Genen und der Modulation der Genexpression in Zygomyceten.

1. Introduction

1.1 Zygomycetes: an overview

The zygomycetes are heterothallic fungi that are named for their sexual reproductive structures, the zygospores. More common is the asexual reproduction. This occurs in most cases by the formation of numerous nonmotile, unicellular but multinucleate sporangiospores in uni- or multispored sporangia or merosporangia. The mature asexual spores are dispersed singly or with the intact sporangium through air currents, water droplets or foraging by small animals (Ingold 1978; Zoberi 1985).

Zygomycetes are mostly non-pathogenic, but as opportunists may affect those with low immunity and those prone to infections (Ribes et al. 2000). Some species, especially from the genera *Mucor*, *Rhizopus* and *Gilbertella* are responsible for economic damage due to food spoilage and post-harvest losses (Tako and Csernetics 2005). Commercially, various zygomycete species are used in biotransformation of steroids and to produce a variety of extracellular enzymes such as aspartate proteases, lipases, amylases and cellulases (Somkuti and Babel 1968; Somkuti 1974; Adams and Deploey 1976; Tonouchi et al. 1986) as well as other substances, e.g. the pharmaceutically used γ-linolenic acid (Barber 1988; Jantti et al. 1989). Equally interesting is their use as model organisms to study basic biological features as e.g. blue light sensing, dimorphism and developmental regulation via low-molecular signals, the trisporoids. Both facets are somewhat hampered by the still existing limitations to manipulative molecular biology, especially to recombinant DNA technology. Although successful transformation has been achieved in few species, e.g. *Absidia glauca* and *Mucor circinelloides*, a truly efficient gene transfer system has not been developed yet. The present study deals with a strategy to overcome these limitations.

1.2 Development and reproduction in zygomycetes

In heterothallic species, the sexual zygospores develop from the fusion of two hyphae, each belonging to one of the two mating types, + and −. Sexual reproduction begins, when two adjacent individuals of opposite mating types sense the sexual hormones or pheromones. The sexual pheromone pathway of *M. mucedo* and *Blakeslea trispora* has been well studied (e.g. Nieuwenhuis 1975; Bu'Lock et al. 1976; Schimek et al. 2005, reviewed by Gooday 1994; Schimek and Wöstemeyer 2006; Wöstemeyer and Schimek 2007; Schimek and Wöstemeyer 2009). The pheromones trigger the fungi to produce branch hyphae, which grow to contact the partner hypha and develop into gametangia at their tips. At the point of fusion the nuclei of the + and the − gametangium are mixed. In the gametangial fusion region the nuclei presumably pair and fuse. The multinucleate structure then develops into a zygosporangium, bearing the single-celled zygospore (Figure 1.1). In homothallic strains, e.g. *Zygorhynchus* sp. and *Mucor* sp., single spore isolates may give rise to zygospore-producing colonies because the zygospores develop between branches of the same individual (Blakeslee 1906).

The zygospores can remain dormant for months and can resist adverse conditions. The fate of most nuclei within the zygospore is still unclear, but meiosis takes place during germination, as was demonstrated genetically for *Phycomyces blakesleeanus* (Eslava et al. 1975). Therefore, diploidy is aberrant in this class of fungi and is seen only in the zygospore maturation stage (Figure 1.2). The progeny usually include a small proportion of uncustomary segregants that show signs of sexual stimulation without a partner. One of the resulting recombinant nuclei survives, and germinates to form a new haploid mycelium or sporangium.

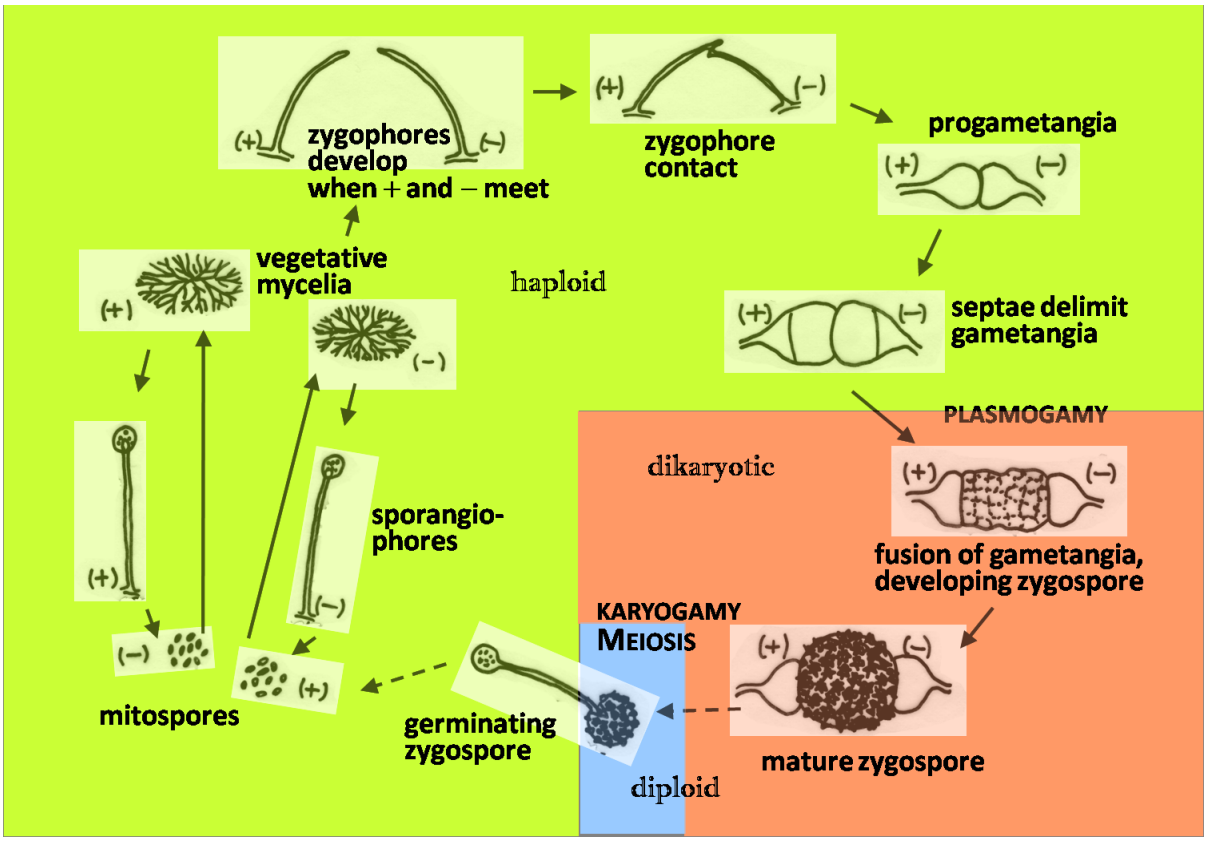

Figure 1.1 **Life cycle of *Mucor mucedo***. (by C. Schimek, with permission)

Compared to ascomycetes, zygomycetes are considerably more refractory to genetic analysis. The germination rate of zygomycete zygospores is very low, which severely hampers Mendelian genetics, as it is hard to obtain sufficient progeny for genetic studies. Studies are also complicated by the fact that in heterothallic species, there is generally no complete recovery of all meiotic products.

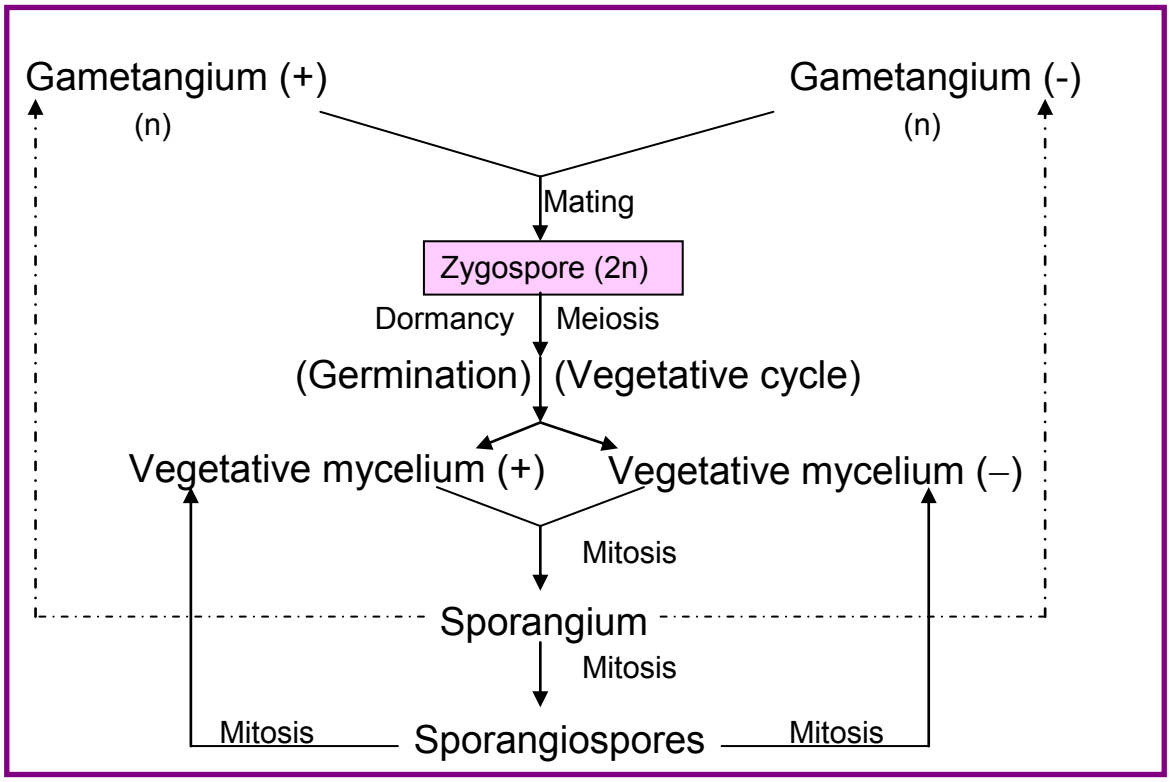

Figure 1.2 **Nuclear phases during the zygomycete developmental cycle.**

In general, zygomycetes, especially the Mucorales possess a coenocytic mycelium (Benny et al. 2001). During asexual reproduction, large numbers of aerial hyphae are produced from the substrate mycelium. Nuclei and cytoplasm are transported to the tips of these hyphae, which swell up and form rounded structures. The nuclei undergo repeated mitosis while the cytoplasm becomes increasingly fragmented. Around each nucleus or group of nuclei, as in many species the spores are multi-nucleated, some cytoplasm and organelles collect to form a pre-spore, which is subsequently separated from the next by a plasma membrane. Spore walls begin to form within the spaces created by the cytoplasmic cleavage. These walls are built by the fusion of Golgi vesicles containing cell wall monomers and enzymes with the spore membrane. Thus, a sporangium is formed, which, in some species, is separated by a new wall, the columella, from the lower part of the sporangium-bearing hypha.

Some zygomycetes show morphological dimorphism, which is best understood in *M. circinelloides* (syn. *M. racemosus*) and *M. rouxii*. Depending on a variety of growth conditions like carbon dioxide, oxygen, and hexose concentrations and several other factors, the normally filamentous fungus may switch to a stable yeast form which propagates by budding (Bartnicki-Garcia 1961; 1962a; Lübbehüsen et al. 2003) and shows a completely different composition of the cell wall (Bartnicki-Garcia and Nickerson 1962b; Bartnicki-Garcia and Lippman 1969). This feature makes *M. circinelloides* a rather interesting candidate for biotechnology. Yeast-like growth presents a huge advantage in fermentations, the biomass production is higher and the cells are more easily separated from the media. For that reason, development of strategies for genetic manipulation have somewhat concentrated on this species.

1.2.1 Trisporic acid

In Mucorales and other zygomycetes, the recognition between mating partners and the early sexual morphogenesis and development are regulated by trisporoids (Austin et al. 1969; Sutter 1970; Schimek et al. 2003). Trisporoids at the base of host recognition for the biotrophic fusion parasitism of *Parasitella parasitica* (Wöstemeyer et al. 2002; Schultze et al. 2005). Trisporoids are a family of oxidized, unsaturated C18 or C19 isoprenoid compounds acting as hormones or pheromones (van den Ende 1967; van den Ende 1968; van den Ende and Stegwee 1971). The name-giving compound, trisporic acid (TA; 1,1,3-trimethyl-2-(3'-methyloctyl)-cyclohexane) (Figure 1.3), was first isolated from cross cultures of *B. trispora* (Caglioti et al. 1966).

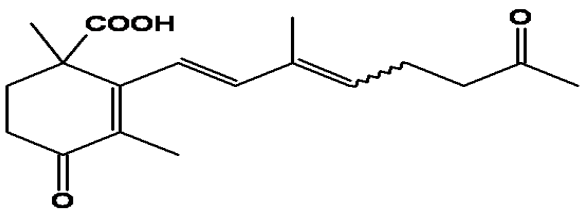

Figure 1.3 **Structure of trisporic acid B**

Trisporoids are biosynthetically derived from β-carotene and the oxidative cleavage of β-carotene by the product of the *TSP3* gene (Burmester et al. 2007) is the first committed step towards TA biosynthesis. From the last common intermediate, 4-dihydrotrisporin, the two mating types produce different trisporoid precursors which can be converted into trisporic acid as the final compound of the biosynthesis pathway only with the help of the complementary mating type (Figure 1.4) (Bu'Lock et al. 1972; Edwards et al. 1971; van den Ende et al. 1970). This was studied preferentially in *Mucor mucedo*, where 4-dihydrotrisporin is converted into trisporin in the − mating type and into 4-dihydromethyltrisporate in the + mating type. These two intermediates are then converted into trisporol and methyltrisporate, respectively, and subsequently into trisporic acid. Besides the carotene oxygenase, the two dehydrogenases involved in triporoid biosynthesis, 4-dihydrotrisporin dehydrogenase and 4-dihydromethyltrisporate dehydrogenase, have been identified and their genes and regulation characterized in detail (Werkman 1976; Schimek et al. 2005; Schultze et al. 2005; Wetzel et al. 2009). A somewhat differing model has been postulated on the same general background for *B. trispora* (Schachtschabel et al. 2008).

In *M. mucedo* and *P. blakesleeanus*, TA and its precursors induce the formation of the specialized contact hyphae for the mating process (van den Ende 1968; Edwards et al. 1971; Bu'Lock et al. 1972; Sutter 1970; Sutter et al. 1973; Sutter et al. 1996). Moreover, in *M. mucedo* and other zygomycetes, the contact region becomes bright yellow due to the accumulation of β-carotene when mycelia of the two mating types meet (Austin et al. 1969; Barnett et al. 1956; Ciegler et al. 1959, Kuzina and Cerda-Olmedo 2006). Similarly, intersexual heterokaryons show a sexual stimulation of carotenogenesis (Murillo and Cerda-Olmedo 1976; Govind and Cerda-Olmedo 1986). This indicates that trisporoids participate in the regulation of carotene production, and indeed, the same effect can be induced by direct application of TA or the precursor trisporoids. Trisporic acid therefore has a positive feed-back effect on its own production.

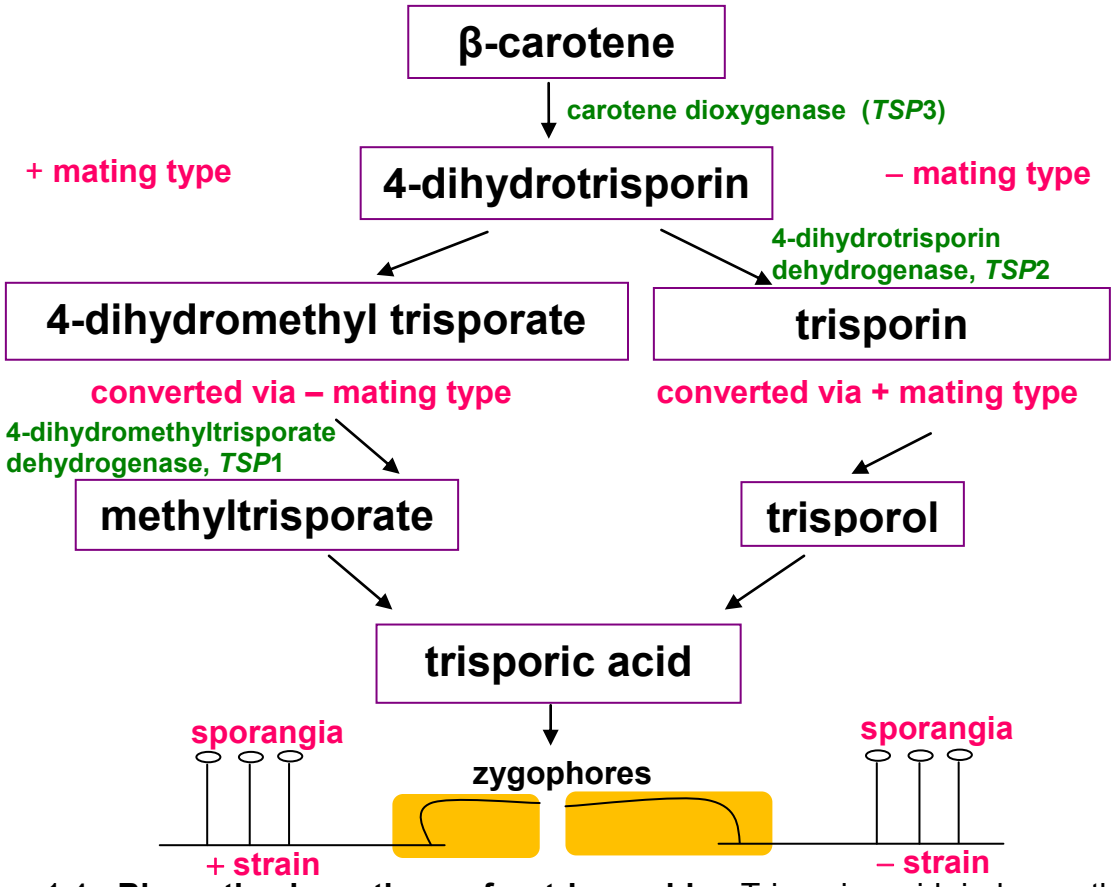

Figure 1.4: **Biosynthesis pathway for trisporoids.** Trisporic acid induces the formation of zygophores in both mating types of *M. mucedo*. green: known enzymes and genes of the pathway, yellow: mating induced-carotene formation.

1.2.2 Carotene biosynthesis in zygomycetes and regulation by *Crg*A

Carotenoids constitute one of the most widely distributed classes of naturally occurring organic pigments (Britton et al. 1995). They are lipid soluble, orange, red and yellow compounds that are found, besides in plants and algae, also in many fungi from all major phylogenetic groups. Within the zygomycetes, almost exclusively β-carotene is produced. Best studied with respect to carotene biosynthesis are *P. blakesleeanus*, *B. trispora* and *M. circinelloides*. In *P. blakesleeanus*, β-carotene is synthesized in the membranes of protein coated oil droplets, the lipid globules, and

most of the β-carotene is also found in these bodies, while a smaller portion occurs in a particulate vacuolar fraction (Ripley and Bramley 1976; 1982).

The biosynthesis pathway follows that known from other fungi, starting from 3-hydroxy-3-methylglutaryl-coenzyme A and mevalonate (e.g. Linden et al. 1997, Linnemanstöns et al. 2002). It comprises the tree enzymatic activities of phytoene synthase, phytoene dehydrogenase/desaturase, and lycopene cyclase, catalyzing the formation of phytoene from geranylgeranyl pyrophosphate, the conversion of phytoene into lycopene by the symmetric introduction of four double bonds, and finally the formation of the ionone rings at both ends of the lycopene molecule, yielding β- and γ-carotene.

In many fungi, carotenoid accumulation is increased by irradiation with blue light: Light induces an increased transcript accumulation of the carotene biosynthesis genes in *N. crassa* (Nelson et al. 1989; Schmidhauser et al. 1990; Schmidhauser et al. 1994), *M. circinelloides* (Velayos et al. 2000), and *P. blakesleeanus* (Ruiz-Hidalgo et al. 1997), indicating the participation of a light-regulated transcription factor. Carotene production in *Mucor*-like fungi is influenced additionally by sexual interactions, which are also to some extent light-regulated. A connection between the two regulatory pathways is therefore highly probable. The *CrgA* gene, which was first identified in *M. circinelloides*, codes for a negative regulator of light-induced carotene biosynthesis (Navarro et al. 2001). Its expression is also activated by light, indicating an as yet unknown regulatory pathway. Over-expression of *CrgA* disrupts the light-regulation and causes over-accumulation of β-carotene in dark- and light grown mycelia (Navarro et al. 2000). The putative product of *CrgA* presents several recognizable structural domains, namely a RING-finger zinc binding domain near the N-terminus, a putative nuclear localization signal, two stretches of acidic amino acids, Glutamine-rich regions, and a putative isoprenylation motif at the C-terminus, all in accordance with its function as transcriptional regulator. At least two of the domains, the RING-finger domain and one of the glutamine-rich regions, are essential for the accurate light-regulation of carotenogenesis (Lorca-Pascual et al.

2004). The *CrgA* gene has also been identified in *B. trispora* (Quiles-Rosillo et al. 2005).

1.3 Transformation in fungi

Integration of vector elements into chromosomal DNA in natural and manipulated gene transfer in most cases occurs by recombination at homologous or heterologous sites of the genome (Ballance et al. 1983; Case et al. 1979). According to Hinnen et al. (1978), three different types of such integration events can be distinguished: in type I, a single cross-over between the transformed and the chromosomal sequences results in integration of the cloned sequence adjacent to the recipient gene. Such transformations generally show a higher mitotic stability over other integration types, even in absence of selective pressure (Hynes 1986; Rambosek and Leach 1987). Type II describes the integration of the transformed element at any other site within the genome. Gene conversion or double crossover events, where the cloned sequence replaces the homologous recipient gene, are classified as type III events. The frequency of any successful transformation event depends on the organism, the strain, or even the different types of plasmids (Ballance 1986; Yelton et al. 1984). An important factor is normally the extent of homology between the transformation vector and the recipient genome (Cullen et al. 1988).

Transformation of a fungus was first reported for *N. crassa* where the requirement for inositol in inositol-deficient mutants was cured with the help of wild type DNA (Mishra et al. 1973; Mishra and Tatum 1973). However, these results were not unequivocal, as the mutant was thought to display an altered porosity of the cell membrane as result of the inositol starvation, leading to facilitated uptake of DNA. The proof for the successful transformation of an eukaryote was present several years later with the transformation of a *Leu2* mutant of *Saccharomyces cerevisiae* with wild type DNA, reinstating leucine-independence in the transformed strain (Hinnen et al. 1978). Later, transformation was also extended to filamentous fungi, e.g. *Aspergillus nidulans* (Tilburn et al. 1983) and several others. But until today the

technique is not applicable to all species, and every newly approached species requires sometimes arduous optimization of strategies and protocols.

1.3.1 Protoplasting as prerequisite for transformation

Intra- and Interspecific protoplast fusion techniques are widely used for the introduction of novel genetic traits (Gadau 1992) as they help to overcome many natural barriers to cross-breeding in fungi. For most fungal transformation systems, the treatment of the recipient prior to the transformation event proved to be a crucial step, as the cell wall otherwise presents an insurmountable barrier to the DNA molecules. Hutchinson and Hartwell (1967) prepared the way by describing a technique to prepare protoplasts from *Saccharomyces cerevisiae* and stabilizing them with sorbitol. Protoplasting is generally done by partial digestion of the cell walls with enzyme preparations obtained from other microorganisms, which are chosen for their specific hydrolytic activities towards the diverse cell wall components, as e.g. chitin and 1,3-glucans. A number of frequently used enzyme mixtures are Helicase (Beggs 1978), Glusulase (Hinnen et al. 1978), and Zymolyase (Hsiao and Carbon 1979). Another one, Novozyme 234, was prepared from *Trichoderma viride* and used to prepare protoplasts from *Schizosacharomyces pombe* (Beach and Nurse 1981).

Protoplast preparation is comparatively easier in yeasts than in filamentous fungi. In the latter, the protoplasts are prepared from various cell types. In *Neurospora*, the protoplasts are prepared preferentially from macroconidia (Rossier et al. 1985) or, alternatively, from young mycelium (Buxton and Radford 1984). In *Aspergillus* and *Penicillium* species both mycelium and germinating conidia are used (Ballance and Turner 1985). In basidiomycetes, basidiospores, dikaryotic mycelium and oidia are the possible sources (Binninger et al. 1987; Munoz-Rivas et al. 1986), while for zygomycetes usually germlings, very young mycelia, are used (Burmester 1992; Schilde et al. 2002; van Heeswijck 1984; Wolff and Arnau 2002), as the spores are very resistant to enzyme attack (Jones et al. 1968). By protoplast fusion between

amino acid auxotrophic + and − mutant strains of *A. glauca,* Wöstemeyer and Brockhausen-Rohdemann (1987) could show that both mating type-specific rRNA gene clusters were maintained in the fusion strains.

1.3.2 Transformation and genetic manipulation systems for zygomycetes

In zygomycete fungi, genetic manipulation has been applied since the early 1980's with considerable effort and varying techniques. All attempts were insofar successful, as the introduction of foreign DNA into the organisms was never a real problem. The different techniques used to deliver the vectors also seem to have only little effect on the transformation efficiency. In $CaCl_2$-PEG-mediated incorporation (van Heeswijck and Roncero 1984; Revuelta and Jayaram 1986; Wöstemeyer et al. 1987; Yanai et al. 1990), the DNA binds to the protoplast membrane pretreated with PEG in order to manipulate the membrane properties and thus facilitate endocytosis (Kawai et al. 2010). In biolistic transformation, DNA is delivered by bombardment with plasmid-coated metal particles (Bartsch et. al 2002; Gonzalez-Hernandez at al. 1997; Skory 2002). In electroporation, the permeability and organization of the cell membrane is transiently altered by an electric pulse and macromolecules may be incorporated into the cell during the reorganization process (Burmester et al. 1990; Gutierrez et al. 2011). Agrobacterium-mediated transformation uses the bacterial conjugation system for vector transfer (Michielse et al. 2004, Wei et al. 2010).

In contrast, establishing mitotically stable, efficient, and integrative mutations is still a problem. Over time, a number of techniques and systems have been proposed and optimized for the specific research or biotechnological purposes.

The transformed vector plasmids are usually maintained by autonomous replication within the recipient (Benito et al. 1992; Burmester and Wöstemeyer 1987; Horiuchi et al. 1995; Iturriaga et al. 1992; Liou et al. 1992; Revuelta and Jayaram 1986; Roncero et al. 1989; Takaya et al. 1996; van Heeswijck 1986; Wöstemeyer et al. 1987). This

is due to the presence of ARS sequences, first described in yeast (Struhl et al. 1979), which allow for chromosome independent replication of vector DNA. Functional ARS elements have been identified in *M. circinelloides* (Roncero et al. 1989) and *A. glauca* (Burmester and Wöstemeyer 1987) and have been used in several transformation systems.

The transformation of a leucine auxotrophic mutant of *M. circinelloides* with the corresponding functional gene present on the yeast *E. coli* - *Mucor* shuttle vector pMcLt302 was the first report on direct cloning of a gene from a filamentous fungus by positive selection for the corresponding prototrophic phenotype (van Heeswijck and Roncero 1984). Other approaches based on the complementation of auxotrophic markers, have been worked out for *M. circinelloides* (Acs et al. 2002; Anaya and Roncero 1991; Ruiz-Diez 2002).

Transformation using a dominantly selectable marker gene such as the neomycin resistance gene were successful in *A. glauca* (Wöstemeyer et al. 1987), where plasmid pAmN61 containing the NPT II neomycin phosphotransferase structural gene fused to the N-terminal region of a homologous actin gene was transformed into *A. glauca* protoplasts. Neomycin resistant transformants were selected on neomycin containing complete medium. Southern blot analysis showed that the pAmN61 DNA was autonomously replicated in *A. glauca*.

Chromosomal integration could be enforced by the combination of the genes to be introduced and selective pressure on chosen mutants, as in the transformation of *M. circinelloides* with the plasmid pTL42. Different integrative events derived from either homologous or heterologous recombination or a recombination by gene replacement were observed in this case (Arnau et al. 1991; Arnau and Stroman 1993). The inclusion of repetitive DNA elements into an autonomously replicating plasmid also resulted in chromosomal integration in *A. glauca*, but these integration events were often associated with rearrangements of the introduced DNA and with the appearance of mutant phenotypes (Burmester et al. 1990). Similar effects were noted by Skory in *Rhizopus oryzae* (2002). The inclusion of the *SEG1* element to the

introduced DNA increased the mitotic stability of *A. glauca* transformants, resulting in mitotic stabilization of the autonomously replicating DNA even under non-selective conditions without chromosomal integration (Burmester et al. 1992).

Stable integrative transformants were obtained with the *Agrobacterium*-mediated transformation of *pyr4* into an uracil auxotroph strain of *Rhizopus oryzae* (Michielse et al. 2004), where also the advantage of an auxotrophy over a dominant selection marker was shown. Nevertheless, all integrations occurred at the same locus, targeting was not possible.

The original difficulties in transforming zygomycete fungi due to inefficient integration or truncation of the introduced DNA (Michielse et al. 2004), or caused by low mitotic stability of the transformants (Arnau and Stroman 1993; Burmester 1992; Benito et al. 1995; Gonzalez-Hernandez et al. 1997; Horiuchi et al. 1995; Suarez and Eslava 1988) suggested that zygomycetes have specific mechanisms for the detection and elimination of foreign DNA. The resulting problems have been mostly overcome by the construction of suitable vectors and recipient strains, and several other tools to functional genetic analysis were developed on that base. Constructs combining the green fluorescent protein to homologous promoters constituted the first reporter system that allowed the monitoring of gene expression in a zygomycete, *A. glauca* (Schilde et al. 2001).

Targeted replacement of genes by homologous recombination and thus the possibilities for creating either true knockout mutants or inserting mutations at will and at any desired locus to study the function of genes and gene products still poses a problem. The possibility of RNA interference (RNAi) to modulate gene function is one of the more recent advances in zygomycete research and was first introduced for *M. circinelloides* (Nicolas et al. 2003), and a dicer-like gene could be identified later in this species (Nicolas et al. 2007). In ascomycetes, similar problems with low frequency of homologous integration events were successfully overcome using strains impaired in one of the genes involved in the non-homologous end joining pathway (Krappmann 2007).

1.3.3 The *ku* gene and its function in stable transformation

Other adverse effects on transformation efficiency come from intracellular and intranuclear defence systems directed against foreign DNA as well as endogeneous repair systems involved in the maintenance of genome integrity throughout the cell cycle. One possible hazard are DNA double strand breaks. These have to be repaired fast to prevent chromosomal rearrangements or mutations that might ultimately result in cell death. The double strand breaks in eukaryotes are repaired by two different recombination pathways, namely homologous recombination (HR) and non-homologous end joining (NHEJ). By HR, the double strand breaks are repaired by retrieving genetic information from undamaged homologous sequences, while the NHEJ pathway involves direct ligation of the strands and does not rely on homologous recombination (Pastwa and Blasiak 2003). The direct ligation of DNA strands in the NHEJ process is mediated by the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), the DNA ligase IV-XRCC4 complex, and the Ku70-Ku80 heterodimer (Ochi et al. 2010). The latter first binds to the broken DNA ends before the rest of the multi-protein repair complex is recruited. Nucleases and the DNA-PKcs are then active in joining the DNA fragments before the DNA ligase complex finally seals the break. The Ku-complex is also required for somatic recombination to promote antigen diversity in the mammalian immune system by utilizing the NHEJ pathway, and for telomere length maintenance and subtelomeric gene silencing (Boulton and Jackson 1998).

A considerable increase in gene targeting frequency in transformations was reported for mutants defective in one of the *ku* genes in various fungi: *Neurospora crassa* (Ninomiya et al. 2004), *Aspergillus fumigatus* (da Silva Ferreira et al. 2006; Krappmann et al. 2006), *Aspergillus nidulans* (Nayak et al. 2006), *Aspergillus sojae* and *Aspergillus oryzae* (Takahashi et al. 2006a; 2006b), *Aspergillus niger* (Meyer et al. 2007), *Aspergillus parasiticus* (Chang 2008), *Botrytis cinerea* (Choquer et al. 2008), *Claviceps purpurea* (Haarmann et al. 2008), *Hypocrea jecorina* (Guangtao et al. 2009), *Penicillium chrysogenum* (Snoek et al. 2009; Hoff et al. 2010), *Sclerotinia*

sclerotiorum (Levy et al. 2008), *Sordaria macrospora* (Pöggeler and Kück 2006), *Cryptococcus neoformans* (Goins et al. 2006), *Trichoderma virens* (Valentina et al. 2011), and *Trichophyton mentagrophytes* (Yamada et al. 2009), indicating that *ku* disruption strains are efficient recipients for gene targeting.

1.4 Gene knockdown agents

In another approach to the manipulation of gene expression, the gene of interest is neither to be knocked out nor replaced with a mutated version. Instead, gene expression is sought to be manipulated by interfering with the stability or processing of the transcript. This may be the technique of choice when gene targeting is not possible or inefficient. Knockdown or gene silencing strategies usually causes only partial loss of gene expression, but this may also be advantageous, as also the effects of essential genes on a given phenotype may be studied. In other systems, even partial loss will help to obtain new information on the function of the gene of interest (Nakayashiki and Nguyen, 2008).

As general property, gene knockdown agents should have high sequence specificity to the target sequence. They also should lack off-target effects and non-antisense effects due to interactions with structures other than gene transcripts. The major knockdown agents fulfilling these requirements are phosphorothioate-linked DNA (S-DNA), small interfering RNA, and Morpholino oligonucleotides, the latter two shall be compared below.

1.4.1 RNA interference by small interfering RNAs (siRNA)

Naturally, RNA interference (RNAi) is a way to cope with the presence of cytoplasmatic double stranded RNA (dsRNA), which may be generated by a number of mechanisms. RNAi also takes place in the cytoplasm, where the dsRNAs are processed into smaller units, among them the siRNA with 21 – 25 bp in length, and a 2-nucleotide overhang at the 3'-end (Meister and Tuschl 2004). RNAi mechanisms

can also be initiated by incorporating suitable manufactured dsRNAs into the cell and have thus become a valuable tool in genetic research. One of the processing enzymes is the ATP-dependent dicer, a RNAse specific for dsRNA containing two RNAse III motifs (Dorsett and Tuschl 2004). The short dsRNA fragments are bound by effectors, which mediate the transfer of a single strand to one of the protein complexes responsible for the specificity of the further silencing mechanism: siRNA binds to the RNA-induced-silencing-complex RISC that catalyzes the degradation of specific mRNA. The bound strand of siRNA serves as template and hybridizes to the mRNA to be degraded. The hybrid dsRNA is then cleaved in the middle of the fragment and the gene expression thus stopped (Dorsett and Tuschl 2004; Scherer and Rossi 2004). siRNAs are the RNAi approach mostly used in fungi. Small RNA fragments of sizes differing from siRNA are processed by other mechanisms, binding to either the protein complex for RNA-induced initiation of transcriptional gene silencing RITS, which is involved in sequence-specific methylation of chromatin (Verdel et al. 2004) or the microribonucleoprotein complex miRNP, which inhibits translation of the complementary mRNA at the elongation- or termination step (Nelson et al. 2004, Meister and Tuschl 2004).

1.4.2 Morpholino oligonucleotides (MO)

MO are water-soluble, synthetic molecules that are the product of a redesign of the natural nucleic acid structure (Summerton and Weller 1997). Structurally, the difference between DNA and MO (Figure 1.5) is that while in DNA the deoxyribose rings are linked by anionic phosphodiester bonds, MOs have the standard nucleic acid bases, but contain morpholine rings instead of deoxyribose and are linked through uncharged phosphorodiamidate groups (Summerton et al. 1997) which renders the complete molecule uncharged in the physiological pH range and helps to minimize non-specific interactions inside the cell (Heasman et al. 2002). MOs hybridize to RNA or single-stranded DNA by conventional base-pairing but are immune to degradation by nucleases (Hudziak et al. 1996). They are therefore much more stable than any oligonucleotide used for interference strategies and have

become a powerful and widely used tool in knockdown experiments to analyze gene function, especially for higher eukaryotes like mice, frogs, sea urchin, and zebra fish (Heasman 2002). Compared to siRNA, MO provide some additional advantages, as they may be used on DNA and RNA, and are also applicable for manipulations within the nucleus.

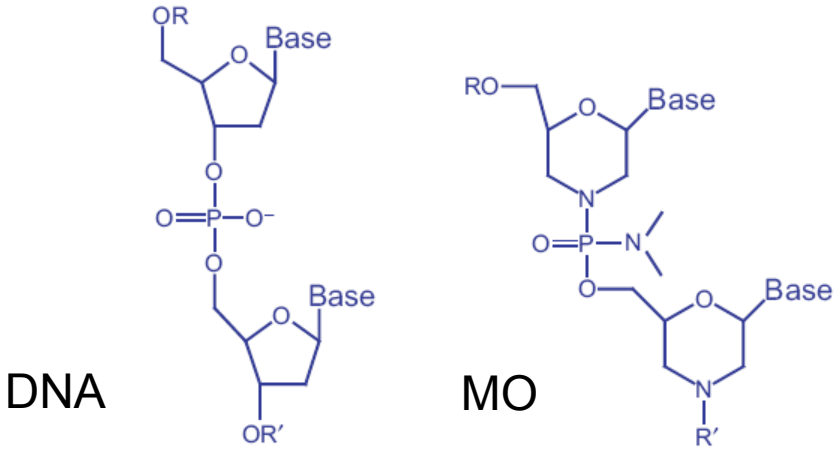

Figure 1.5: **Backbone structures of DNA and MO.** R and R' denote the continuation of the oligomer chain in the 5' and 3' directions, respectively (adapted and modified from Corey and Abrams 2001).

MO can be used for manipulating expression of any given gene for which the sequence is known. Usually they are synthesized to a length of about 25 bases and then bind to the 5'-untranslated region (UTR) of the complementary mRNA. They interfere with the progression of the ribosomal initiation complex from the 5' cap to the start codon and thus prevent translation of the targeted transcript (Figure 1.6) (Summerton 1999). They may also be used to block translation elongation of to modify the splicing of pre-mRNA and so help to study the effect of alterations to specific exons (Draper et al. 2001).

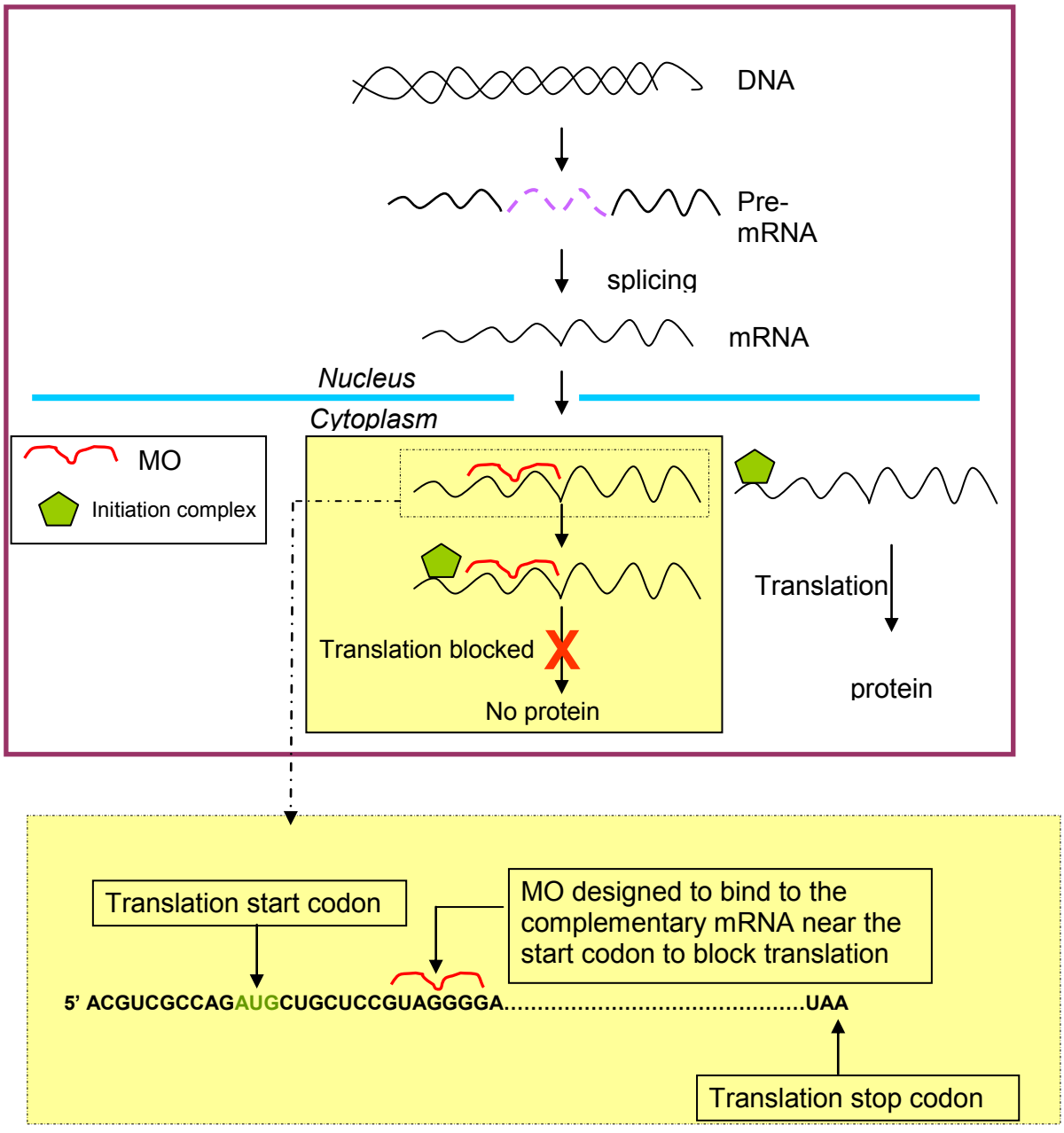

Figure 1.6: **Mode of action of morpholino oligonucleotides (MO) in blocking translation.**

MO are most commonly applied as single strands but may also be used in form of heteroduplexes with DNA (Draper et al. 2001). They can be delivered into cells or tissues at various developmental stages by either microinjection (Rosen et al. 2009), electroporation (Cerda et al. 2006; Jubin 2005), endocytosis facilitated by the Endo-Porter peptide (Morcos 2001), or scrape loading (Partridge et al. 1996).

1.5 Aim of the research project

The aim of the present study was to apply antisense morpholino-oligonucleotides as tool to enhance the frequency of integrative transformation in zygomycetes. The rate of stable genetic manipulations in zygomycetes is generally low, in almost all attempts at transformation, the transformed vectors are autonomously replicated within the transformants. Establishing ectopic as well as homologous integrations usually require analysis of hundreds of transformants, severely impairing manipulative molecular genetical approaches in this biotechnologically important group of fungi.

Morpholino oligonucleotides, mimicking natural nucleic acid structure but with a backbone non-degradable by nucleases, have been introduced as a genetic tool for knockdown of genes and other post-transcriptional modifications in higher eukaryotes but not yet in fungi.

To prove their applicability in zygomycetes, in a first round of experiments their effect on the down-regulation of a repressor of carotene biosynthesis, *crgA*, in *Mucor mucedo* was to be analyzed. *M. mucedo* was used for this attempt, as in this species the carotene production can be triggered under experimental conditions with the zygomycete sexual signal molecule, trisporic acid. Before that, the *crgA* gene of *M. mucedo* needed to be identified, based on sequence information for the corresponding gene in *M. circinelloides*, and a protocol for protoplasting *M. mucedo* had to be optimized. The *M. mucedo crgA* sequence was then used to design a suitable antisense MO to block translation of the *crgA* transcript.

After proving the knockdown effect of antisense MOs in *M. mucedo*, this technique should be used to modify the transformation efficiency for zygomycetes. Successful transformation has never been reported for *M. mucedo*, but for the related species, *M. circinelloides*, several well-defined transformation systems exist. For the intended experiment, the leucine auxotrophic mutant R7B was chosen, and was to be transformed with the plasmid pEUKA400 containing a copy of the homologous

functional *LeuA* gene. The *ku70 gene*, involved in the NHEJ-pathway of DNA double strand break repair, was chosen as target for the MO approach. Down-regulation of *ku70* was found to increase integrative transformation in many other fungi. An antisense *ku70* MO should therefore be introduced together with the plasmid and its effect of the transformation efficiency should be analyzed.

2. Materials and Methods

2.1 Culture strains and plasmid

In the present study, strains of *M. mucedo* and *M. circinelloides* were used to show the effects of morpholino oligonucleotides on gene expression. The leucine auxotrophic mutant *M. circinelloides* R7B used for the Ku70 experiments was a gift from José Arnau (Copenhagen, Denmark). This strain was originally derived of *M. circinelloides* CBS 277.49 (Appel et al. 2004). Trisporic acid for the sexual induction of carotene production in *M. mucedo* was purified from mated cultures of *B. trispora*. The + and – mating type strains of *M. mucedo* and *B. trispora* were gifts from H. van den Ende (Amsterdam, The Netherlands). The *Streptomyces* No. 6 strain, which was used for the preparation of cell-wall lytic enzyme, was a gift from T. Beppu (Tokyo). *E. coli* XL 1-Blue strains (Stratagene) were used for transformation and plasmid preparation (Bullock et al. 1987). Table 2.1 lists all fungal and bacterial strains used in this work. The vector pDrive (Qiagen, Hilden, Germany) was used for the cloning experiments. The vector pEUKA400 was also a gift from José Arnau (Copenhagen, Denmark).

Table 2.1: **Fungal and bacterial strains used in this research**

Species/Strain	**Mating type**	**Culture Collection Number**	
		FSU°	**CBS*/ ATCC"**
Mucor mucedo	+	FSU 621	CBS 144.24
Mucor mucedo	–	FSU 620	CBS 109.16
Blakeslea trispora	+	FSU 331	CBS 130.49
Blakeslea trispora	–	FSU 332	CBS 131.49
Mucor circinelloides R7B *leu*$^-$	–	FSU 623	ATCC 90680
Mucor circinelloides wt	–	FSU 5860	CBS 277.49
Streptomyces sp. No.6		-	-
Escherichia coli XL *1-Blue*			

° FSU – Fungal Reference Centre at the Friedrich-Schiller-University Jena
* CBS – Centraalbureau foor Schimmelcultures, Utrecht, The Netherlands
" ATCC – American Type Culture Collection, Tedington, UK

2.2 Biochemicals

The restriction enzymes were from Fermentas (St. Leon-Rot) and New England Biolabs (Frankfurt/Main). The DNA ligase and the 1 kb size markers were from Fermentas (St. Leon-Rot). The *Taq* polymerase was obtained from Invitek (Berlin). The primers used in this work were synthesized by Biomers (Ulm).

2.3 Media and cultivation

2.3.1 Media

For the cultivation of *M. mucedo, M. circinelloides,* and *B. trispora* different media were used depending on which experiments were performed. For growth in liquid culture and for the isolation of spores on solid medium supplemented minimal medium (SUP) after Wöstemeyer (1985) was used. This medium contains 50 mM glucose monohydrate, 20 mM ammonium chloride, 30 mM potassium dihydrogen phosphate, 5 mM di-potassium hydrogen phosphate, 1 mM magnesium sulfate heptahydrate, 0.5 g / 100 mL yeast extract, and for solid medium 1.2 g / 100 mL agar. For the regeneration of protoplasts after electroporation, liquid induction medium (Schimek et al. 2005) consisting of 55 mM maltose, 100 mM potassium nitrate, 37 mM potassium dihydrogen phosphate, 10 mM magnesium sulfate heptahydrate, and 0.1 g / 100 mL yeast extract was used. For the cultivation of *Streptomyces* No.6, SUP medium was used.

For plating of the *M. circinelloides* auxotrophic mutants after transformation, minimal medium with 0.6 M sorbitol and with and without leucine was used. This medium contains 50 mM glucose monohydrate, 20 mM ammonium chloride, 30 mM potassium dihydrogen phosphate, 5 mM di-potassium hydrogen phosphate, 1 mM magnesium sulfate heptahydrate, 0.6 M sorbitol and for solid medium 1.5 g / 100 mL agar. Leucine was added to the final concentration of 3.0 mg / 100 mL from a stock solution containing 360 mg / 100 mL.

E. coli XL-1 Blue was used for transformation and plasmid preparation and was cultivated in Luria Bertani (LB) medium (Bertani 1951). The liquid transformation

medium contained 1 g / 100 mL tryptone, 0.5 g / 100 mL yeast extract, and 170 mM NaCl plus 1.5 g / 100 mL agar for solid medium and was supplemented with 50 µl 100 mM IPTG, 100 µl of 20 mg X-Gal / mL and 50 µg / mL ampicillin.

2.3.2 Culture maintenance

For culture maintenance, single spore isolates of the zygomycetes were allowed to sporulate on supplemented minimal medium. The resulting spores were harvested into 20 % glycerol, aliquoted and stored at - 20 °C. The *E. coli* strains were maintained on M9 medium (Sambrook and Russell 2001) containing 0.6 g / 100 mL di-sodium hydrogen phosphate, 0.3 g / 100 mL potassium dihydrogen phosphate, 0.05 g / 100 mL sodium chloride, 0.1 g / 100 mL ammonium chloride, and 1.5 g / 100 mL agar. After autoclaving, the medium was cooled to around 50 °C and 2 ml 1M $MgSO_4$, 0.1 ml 1M $CaCl_2$, 10 ml 20 % glucose, 1 ml 1M Thiamine-HCl where added per litre prior to pouring into Petri dishes. For *E. coli* XL1-Blue, 20 µg / mL of tetracycline was added. In addition, *E. coli* was kept as glycerol stocks in 16 % glycerol, 1 g / 100 mL tryptone, 0.5 g / 100 mL yeast extract and 170 mM NaCl) at -20 °C. *Streptomyces* spores were harvested into 20 % glycerol, aliquoted and stored at -20 °C.

2.4 Isolation of genomic DNA from *M. mucedo* and *M. circinelloides*

For use in polymerase chain reaction (PCR), genomic DNA isolated over a cesium chloride gradient was used (Schilde et al. 2001; see 2.4.3). From the transformant strains, DNA was isolated using a faster DNA isolation protocol (see 2.4.2).

2.4.1 Cultivation in liquid medium

For the isolation of genomic DNA from *M. mucedo* and *M. circinelloides*, volumes of 100 mL of SUP liquid medium were inoculated with 1 million spores each.

For the transformants, selective minimal medium (MM) was used and inoculated with agar blocks. The cultures were incubated at 20 °C on a shaker at 120 rpm for 3 - 5 days under natural light conditions. The mycelium was harvested by filtration using a Buchner funnel connected to a water jet pump. The mycelium was transferred to a pre-cooled mortar, where it was ground into a fine powder in liquid nitrogen. The mycelial powder was stored at - 80 °C till further processing.

2.4.2 Fast isolation of genomic DNA

The mycelial powder from one flask was gently mixed with 5 mL of buffer containing 150 mM EDTA, 50 mM Tris-Cl pH 8.0, 20 mM sodium chloride, and 1 g / 100 mL SDS and incubated at 70 °C for one hour in a water bath. One fourth volume of 4 M sodium chloride was added to the samples, mixed gently and incubated on ice for 30 minutes and then centrifuged for 10 minutes at 4000 rpm at 5 °C. The supernatant was transferred to a new Greiner tube and solid PEG 6000 (Serva) at a ratio of 100 mg / 1 mL supernatant was added and mixed gently at room temperature until the PEG was completely dissolved and then incubated on ice for 1 hour. The samples were then centrifuged for 10 minutes at 4000 rpm at 5° C. The supernatant was removed carefully and the pellet was dissolved in 0.8 mL of 0.3 M sodium acetate, 50 mM Tris-Cl, pH 8.0. The solution was then transferred to an Eppendorf tube. 50 µl of heat treated RNaseA (1 mg / mL) was added and incubated at room temperature for 20 minutes. Then, 0.4 mL of trichloromethane was added and mixed gently and centrifuged at 12000 rpm for one minute. The upper phase was transferred to a new Eppendorf tube and one volume of 2-propanol was added and stored overnight at – 20 °C. The next day, the samples were centrifuged first for 5 minutes at 12000 rpm, then the supernatant was removed and 1 mL of ice cold 70 % ethanol was added to the pellet. This was then centrifuged again at 12000 rpm for 2 minutes and the supernatant was removed. The pellets were dried in a vacuum centrifuge for 5 minutes and then dissolved in 40 µl sterile water each. The DNA amount and quality was checked by agarose gel electrophoresis.

2.4.3 Isolation of genomic DNA on a cesium chloride gradient

The mycelium powder from one flask was gently mixed with 40 mL of buffer containing 150 mM EDTA, 50 mM Tris-Cl pH 8.0, 20 mM sodium chloride, 1 g / 100 mL SDS and 100 µg / mL Pronase E solution and incubated for one hour at 55 °C. 0.58 g of sodium chloride were added per each 10 mL of that solution and incubated on ice for 30 minutes. To remove the remaining cell components, the suspension was centrifuged at 6,000 g at 4 °C for 10 minutes (Sorvall RC6, rotor: SLA-1500). To precipitate the DNA, the supernatant was mixed with 10 g / 100 mL polyethylene glycol 6000 and incubated for 1 hour on ice and then centrifuged at 12 000 g at 4 °C for 20 minutes (Sorvall RC6, rotor SS-34). The supernatant was discarded, the precipitate was dissolved in 15 mL 25 mM Tris-Cl pH 8.0, 5 mM EDTA, 10 mM sodium chloride and 1 % Triton X - 100. After addition of 1 g / mL cesium chloride and 20 µg / mL ethidium bromide for visualisation to the mixture, it was centrifuged for 24 hours at 183 000 g at 25 °C (Sorvall UltraPro 80, rotor: TV865B). The genomic DNA was then removed with a syringe. To remove the ethidium bromide, genomic DNA was repeatedly washed with anhydrous 2-propanol. To remove the cesium chloride, the DNA was dialysed three times for 12 hours against 1 L of 10 mM Tris-Cl pH 8.0, 1 mM EDTA at 4 °C.

2.4.4 Electrophoretic separation of DNA

Agarose gel electrophoresis allows the separation of DNA molecules according to their size. Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight.

For electrophoresis, gels containing either 1 or 1.5 g / 100 mL agarose were used. The DNA samples were mixed with 5 µl of loading dye: 50 % glycerol, 10 mM EDTA, 0.02 g / 100 mL Bromophenol blue and 0.02 g / 100 mL Xylene xylol. The separation was carried out in 40 mM Tris, 20 mM sodium acetate and 1 mM EDTA, pH 7.8 or in 89 mM Tris, 89 mM boric acid and 2.5 mM EDTA, pH 8.3 buffer. The field strength was 2.5 V / cm. To determine the size of DNA fragments, a size marker (1 kb-ladder or 1 kb-plus, Fermentas) was used. The

gels were stained in a solution of 0.01 g / mL ethidium bromide in water for 30 minutes and then visualised by UV illumination (Spectroline ® Model TL-312A, 312 nm).

2.4.5 Estimation of DNA purity from absorbance measurements

The absorption of the diluted DNA was measured at 260 nm with a UV-Vis spectrophotometer (Jasco V-560). To estimate the DNA concentration the following formula was used:

DNA (g / mL) = Absorption 260 nm • 50 • dilution factor

In order to check the purity of the isolated nucleic acids, the absorption at 280 nm was also measured. The quotient of 260 nm to 280 nm should be from 1.8 to 2.0 for pure nucleic acids (Sambrook et al. 1989).

2.5 Extraction of trisporic acid (TA) and determination of the concentration

2.5.1 Extraction of TA

For sexual stimulation in *M. mucedo* + and – strains, TA enriched from mated cultures of *B. trispora* + and – was used. The isolation of TA was carried out following the Schimek et al. (2003) method:

100 mL of SUP broth were inoculated with small mycelial agar blocks (0.5 x 0.5 cm) of *B. trispora* + or – grown on solid SUP medium and the cultures were grown under natural light conditions at 120 rpm and 20 °C on a shaker. After three days the mycelia of one flask each of B. *trispora* + and – were strained through a sterile tea strainer, and then washed with 100 mL of maltose solution (55 mM maltose and 0.9 mM ammonium dihydrogen phosphate). The combined mycelium was then transferred into a new culture flask containing 100 mL of maltose solution and incubated in darkness at 20 °C and 120 rpm on a shaker. After 5 - 6 days, the mycelium was removed using a Büchner funnel and the filtrate was collected. The pH of the filtrate was adjusted to 8.0 using 10 M sodium hydroxide. Subsequently, the filtrate was extracted with 1/2 volume of

trichloromethane: 2 - propanol, 100 : 5, in a separatory funnel. The neutral trisporoids accumulate in the organic (lower) phase, which was collected. The watery phase was then adjusted with 32 % hydrochloric acid to pH 2.0 and extracted again to obtain the TA. Both organic extracts were dried over water-free sodium sulfate. The following day, the two extracts were concentrated by evaporating the solvent at 340 mbar and 50 °C in a rotary vacuum evaporator (Heidolph WB 2001 or 4003 - Heidolph Laborota digital). The TA was dissolved in pure ethanol and stored at - 20 °C in brown glass bottles. All work was conducted in a darkened room as trisporoids are sensitive to light.

2.5.2 Determination of the concentration of TA

Concentration of the TA from *B. trispora* was determined on the basis of the absorbance at the absorbance maximum (325 nm in ethanol; Sutter and Whitaker 1981). To calculate the concentration of the TA, the following formula was used:
c (mg / mL) = dilution factor • Eλ • 10 mg / mL • ελ

c = concentration, E = extinction at wavelenght λ, ελ = specific extinction coefficient.

The concentration was determined using the specific extinction coefficient E_{325nm} = 575 (Sutter and Whitaker 1981).

2.6 Effect of concentration of TA on the production of β-carotene in *M. mucedo* FSU 621 + and FSU 620 –.

Spores of *M. mucedo* FSU 621 + and FSU 620 − were harvested by rinsing the mature cultures with distilled water. 2 mL reagent tube cultures in liquid induction medium were inoculated with 10^3 spores each. The cultures were incubated at 20 °C on a roller in the dark. To study the effect of concentration of TA on β-carotene synthesis in *M. mucedo* + and (−) strains, various amounts of TA were added to the cultures on the 2nd day. For each series, 0.2 µg, 2 µg and 20 µg TA in a volume of 20 µl / culture was used. On the next day, the cultures were passed through a Whatman No.1 filter paper to remove the medium and to make the

mycelium as dry as possible prior to extraction. The wet weight of the mycelia was determined for each strain. Two different controls were maintained, namely, one with the addition of only the solvent ethanol and the other without the addition of either ethanol or TA.

2.7 β-Carotene analysis

2.7.1 Extraction and estimation of β-carotene

β-Carotene was extracted from the mycelium following the procedure described by Govind and Cerda-Olmedo (1986) but using methanol as extraction solvent. The concentration of β-carotene in the extracts was determined based on the absorbance at the absorbance maximum. The purity of the β-carotene was assessed based on the full absorbance spectra in the range from 350 to 500 nm recorded on a Jasco V-560 UV/VIS Spectrophotometer (Jasco, Germany).

Concentration of β-carotene was evaluated from the peak at 448 nm, using the molar extinction coefficient of β-carotene in ethanol at 453 nm, $141x10^3$ M^{-1} cm^{-1} (http://epic.awi.de/publications/jef1997f.pdf).

$E = \varepsilon \cdot c \cdot d$

E= extinction, ε= molar extinction coefficient, c= concentration, d= distance =1)

To determine concentration the following formula was used:

$c = E / \varepsilon$

2.7.2 Preparation of β-carotene for control readings

20 mg β-carotene were dissolved in 10 mL petrol ether (40 – 60 °C) and stored in darkness (brown glass bottle) at -20 °C. 200 µl of this solution was mixed with 150 µl of 0.7 % Triton X-100 and 1.6 % Triton X - 405 in ethanol. The solvents were removed with a vacuum concentrator. The sample dried down to a gel containing the carotene. This gel was suspended in 200 µl of water or liquid medium and later mixed well with methanol. The absorbance of the diluted β-

carotene was measured in the range from 350 to 500 nm in an UV/VIS Spectrophotometer (Jasco V-560).

2.8 Streptozyme Preparation: Growth of cultures and purification of the enzyme

2.8.1 Culture medium

To six 3-L Erlenmeyer flasks containing each 750 mL de-ionized water, 1 mL 1 M $MgSO_4$, 1 mL 0.1 M $CaCl_2$, 1 mL trace element solution SL8 (Biebl and Pfennig 1978), 2 g chitin (Sigma, practical grade) was added and autoclaved. Sterilized stock solutions for the salts and trace elements were used. Also 20 X *Streptomyces* Basal Medium (di-potassium hydrogen phosphate 16 g, potassium di-hydrogen phosphate 4 g, di-ammonium sulphate 10 g, distilled water 1 litre) was prepared and autoclaved.

2.8.2 Purification of chitosan

30 g of chitosan (Sigma, practical grade) was stirred into 1.5 L of 2 % acetic acid continuously for 1 hour till the material dissolved almost completely. While stirring continuously, 10 M NaOH was added very slowly until pH 7.0 was reached, and the pH was controlled using pH indicator sticks. The chitosan precipitated again was sedimented by 20 minutes centrifugation at 10000 rpm (Rotor SLA). The sediment was washed twice with 750 mL water and centrifuged again after each step. Finally the sediment was suspended in 500 mL water, and distributed as evenly as possible into ten 200 mL flasks, and the volume was made up to 200 mL with water, and autoclaved.

2.8.3 Starter culture of *Streptomyces* No. 6

Two 1-L Erlenmeyer flasks containing each 300 mL SUP medium were prepared. Approximately 10^6 spores of *Streptomyces* No.6 were inoculated per flask and incubated for 2 days on a shaker (100 rpm) at 19 – 22 °C. 100 mL of this culture was used as inoculum for each 3- L Erlenmeyer flask containing 750 mL medium.

2.8.4 Chitosan medium

Prior to use 50 mL *Streptomyces* Basal Medium and 1 flask (200 mL) of purified chitosan was added to each 3 - L Erlenmeyer flask. Each 3 - L Erlenmeyer flask containing 750 mL medium was inoculated with 100 mL starter culture and incubated for 3 - 5 days at 19 – 22 °C.

2.8.5 Purification of streptozyme

All steps were performed as strictly as possible in an ice bath. The mycelium was removed from the culture liquid by filtration with a Büchner funnel. The spores and small particles were removed by filtration through a 0.45 µm membrane filter. The volume of the filtrate was determined. The filtrate was cooled to ice bath temperature by stirring continuously on an ice bath. Ammonium sulfate was slowly added to 90 % saturation while continuously stirring. After all ammonium sulfate had dissolved, stirring was continued for at least 1 hour, then the solution was centrifuged 15 minutes at 10000 rpm (Rotor SLA) at 4 °C. The precipitates were dissolved in a total volume of ≤ 30 mL ice cold 20 mM Na-phosphate buffer pH 7.0 and dialyzed 3 times for 30 minutes each against 1.5 L of ice cold 20 mM Na-phosphate buffer. The preparation was freeze-dried in 1 mL portions using a vacuum centrifuge and stored at –70 °C. Prior to use in the experiments, the powder was reconstituted to the appropriate buffer concentration.

2.8.6 Determination of protein concentration with Bicinchoninic acid (BCA)

The BCA (Smith et al. 1985) method is a biochemical assay for determining the total level of protein in a solution in the concentration range from 0.5 µg / mL to 1.5 mg / mL, similar to the Lowry protein assay. BCA is a highly chromogenic reagent when complexed with Cu^{+1}, forming a purple blue complex with an absorbance maximum at 562 nm. The absorbance is directly proportional to the protein concentration.

5 different dilutions of the enzyme samples containing 1, 5, 10, 25 and 50 µl of the reconstituted enzyme preparation in a total volume of 50 µl were mixed with 1 mL of the BCA working solution prepared according to the instructions of the manufacturer (Thermo Scientific / Sigma) and then incubated at 37 °C for 30 minutes in a water bath. For calibration, a dilution series of bovine serum albumin containing 6.3, 12.5, 25, 37.5 and 50 µg in a total volume of 50 µl was prepared using 20 mM sodium phosphate buffer were incubated and measured similarly. Pure 20 mM sodium phosphate buffer pH 7 was used as a blank. The absorbance was read at 562 nm (Jasco V-560) and the protein concentrations were determined with the help of the calibration curve.

2.9 Cloning of the *CrgA* gene

2.9.1 Accession of the *CrgA* gene from NCBI

In order to find conserved regions of the *CrgA* gene, GenBank accession number AJ250998.1, homology search and alignment analysis was performed by BLAST search and CLUSTAL W (www.ebi.ac. uk/clustalw), respectively. The *M. mucedo CrgA* gene was cloned as shown below in order to obtain the sequence for designing the CrgA morpholino oligonucleotide for down regulation of the *CrgA* gene expression in *M. mucedo*.

Table 2.2: **Primer sequences used in this work**

Primer Name	Primer Sequence
CrgA F	5’tgyyt5gt5mg5ws5yt5gaycayca3’
CrgA R	5’ttytcytcytc5bh5ar5ggcat3’
pEUKA400 leuA si-F	5’ GTCCAACGTGCTCT**A**GACTACATTGGTATTGCTCCC 3’
pEUKA400-R	5’ AGCAGAGCGACGAATGGGATCCTCAATCTTGGCAGG 3’
Seq pri-R	5’ CGCTTGACTAAACCAGAGCCAGGCACCACC 3’
leuA-F	5’GGCTCATGATCTTCATGGCGCTCAC 3’
leuA-R	5’ GGAAGCTGATTCAAGAACTCAATGTG 3’
M13-20 forward	5 'GTAAAACGACGGCCAGT 3'
T7 promoter	5 'TAATACGACTCACTATAGGG 3'

5 = 2’ Inosin; silent mutated base in bold letter (**A**).

2.9.2 Polymerase Chain Reaction

2.9.2.1 Amplification of the DNA fragments

On the basis of the previously obtained *CrgA* gene sequence of *M. circinelloides*, the degenerated primers CrgA F and CrgA R were designed (Table 2.2) and the gene was amplified by PCR using genomic DNA from *M. mucedo* –. 25 µl of reaction mixture contained: 12.5 ng genomic DNA, 10 pmol of each primer (CrgA F and CrgA R), 3 mM $MgCl_2$, 2.5 mM dNTPs, 10 x PCR buffer (100 mM Tris-Cl pH 8.8 and 500 mM KCl) and 0.5 - 1 U Taq polymerase (Invitek). The PCR was performed in a programmable thermo block under the following conditions: initial melting at 94 °C for 5 minutes, annealing at 45 °C for 0.3 minutes and elongation at 72 °C for 1 minute for the first cycle and denaturation at 94 °C for 0.3 minutes, annealing at 45 °C for 0.3 minutes and elongation at 72 °C for 1 minute for cycles 2-30.

2.9.2.2 Purification, cloning and sequencing of PCR Products

2.9.2.2.1 Gel purification of PCR products

The PCR products were checked for the right size by agarose gel electrophoresis. The PCR fragments were isolated from the gels using the modified method of Vogelstein and Gillespie (1979). The PCR band from the agarose was excised from the gel, transferred into 6 M sodium iodide and incubated at 55 °C until the agarose was dissolved completely. Then 5 µl of glass milk were added followed by incubation on ice for 5 minutes. Subsequently, the samples were shaken at 250 rpm for 20 minutes, incubated on ice for 5 minutes and then centrifuged at 16 000 g for 2 minutes. The supernatant was removed and the pellet was washed twice with a solution containing 20 mM Tris-Cl pH 7.2, 200 mM NaCl, 2 mM EDTA and 51 % ethanol and centrifuged at 16 000 g for 2 minutes. The pellet was dissolved in 20 µl deionised water, incubated at 65 °C for 20 minutes and centrifuged at 16 000 g for 2 minutes. The supernatant was removed and residual water was evaporated in a vacuum concentrator for

10 minutes. Finally the DNA pellet was dissolved in 3 - 4 µl of deionised water and stored at -20 °C.

2.9.2.2.2 Transformation of the *CrgA* gene PCR fragment into *Escherichia coli*

Plasmid DNA and *CrgA* PCR fragments were ligated using T4 DNA ligase in ligation buffer: 10X ligation buffer stock: 400 mM Tris-Cl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, and transformed into *E. coli* using the basic techniques from Sambrook and Russell (2001), that is the purified PCR products were ligated into pDrive vector (Qiagen, Germany). *E. coli* XL-1 Blue was transformed with this plasmid and transformants were selected by blue-white selection on LB agar plates supplemented with 50 µg mL^{-1} ampicillin, 50 µl 100 mM IPTG and 100 µl of 20 mg X-Gal mL^{-1}.

2.9.2.3 Isolation and sequencing of the plasmid DNA

The plasmids were isolated using the modified method of Birnboim and Doly (1979). The transformants were grown overnight at 37 °C in LB medium supplemented with 50 µg / mL ampicillin. The cells were centrifuged at 16 000 g for 2 minutes and the supernatant was removed. The pellet was resolved in 50 mM glucose, 10 mM EDTA and 25 mM Tris-Cl pH 8.0. For the digestion of the cell wall 0.5 µg / µl lysozyme was added, and incubated for 10 minutes at room temperature. For lysis of the cells, 1.5 volume 0.2 M NaOH and 1 g / 100 mL SDS was added and for the precipitation of the proteins, 1.5 volume 3 M potassium acetate pH 4.8 was added and mixed very slowly and incubated on ice for 20 minutes and then centrifuged at 16,000 g for 10 minutes. The supernatant was transferred to a new Eppendorf tube and 25 ng / µl RNase was added and incubated at room temperature for 10 minutes. After adding 1 volume of chloroform and brief mixing, the sample was centrifuged at 16 000 g for 2 minutes. The aqueous phase was transferred to a new Eppendorf tube, mixed with 1 volume of 100 % 2-propanol, incubated at room temperature for 30 minutes and centrifuged at 16 000 g for 15 minutes. The supernatant was

removed and the pellet was washed twice with 200 µl of 70 % ethanol and centrifuged at 16 000 g for 5 minutes each time to precipitate the plasmid DNA. The pellet was dried and then resolved in 25 µl deionised water. The 1430 bp insert was checked by digesting the transformant plasmid DNA with *Eco*RI restriction enzyme at 37 °C for 1.5 hours. The restriction enzyme was inactivated by incubating at 70 °C for 10 minutes, then the digested transformant was loaded onto an 1 % agarose gel for electrophoresis. Size determination was based on comparison with a 1 kb DNA ladder. The clone was confirmed by sequencing (JenaGen GmbH, Jena).

2.9.2.4 Computer analysis of the *CrgA* Sequence

Homology search and alignment analysis were performed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and CLUSTAL W (www.ebi.ac.uk/clustalw) respectively. The Bio Edit Sequence Alignment Editor version 7.0.5.2 software (Hall 1999) was used for the nucleotide sequence data analysis and sequence comparison. According to related protein sequences in the protein databases of NCBI (http://www.ncbi.nlm.nhi.gov), the genomes of *R. oryzae* (http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae), *M. circinelloides* (http://M. gen. uM. es/) and *P. blakesleeanus* (http://genome.jgi-psf.org/Phybl1/Phybl1.home.html) were used. As sequencing primers the M 13 - 20 forward and T7 promoter (Table 2.2) were used.

2.10 Morpholino Oligonucleotide Design

2.10.1 *M. mucedo* CrgA MO

MO intended to interfere with the translation of the *CrgA* gene transcript were designed based on the sequence result of the *CrgA* gene of *M. mucedo* - FSU 620 and synthesized by Gene Tools (Philomath, Oregon, USA). The MO was designed to a region within the sequenced fragment of the *M. mucedo CrgA* gene close to the 5’ end. The MO sequence was designed as follows:

Sense strand of *M. mucedo* DNA

5'…TCCGCCACCCACCACTGTCCTTACC…

⇓ Transcription

Sense mRNA

5'…UCCGCCACCCACCACUGUCCUUACC…3'

CrgA-MO sequence, complementary to the target mRNA, 5' → 3'

5'-GGTAAGGACAGTGGTGGGTGGCGGA-3'

The MOs were dissolved to a stock concentration of 1 mM in de-ionized water.

2.10.2 *M. circinelloides* Ku70 MO

The MO for blocking *ku70* gene transcript translation was synthesized by Gene Tools (Philomath, Oregon, USA), and the sequence was designed as follows:

Sense strand of *M. circinelloides* DNA

5'…ATGTCATACGAATATACCAGTGTAT …3'

⇓ Transcription

Sense mRNA

5'… AUGUCAUACGAAUAUACCAGUGUAU …3'

Ku70-MO sequence complementary to the target mRNA, 5' → 3'

5'- ATACACTGGTATATTCGTATGACAT-3'

MOs were dissolved to a stock concentration of 1 mM in distilled water.

2.11 Endoporter

1.0 mL of 1 mM Endo-Porter in DMSO was purchased from Gene Tools, (Philomath, Oregon, USA), and was aliquoted and stored at room temperature.

2.12 Whole plasmid amplification by site directed mutagenesis

PCR based site directed mutagenesis creates mutants with > 50 % efficiency by using a single mutagenesis primer set. It can be done with any double stranded plasmid, which allows site-specific mutation (Weiner et al. 1994). In order to prevent or decrease second-site mutations during PCR, this process employs increased template concentration and a reduced number of cycles. Also, a polymerase with proof reading activity is mainly employed for this technique, in this case the *Pfu* DNA polymerase. Furthermore, as DNA isolated from almost all *E. coli* strains is Dam-methylated, *Dpn*I endonuclease, which acts specifically on methylated and hemimethylated DNA, is used to digest the parental methylated DNA and thus select for the PCR amplified DNA containing the mutation (Weiner et al. 1994). The target sequence for *Dpn*I is 5'-Gm6ATC-3'.

In order to insert a unique restriction enzyme site into the leucine gene of the pEUKA400 vector (figure 2.1, left) by site directed mutagenesis, the entire pEUKA400 plasmid was amplified by PCR using the mutagenic back-to-back primers pEUKA400 leuA si-F and pEUKA400-R (Table 2.2) and *Pfu* polymerase at an extension time of 1000 bases/minute. In order to create a silent mutation with a unique restriction site within the *LeuA* gene, a single nucleotide change was designed, changing CTC to CTA, but both variations coding for the amino acid leucine. The site directed mutagenesis was performed at the following PCR conditions: initial melting at 94 °C for 5 minutes, annealing at 62 °C for 0.3 min and elongation at 68 °C for 8 minutes for the first cycle and denaturation at 94 °C for 0.3 minutes, annealing at 62 °C for 0.3 minutes and elongation at 68 °C for 1 minute for cycle 2 -18. This generated nicked, circular DNAs. The template DNA was eliminated from the reaction mix by digestion with *Dpn*I leaving only mutated plasmids which, as they were generated *in vitro,* are therefore unmethylated. These were then transformed into competent *E. coli* XL 1-Blue cells by heat-shock treatment and left to be repaired by the endogenous bacterial repair machinery. The plasmid was purified and its sequence confirmed by sequencing (JenaGen GmbH,Jena).

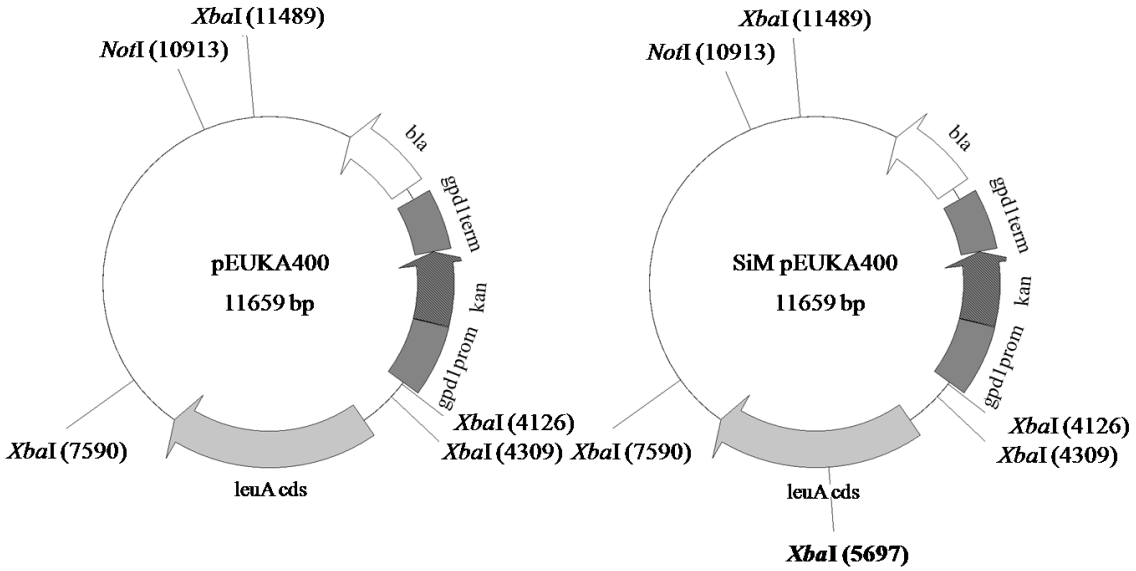

Figure 2.1: **Map of the original pEUKA400 and the silently mutated pEUKA400 (SiM pEUKA400) vector showing the relevant features.**
left: original pEUKA400 containing the coding sequence of leucine A gene of *M. circinelloides* coding for the α-isopropylmalate isomerase (leuA cds), and the kanamycin resistance gene (kan) under the control of the *M. circinelloides* glyceraldehyde 3-phosphate dehydrogenase gene 1 promoter and terminator regions (gdp1prom and gdp1term, respectively), as well as the β-lactamase gene (bla) conferring resistance to ampicillin (Appel et al. 2004). The plasmid contains 4 *Xba*I restriction sites. Vector analysis and mapping was done using SIM Vector 4.5. right: silently mutated pEUKA400 with an additional ***Xba*I** restriction site at position 5697, within the coding region of the *LeuA* gene.

2.13 Preparation of protoplasts and transformation

Before the actual experiments, the conditions for the formation and regeneration of protoplasts from *M. mucedo* and *M. circinelloides* were optimized with special focus on the concentration of the osmotic stabilizer, age of the mycelium and incubation time. Protoplast formation efficiency was observed with a light microscope and quantified using a haematocytometer cell counting chamber.

2.13.1 Preparation of protoplasts from *M. mucedo* and introduction of CrgA-MO by electroporation

The ideal developmental stage for obtaining protoplasts of zygomycetes is shortly after germination when the germ tube is just about to from the first branch. *For M.*

mucedo, this stage was reproducibly reached when 100 mL of SUP broth were inoculated with 1 x 10^7 spores and incubated in a shaking water bath overnight. To slow down germination, the water bath was first filled with ice, which was allowed to melt during the night. Shaking was only switched on for the last 6 hours, when the bath had reached room temperature. In the morning, germination was controlled by microscopy before the germlings were concentrated using a sterile filter unit and very weak vacuum produced by a water jet pump. The collected germlings were washed two times with 10 mL each of a solution containing 0.6 M Sorbitol, 10 mM MOPS / KOH pH 6.3, and 3 mM $CaCl_2$ and finally transferred to a 100 mL Erlenmeyer flask in 10 mL of the same solution. Streptozyme was added taking 300 µl from the 1 mg / mL stock along with 100 µL of Novozym 234 (Calbiochem, CA, USA) from a 50 mg / mL stock and the protoplast formation was followed by microscopy every 15 minutes. After one hour of enzyme treatment, the protoplasts were separated from the parental hyphae by filtration through 3 layers of Miracloth and concentrated by centrifugation for 10 minutes at 1500 rpm. The protoplast pellet was washed twice with a solution containing 0.6 M Sorbitol, 10 mM MOPS / KOH pH 6.3 and 3 mM $CaCl_2$ and finally suspended in 2 mL of 0.6 M Sorbitol / MOPS / $CaCl_2$. 1 mL of this suspension was filled into a electroporation cuvette, mixed with 10 µmoles of CrgA-MO and kept on ice for 10 minutes. The other 1 mL served as a control without CrgA-MO and was kept on ice for 10 minutes. Electroporation was performed with a GenePulser / Pulse Controller system (BioRad, München). The system was set at: 300 V, 25 µF, $\Omega = \infty$, and the time constant values were noted for each electroporation. After the pulse, the cells were again kept on ice for 10 minutes, and then transferred into a sterile reagent tube together with 2 mL of liquid induction medium, 70 µg of TA, and endoporter to the concentration of 18 µM. The cells were then gently agitated on a culture roller for 12 hours at 25 °C in the dark.

2.13.2 Preparation of protoplasts from *M. circinelloides* R7B and electroporation

The spores were harvested using 100 µl of 0.1 % Tween 20 in sterile water. All the other steps were the same as in the protocol for *M. mucedo*, except that the protoplast pellets were finally suspended in 3 mL of 0.6 M sorbitol / MOPS / $CaCl_2$. 10 µmoles of Ku70 MO and 2 µg of SiM pEUKA400 plasmid (64 ng / µl) was added to 1 mL of protoplast suspension. One mL of the remaining suspension was mixed with only 2 µg of SiM pEUKA400 plasmid and the last 1 mL served as control, with neither Ku70 MO or SiM pEUKA400 plasmid. All were kept on ice for 10 minutes. The electroporation settings were: 300 V, 25 µF, $\Omega = \infty$, and the time constant values were noted for each electroporation. After the pulse, the cells were again kept on ice for 10 minutes, and then transferred into sterile reagent tubes together with 500 µl of liquid induction medium each. They were then agitated on a rolling machine for 2 hours at 25 °C. Serial dilutions of the regenerated protoplasts were made in 0.6 M sorbitol solution. Dilutions 10^{-1} to 10^{-4} were plated on minimal medium with 0.6 M sorbitol and with or without added leucine and incubated at 25 °C for 3 - 5 days.

2.14 DAPI staining of the protoplasts

Protoplast nuclei were stained with DAPI for fluorescence microscopy. A stock solution of DAPI (1 mg / mL) was prepared in water. 0.5 µl DAPI was taken from the stock solution and mixed with 500 µl of a solution containing 40 mM sodium phosphate buffer pH 7.0 and 50 mM sorbitol. The cells were incubated in this solution for 30 minutes at room temperature and in darkness before microscopy.

2.15 Regeneration of protoplasts

For regeneration of protoplasts on solid medium, the suspension of lysed mycelium enzyme treatment was passed through 3 layers of Miracloth to separate the protoplasts and ungerminated spores from remnants of mycelium. The filtrate was diluted 10^{1}-10^{6} fold in 0.6 M sorbitol and the dilutions were plated

onto solid minimal medium containing 0.6 M sorbitol. The Petri dishes were incubated at 25 °C for 5 days, and then protoplast regeneration was checked by observation of colony formation. As a control, the filtrate was also diluted 10^1 - 10^6 fold in distilled water and plated in the same way, but here the cells burst because of osmotic instability.

2.16 Calculation of reversion frequencies

To assess the transformation efficiency of the *M. circinelloides* R7B leu$^-$ mutant strain, the rate of reversion was determined. A total of 1.25 x 10^8 spores were examined on minimal medium, with 5 x 10^6 spores plated on each plate. Regeneration frequencies were estimated by comparison of direct microscopic counts of the protoplasts with plate counts on regeneration agar. The regeneration frequencies were estimated by dividing the total number of colonies grown on the regeneration media by the total spore count.

2.17 Screening of the transformants

2.17.1 Selection of the transformants of *M. circinelloides*

Transformant colonies selected on minimal medium were transferred to fresh minimal medium and incubated for 5 - 7 days at room temperature. When the mycelium covered the whole plate, a small block was cut and inoculated into liquid minimal medium. DNA was isolated from the mycelium after 7 – 10 days of growth (see 2.4.1.) and used for Southern hybridization analysis.

2.18 Blotting and hybridization techniques

2.18.1 Southern hybridization of the transformants

The detection of genomic DNA with labelled DNA fragments was carried out according to Southern (1975). About 6 µg genomic DNA of the auxotrophic strain of *M. circinelloides* R7B, wild type *M. circinelloides* and the transformant were each digested with 2 U / µg DNA of the *Xba*I restriction enzyme in 1 X Yellow

Tango buffer (Fermentas): 33 mM Tris-acetate (pH 7.9 at 37 °C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA for 6 hours at 37 °C. After that, the restriction enzyme was inactivated by incubating at 70 °C for 20 minutes and the digested DNA was separated by agarose gel electrophoresis. The DNA fragments in the agarose gel were denatured in a solution of 0.5 M NaOH and 1.5 M NaCl in water for 20 minutes. Subsequently, the gel was washed twice for 20 minutes in a solution of 0.5 M Tris-Cl pH 7.0 and 3 M NaCl for neutralisation. After that step, a nylon membrane was placed over the gel for capillary transfer of the DNA in 20 X SSC solution for at least 8 hours (Southern 1975). 20 X SSC contained 3 M sodium chloride and 0.3 M sodium citrate, pH 7.0. Upon completion of the transfer, the membrane was briefly washed with distilled water, dried in air and then in a vacuum oven at 80 °C oven for 3 hours for the DNA to be fixed to the membrane.

In order to block non-specific binding sites on the membrane, the membrane was blocked for 4 hours in pre-hybridisation buffer, containing 50 % formamide, 5 X SSC, 1 X Denhardt, 0.1 g / 100 mL SDS, 50 mM sodium phosphate buffer pH 7.0 and 500 mg / mL denatured DNA, at 42 °C. Here, denatured herring sperm DNA was used, which was dissolved in deionised water at 10 mg / mL and centrifuged at 3000 g for 5 minutes at room temperature (Heraeus Megafuge 1.0 R, Rotor: BS4402/A) to remove insoluble components. The supernatant with the soluble DNA was denatured by incubation at 95 °C for 15 minutes and then cooled on ice. 50X Denhardt consisted of 1 g / 100 mL bovine serum albumin, 1 g / 100 mL Ficoll and 1 g / 100 mL polyvinylpyrrolidone. The pre-hybridisation buffer was discarded and hybridisation was carried out at 42 °C overnight in 50 % formamide, 5 X SSC, and 1 X Denhardt, 0.1 g / 100 mL SDS, 20 mM sodium phosphate buffer pH 7.0 and 100 mg / mL denatured DNA. The vector probe was denatured at 95 °C for 5 minutes and added to the hybridisation buffer.

2.18.2 Preparation of digoxigenin-labelled probes

100 ng of 1 kb DNA marker in 7 µl deionised water was denatured by incubation at 95 °C for 10 minutes and placed immediately on ice. After addition of 1 µl hexanucleotide, 1 µl dNTP labelling mix and 0.5 µl Klenow fragment (DIG DNA

Labelling Kit, Roche, Heidelberg), the mixture was incubated at 37 °C for 1 hour and then inactivated by incubation at 70 °C for 10 minutes. The same labelling procedure was followed for pTZ19R and for the PCR amplified leucine A gene fragment, for the later use in the detection of the transformed gene fragments. The vector pTZ19R comprised the bacterial parts including the ampicillin resistance gene from the pEUKA400 vector used for transformation.

2.18.3 Cleaning the probes

For the removal of the remaining components of the Klenow approach the digoxigenin-labelled DNA probes were purified by column chromatography on Sephadex G50. The column volume was 1.6 mL, the probes were added and then the column was washed with 0.4 mL 10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0 and 0.1 g / 100 mL SDS. The first fraction of 0.4 mL was discarded, the second 0.4 mL fraction contained the digoxigenin-labelled DNA probe.

2.18.4 Washing and detection of the transformants

After hybridisation, the membrane was washed first twice in 2 X SSC and 0.1 g / 100 mL SDS at 42 °C for 20 minutes and then twice with 2 X SSC and 0.1 g / 100 mL SDS at 50 °C for 20 minutes to remove excess probe. Then it was washed twice with 0.2 X SSC and 0.1 g / 100 mL SDS at 50 °C for 10 minutes.

For detection, the membrane was washed with 100 mM maleic acid pH 7.5, 150 mM NaCl and 0.1 % Triton X - 100 for 5 minutes. In order to block non-specific binding sites, the membrane was incubated for 30 minutes in block buffer consisting of 100 mM maleic acid pH 7.5, 150 mM NaCl and 1 g / 100 mL skimmed milk powder. The membrane was incubated for 30 minutes in fresh block buffer containing the anti-digoxigenin-alkaline phosphatase conjugate (Roche, Heidelberg) in a dilution of 1:10000. and then washed twice for 15 minutes in a solution containing 0.1 M maleic acid pH 7.5, 0.15 M NaCl and 0.1 % Triton X - 100. For the detection of the fragment containing the leucine A gene by chemiluminescence, the membrane was incubated in detection buffer: 100 mM Tris-Cl pH 9.5 and 100 mM sodium chloride for 5 minutes. Subsequently, the

membrane was incubated with 250 µM CSPD (Bronstein et al. 1991) in 100 mM Tris-Cl pH 9.5 and 100 mM sodium chloride for 5 minutes. The membrane was sealed bubble-free into a plastic bag and incubated at 37 °C for 15 minutes before the film (Kodak XAR-5) was placed on top of the membrane. The film was exposed depending on the signal strength from 30 minutes to 2 hours. In the case of the vector probe, the film was developed after one hour of exposure.

3. Results

3.1 Preparation of protoplasts in *M. mucedo*

This is the first report about protoplast formation and introduction of regulatory genetic elements in *M. mucedo*. At the beginning, the conditions for the formation and maintenance of stable protoplasts had to be optimized for this species. To prepare protoplasts, ideally a stage with only the germinating hyphae but no branches was needed (Figure 3.1). Development was documented and followed by microscopy. *M. mucedo* forms usually two to three germinating hyphae from different areas of the spore.

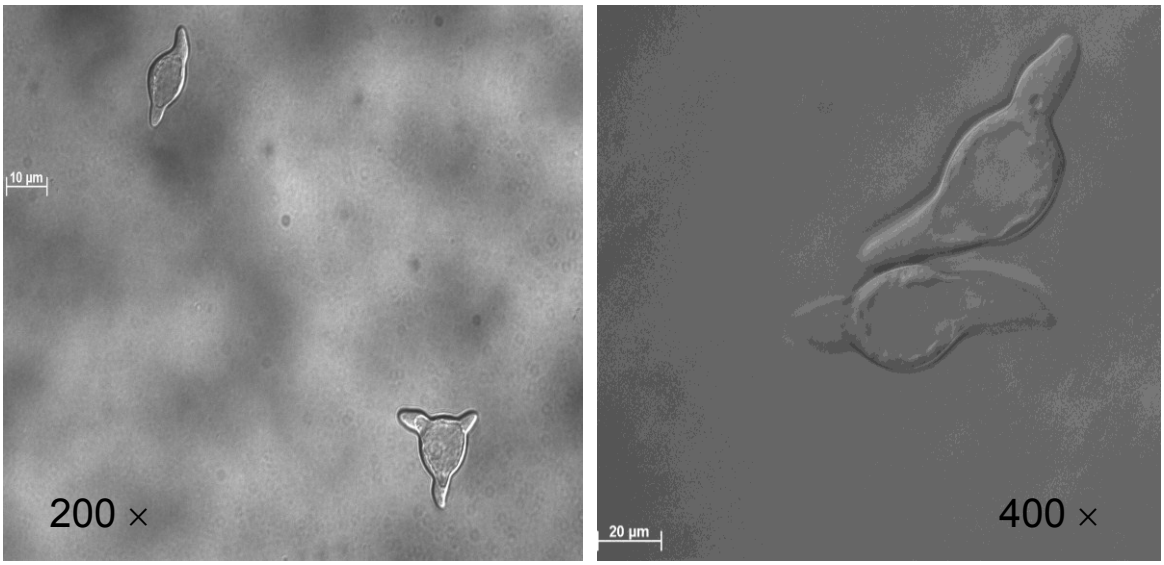

Figure 3.1: **Germinating spores of *M. mucedo* at the stage ideal for protoplast formation.** Bright field micrographs with magnification and size bars.

Following treatment of the young mycelia with the cell wall lytic enzymes Novozym and an enzyme preparation from *Streptomyces* No.6 in osmotically stabilized sodium phosphate buffer, the first protoplasts appeared after approximately 20 minutes. When the young mycelia were exposed to the lytic enzymes, the hyphae first swelled at certain points where there was greatest sensitivity to the enzymatic attack. The hyphal wall lysed and and the protoplasts were released from pores formed in the cell wall. Most of the protoplasts were released from the growing tip of the hyphae. As seen in the microscope, nothing or only a small indefinite amount of the protoplasmic content was retained inside

the germlings. The protoplasts formed as globular structures which were either small (approximately 5 µm) or large (approximately 15 µm), depending on the exposure time (Figure 3.2).

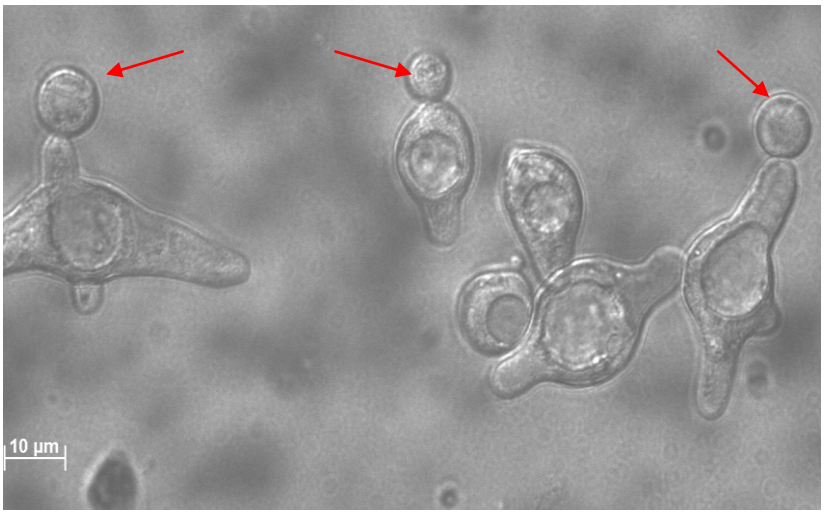

Figure 3.2: **Germinating spores after treatment of young mycelia with cell wall lytic-enzymes for 1 hour;** Bright field micrographs with size bars, red arrows indicate budding protoplasts.

With 30 minutes exposure time to the lytic enzyme, the protoplasts emerged exclusively from the hyphal tips and were large and more uniform in size, while at long exposure time of 1-2 hours, the protoplasts additionally emerged from other regions of the hyphae and were found to be generally smaller but more variable in size. Some protoplasts were irregular; and of various appearance, ranging from very dark, to grey and transparent, and from appearing more fragile to refractive. The contents of the protoplasts was also not evenly distributed. After emergence from the hyphae, each protoplast gradually increased in size rapidly at the expense of the mycelial contents, which simultaneously decreased in size and developed a single large vacuole. The osmotic sensitivity of the protoplasts was demonstrated by their rapid lysis following dilution with water. It was found that the protoplasts swelled slightly and burst suddenly when placed in distilled water.

The length of time required for the formation of protoplast varies with the concentration of the enzyme and the incubation conditions. On the whole, the protoplast formation is a step by step procedure where the protoplasts are not released slowly and gradually, but rather abruptly. Gentle agitation helped this

release of protoplasts. When the lytic enzyme was freshly diluted directly before treatment, the release of protoplasts occurred much more quickly than with a stored dilution. The effect of osmotic stabilizers at different concentration between 0 and 0.8 M was studied. Generally the protoplasts ruptured at and below 0.1 M stabilizer concentration.

3.1.1 Optimization of protoplast generation

To prepare protoplasts for the transformation experiments, the choice of enzyme for digesting the cell walls is crucial. Many cell wall lytic enzymes are prepared from *Streptomyces* species and are used in protoplast formation in different fungi. As alone among fungi, zygomycetes incorporate some chitosan into their hyphal walls, *Streptomyces* No. 6 was chosen for the production of cell wall lytic enzymes. This strain produces both chitinase and chitosanase when induced with chitin and chitosan, respectively, in the culture medium.

To enhance the generation of protoplasts, the commercially available lytic enzyme mix Novozym with cellulase, protease and chitinase activities was used in combination with the enzyme mix prepared from *Streptomyces* No. 6 in the present study. In both *Mucor* species, no protoplasts were formed with either of the lytic enzymes alone. Protoplast yields were highest with 5 mg of Novozym in combination with 0.3 mg of *Streptomyces* No.6 enzyme mix in a total volume of 10 mL. Protoplast yields were reduced at lytic enzyme concentrations either lower or higher than these concentrations, as higher enzyme concentrations affected the regeneration of the protoplasts, and lower enzyme concentrations affected the efficiency of protoplast formation.

The highest yield of protoplasts at 5×10^6 protoplasts per mL of lysis buffer was obtained when the germlings were incubated with the lysis mixture for 1 hour. Too long exposure to the lytic enzyme resulted in bursting of the protoplasts due to cell membrane damage and generally affected the viability and regeneration of the protoplasts.

Another crucial factor was the developmental stage of the germlings at the beginning of protoplast formation. An optimal stage was obtained with a total

growth period of 16 hours where the spores were agitated only for 6 hours, thus somewhat reducing the oxygen supply and retarding general growth. The morphology of the germinating spores was controlled microscopically and enzyme treatment was started only when the appropriate stage was reached. Maximum yield of protoplasts at about 70% protoplasts from the inoculum spore number were obtained from this 16 hours old mycelium. Using mycelium either older or younger than 16 hours resulted in decreased protoplast yield. The reduction in protoplast yield with increasing mycelial age is probably due to the increase in chitosan content of the fungal cell wall.

The effect of different concentrations of the osmotic stabilizer up to 0.8 M on the maintenance and stability of the protoplasts during release from the mycelium and centrifugation was investigated. It was found that 0.6 M sorbitol was more efficient in producing significantly higher yields of protoplasts than at other concentrations. At other concentrations, the protoplasts were fragile and the yield was very low.

3.1.2 Regeneration of protoplasts

To determine the stability and abilities of regenerated protoplasts, they were diluted either with the osmotic stabilizer 0.6 M sorbitol in 10 mM MOPS buffer pH 6.3 or sterile water. They were then plated on minimal medium containing 0.6 M sorbitol and incubated at 25 °C for 5 days. No colonies appeared when the protoplasts were diluted in water whereas protoplasts normally regenerated after serial dilution in stabilizer solution. This result suggests that the regenerated colonies were indeed obtained from protoplasts. The regeneration frequency was generally about 70 % of the initial protoplast number.

The study on protoplast regeneration allows a tentative evaluation on the effects of enzyme treatment on normal cell growth and development. Protoplasts deficient in the ability to regenerate apparently either lacked nuclei or were damaged at some point during or after enzyme treatment. The concentration of osmotic stabilizer also appeared to influence protoplast regeneration. Therefore,

0.6 M sorbitol was used as the osmotic stabilizer also through this experimental step.

3.1.3 DAPI staining of nuclei

To investigate the distribution of nuclei in the protoplasts, DAPI staining was employed. Each protoplast initially contained at least one nucleus. During germination of the spores, most nuclei were found to be concentrated near the growing hyphal tip (Figure 3.3).

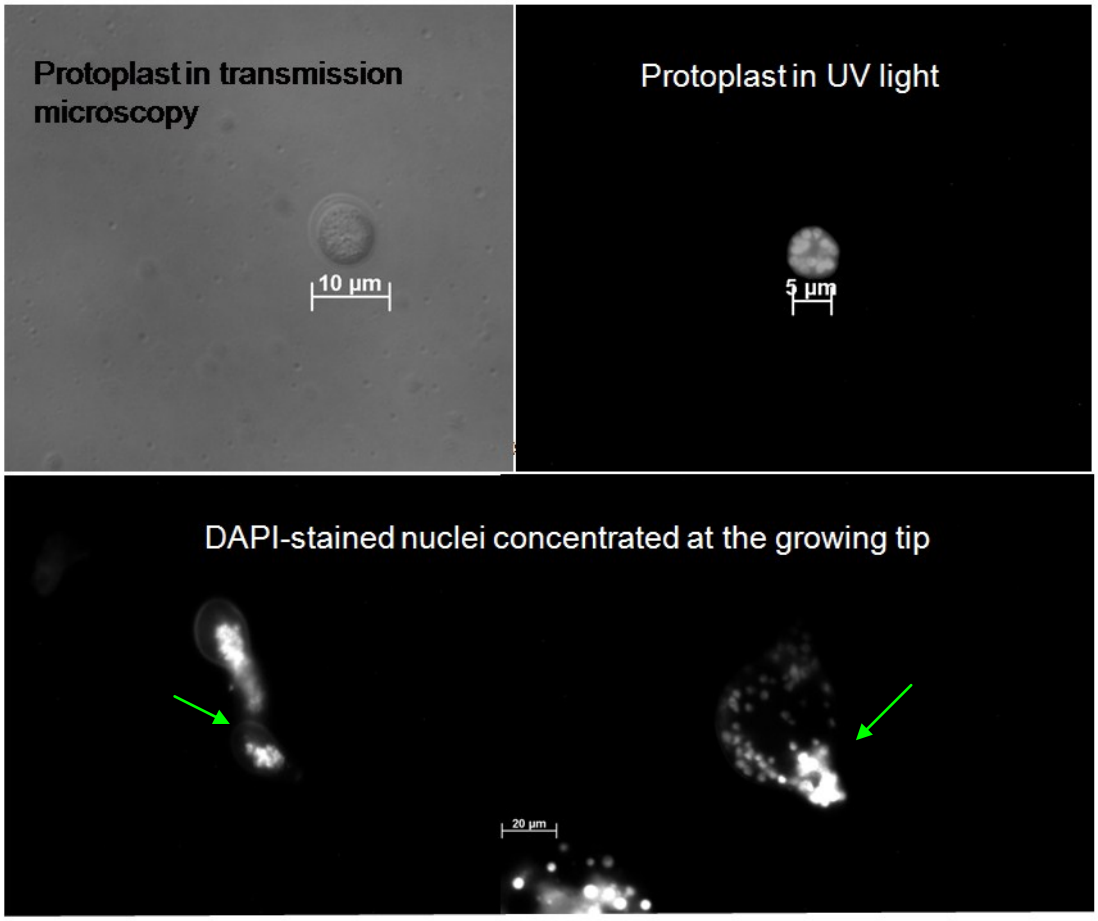

Figure 3.3: **DAPI-stained nuclei in protoplasts and germinating spores of *M. mucedo*.** Bright field and UV fluorescence micrographs with size bars; arrows indicate nuclei.

3.2 Down-regulation of the crgA carotene biosynthesis repressor gene expression results in increased carotene production.

3.2.1 Increase of β - carotene production by trisporic acid

As first step in the optimization of morpholino oligonucleotide-mediated interference of gene expression as a molecular tool, a suitable system for a proof-of-principle experiment needed to be established. This was found in the crgA-mediated regulation of carotene synthesis, which has been shown in *M. circinelloides* (Navarro et al. 2001, Nicolas et al. 2008). CrgA was identified as a light-regulated repressor for carotene synthesis acting mainly in the dark. As a difference in carotene production results in a measurable phenotypic response, this regulatory system was adapted to *M. mucedo*. In a first step, the induceability of carotene synthesis by trisporic acid instead of light was validated and the overall increase of carotene production in the presence of trisporic acid was determined.

Commercially available β-carotene was used as a calibration standard for determination of the carotene concentration in the culture extracts. Spectra were recorded in the absorbance range between 350 and 500 nm, showing the typical carotene spectrum with distinct peaks and shoulders (Figure 3.4 and Table 3.1). The maximum absorbance was at 449 nm. The slight shift in absorbance data from literature data (maximum at 453 nm) are caused by variations in the solvent and sample preparation procedures. This peak was used for the concentration determination.

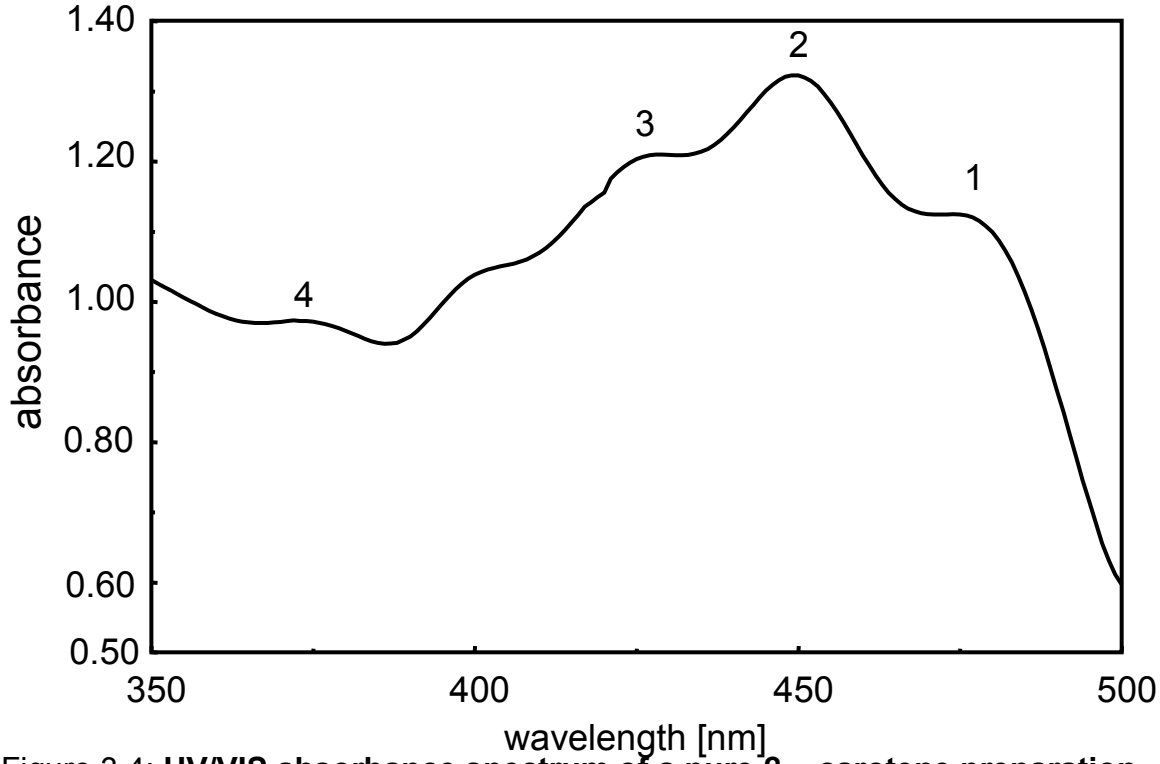

Figure 3.4: **UV/VIS absorbance spectrum of a pure β – carotene preparation.**

Table 3.1: **Calculation of the β-carotene concentration using the molar extinction coefficient ε = 141x10^3 M^{-1} cm^{-1}**

Peak Number	Wavelength (nm)	Absorbance	β-carotene concentration (M)
1	474	1.124	-
2	449	1.322	9.3 x10^{-6}
3	429	1.209	-
4	372	0.973	-

When TA was added to growing cultures of *M. mucedo* on the second day, an at least 2-fold increased carotene amount over the control was measured 24 hours later. β-carotene production was increased by TA in both mating types. The concentration of β-carotene was estimated from absorbance measurements at the absorbance maximum. The increase in β-carotene did not directly correlate with the amount of TA added to the cultures (Figure 3.5). TA was applied at 0.2, 2

an 20 µg, but the data show that in the + mating type, the addition of 0.2 µg TA increased carotene content 2-fold, while the 10 times higher amount 2 µg increased carotene content only 3-fold. The maximum carotene content at 20 µg added TA was only about 3.5 times higher than in the controls, corresponding to 2.2 x 10^{-6} M β-carotene. This proves that TA indeed stimulates carotene production, but the overall capacity of the mycelium for carotene production seems to be limited.

In the – mating type, the increase in carotene production was steeper, but the overall end concentration was very similar to that of the + mating type. Because of the generally lower carotene content in unstimulated *M. mucedo* –, the total increase at 20 µg TA added amounted to about 5-fold (Table 3.2). For that reason, the – mating type was chosen for the MO interference experiments.

Table 3.2: **Supplementation with TA and carotene production**

Amount of TA added [µg]	**OD at 448 nm**		**Concentration of carotene [M]**	
	***M. mucedo* FSU 621 (+)**	***M. mucedo* FSU 620 (−)**	***M. mucedo* FSU 621 (+)**	***M. mucedo* FSU 620 (−)**
0 (control 1) with neither TA nor ethanol	0.076	0.076	$5.3x10^{-7}$	$5.3x10^{-7}$
0 (control 2) with 20µl ethanol	0.096	0.053	$6.8x10^{-7}$	$3.7x10^{-7}$
0.2	0.166	0.242	$1.1x10^{-6}$	$1.7x10^{-6}$
2	0.284	0.260	$2.0x10^{-6}$	$1.8x10^{-6}$
20	0.323	0.333	$2.2x10^{-6}$	$2.3x10^{-6}$

As the TA was dissolved in ethanol, two different controls were used as shown in Table 3.2 to check whether the solvent has some effect on β-carotene production.

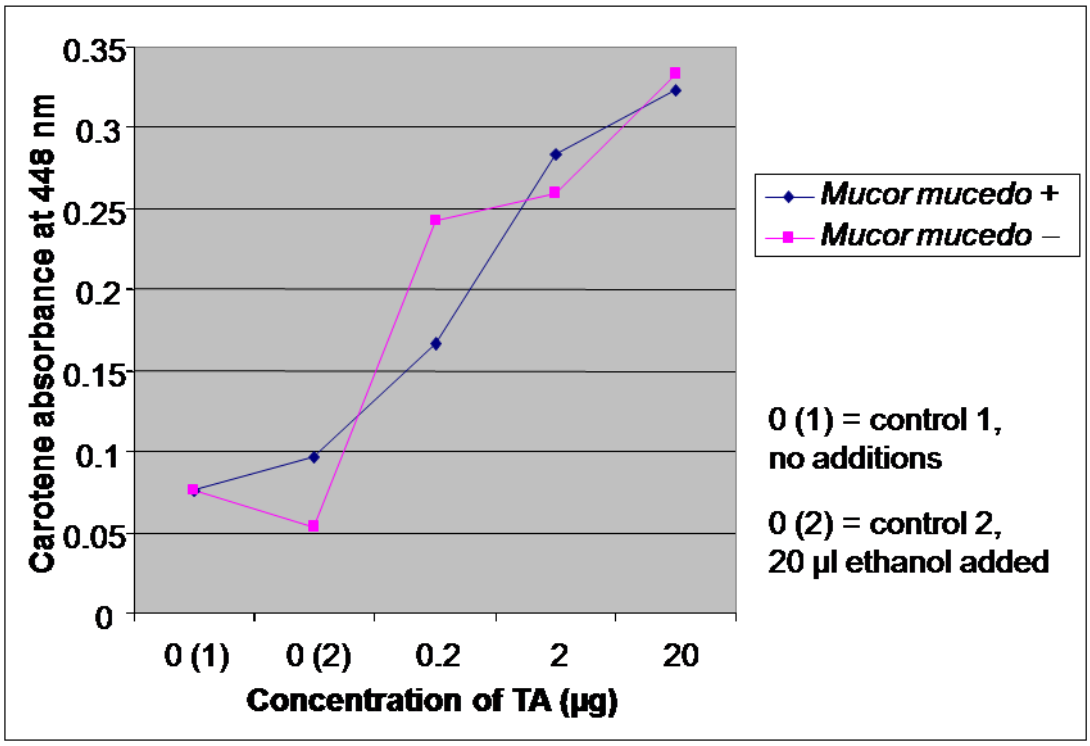

Figure 3.5: **Effect of TA concentration on carotene synthesis**

3.2.2 Cloning of the *crgA* gene from *M. mucedo*

As the *crgA* gene was not yet identified in *M. mucedo*, a search was conducted within the three available genome databases for zygomycetes, of *M. circinelloides, R. oryzae* and *P. blakesleeanus*. A conserved region with high similarity was identified by BLAST homology search and CLUSTAL W alignment analysis as provided by NCBI. Degenerated primers based on the consensus sequence for this region were used to amplify by PCR a fragment of the *crgA* coding region. The resulting PCR product had a size of around 1430 bp and was ligated into the pDrive vector between the *Eco*RI restriction sites. The plasmid was transformed into *E. coli* XL1 Blue. The plasmid was then purified from the transformant and was confirmed by insert release (Figure 3.6) and gene sequencing. When the plasmid was digested with *Eco*RI, two bands appeared after agarose gel electrophoresis, the smaller one at around 1430 bp representing the inserted *crgA* fragment. The undigested plasmid also separated into two bands, the more prominent lower one representing the supercoiled form (Figure 3.6).

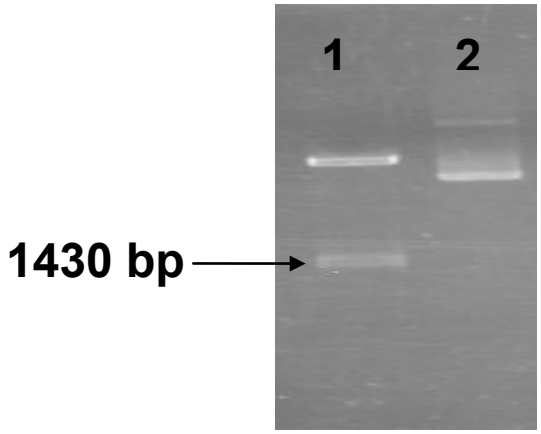

Figure 3.6: **Confirmation of the *crgA* clone by insert release.** Lane 1: *Eco*RI digested plasmid DNA, lane 2: undigested plasmid DNA

The fragment obtained from the *M. mucedo crgA* gene comprises about 65 % of the *M. circinelloides crgA* gene. It shows a sequence identity of 66.5 % at the amino acid level when compared with the corresponding region of *M. circinelloides* (Figure 3.7). The comparison is based on the nucleic acid sequences. When compared to the complete gene, The *crgA* gene sequence of *M. mucedo* was used to design the CrgA MO for down-regulation of the *M. mucedo crgA* gene.

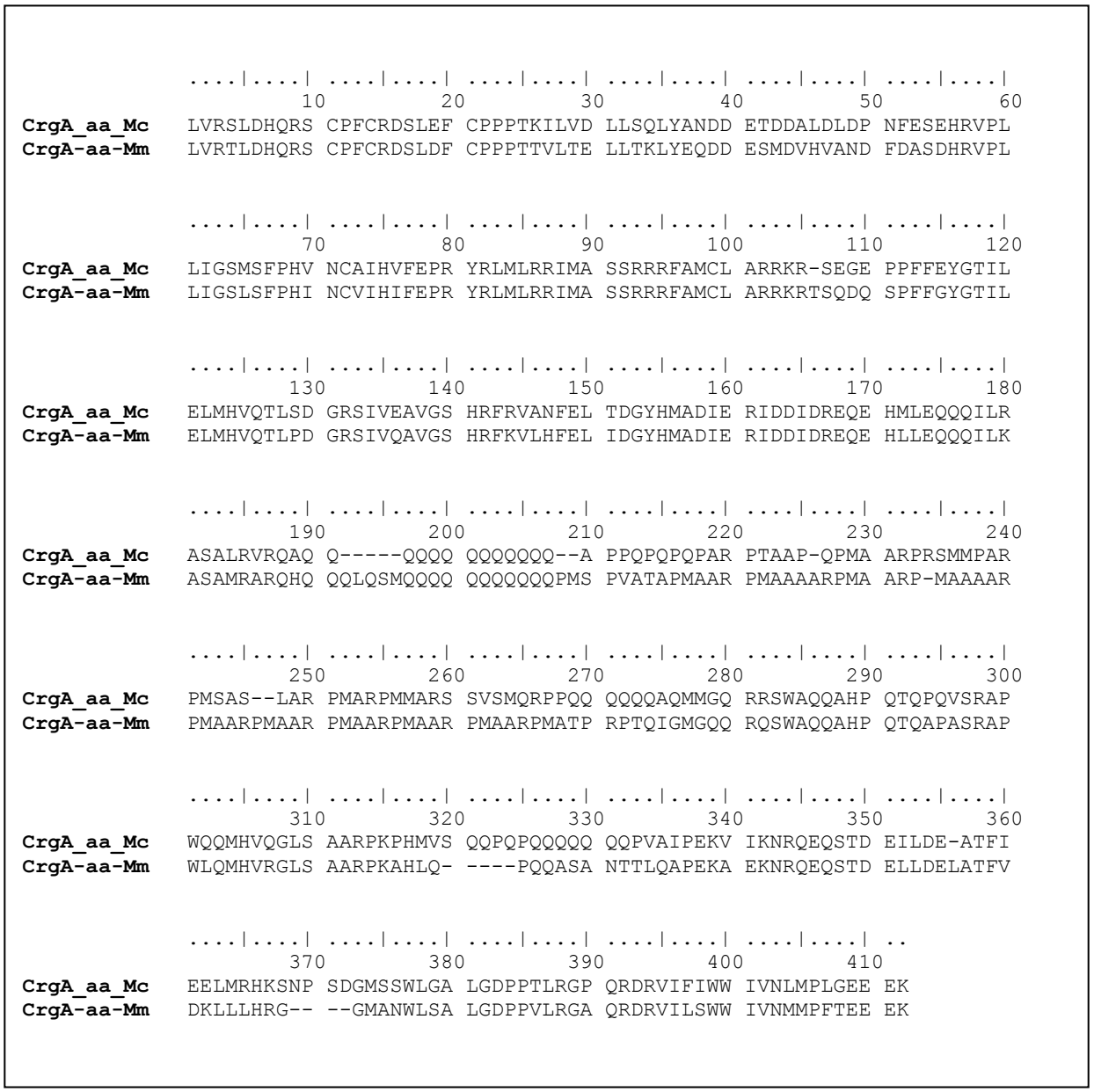

Figure 3.7: **Alignment of the deduced amino acid sequences of *M. mucedo* and *M. circinelloides* CrgA, indicating 66.5% identity.**

3.2.3 Increase of TA-induced β-carotene production after incorporation of CrgA-MO

CrgA-MO were incorporated into the protoplasts of *M. mucedo* via electroporation which were then regenerated in the presence of TA as described in 2.13.1. After 12 hours incubation of the regenerated protoplasts, β-carotene was extracted and the concentration calculated from UV/VIS absorbance spectra recorded in the range between 350 and 500 nm. Although the ratio between the maxima is

altered by co-extracted other cellular components absorbing in the same wavelength region, the main β-carotene absorbance maximum near 448 nm is present. Figure 3.8 and Table 3.3 show the result of a typical experiment.

Table 3.3: **Calculation of β-carotene concentration after treatment with TA and CrgA-MO.**

Peak Number	Wavelength (nm)	Optical Density (OD)	β-carotene concentration (M)
treatment: TA and CrgA-MO			
1	448	0.429	3.0×10^{-6}
2	414	0.513	-
treatment: TA only			
1	445	0.161	1.1×10^{-6}
2	413	0.257	-

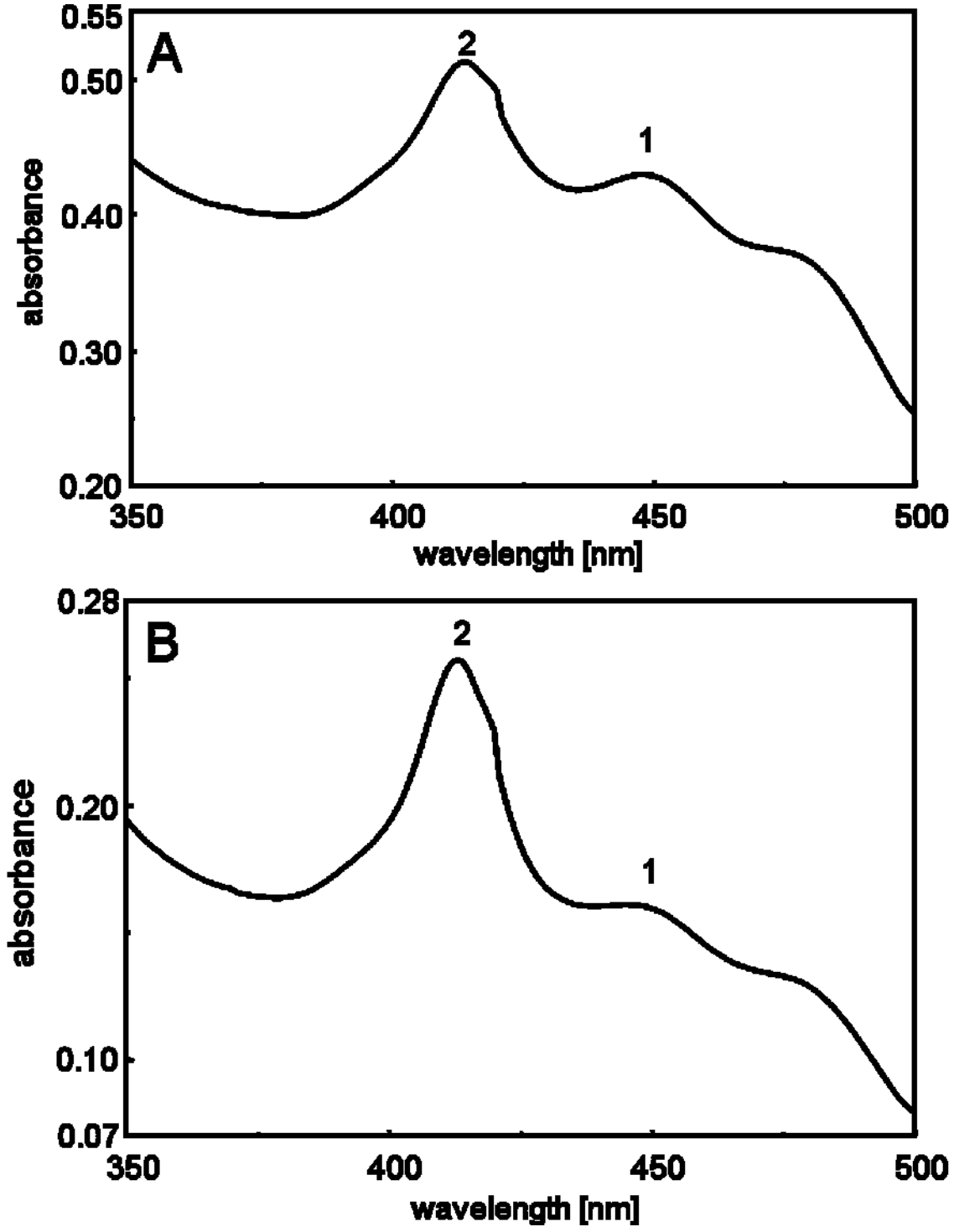

Figure 3.8: **UV/VIS absorbance spectra of cell extracts of *M. mucedo*.** Peak No. 1 is the major absorbance peak of β-carotene. A. Cell extract after treatment with TA and the CrgA-MO; B. Cell extract after treatment with TA but without CrgA-MO.

The effect of the incorporation of the CrgA-MO on the accumulation of β-carotene was studied in several independent experiments. Although the absolute measurements differ between the individual experiments, the general outcome was the same: In all cases, the treatment with CrgA-MO led to an increased production of β-carotene (Figure 3.9), proving the down-regulation of the CrgA gene product and thus the de-repression of β-carotene synthesis. The average increase for the three experiments compared in Figure 3.9 was 28%. MO are therefore an effective tool in the manipulation of gene expression in zygomycete fungi.

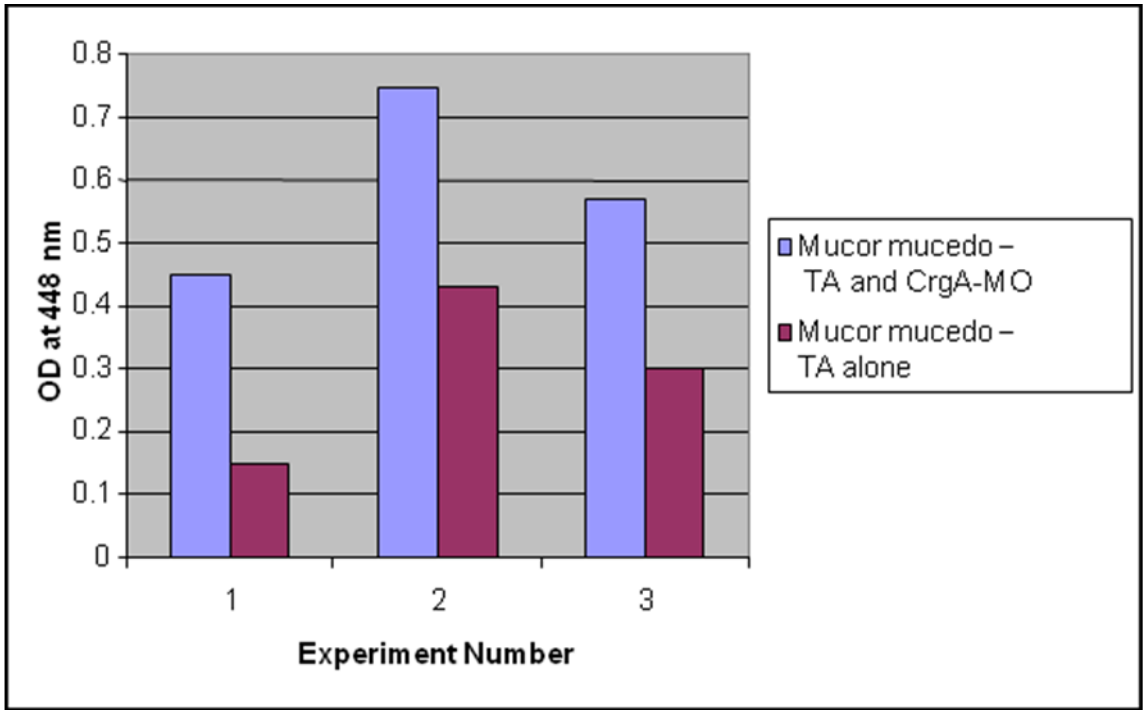

Figure 3.9: **Effect of CrgA-MO on the TA-induced production of β-carotene in *M. mucedo* –.** Comparison of three independent experiments.

3.3 Application of MO-modified gene expression for genetic manipulation of *M. circinelloides*

As the MO approach was found to be successful in the manipulation of gene expression, the second part of this work was committed to the use of MO for establishing an integrative and/or more stable transformation system for *M. circinelloides*. In other fungi, down-regulation of the *ku70* gene product involved in the nonhomologous end joining pathway of DNA repair resulted in the decrease of ectopic integration and thus allowed for better selection of the rare homologous integration events (e.g. Krappmann 2007). Due to the general restrictions to genetic manipulations in zygomycete fungi, the production of knock-out mutants for this or other genes is not possible. We therefore decided to use Ku70-MO to down-regulate the expression of Ku70 simultaneously with the transformation with a vector carrying a desired trait. As a suitable experimental system, *M. circinelloides* was chosen. With the leucine auxotroph mutant strain R7B (*leu*$^-$) (Appel et al. 2004) as recipient and the plasmid pEUKA400 carrying the functional wild type *M. circinelloides Leu*A gene, coding for α-isopropylmalate isomerase, as curative vector (J. Arnau, pers. communication), a well characterized and selectable transformation system already existed.

3.3.1 Introduction of a silent mutation in the *LeuA* gene

In order to allow differentiation between the wild type and the transformed *LeuA* gene and thus discriminate between cured transformants and reverted mutants, a silent mutation introducing an additional *Xba*I restriction site within the coding sequence of the *LeuA* gene was created. The plasmid pEUKA400 contains one copy of the *LeuA* coding sequence with 2244 bp and four restriction sites for *Xba*I as shown in Figure 2.1 and Table 3.4. An additional *Xba*I restriction site at position 5697 of the plasmid and position 992 of the LeuA coding sequence was generated by site directed-mutagenesis using mutagenic primers. In the mutated gene, the C at position 992 is replaced by A (Figure 3.10), thus establishing the restriction site without changing the encoded amino acid.

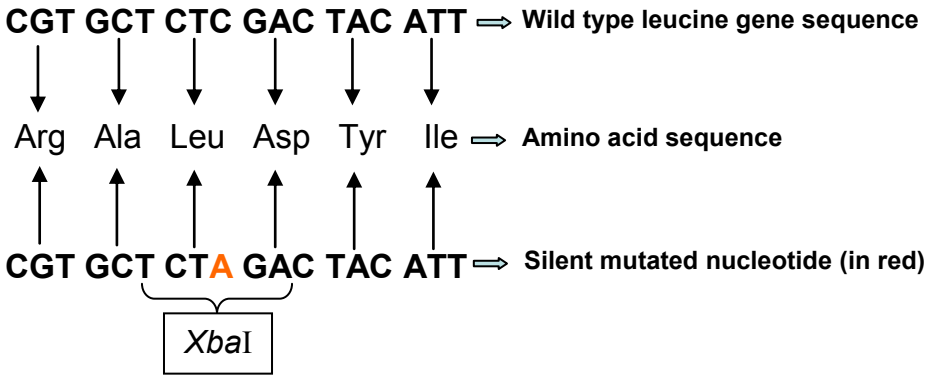

Figure 3.10: **Nucleotide sequences of the wild type and silent mutated *LeuA* gene.**

The mutation was confirmed by digesting both the intact plasmid (Figure 3.11) and the PCR amplified silent mutated leucine gene (Figure 3.12) with *Xba*I and by gene sequencing. For that, the plasmid were purified from the transformed *E. coli* XL-1 Blue.

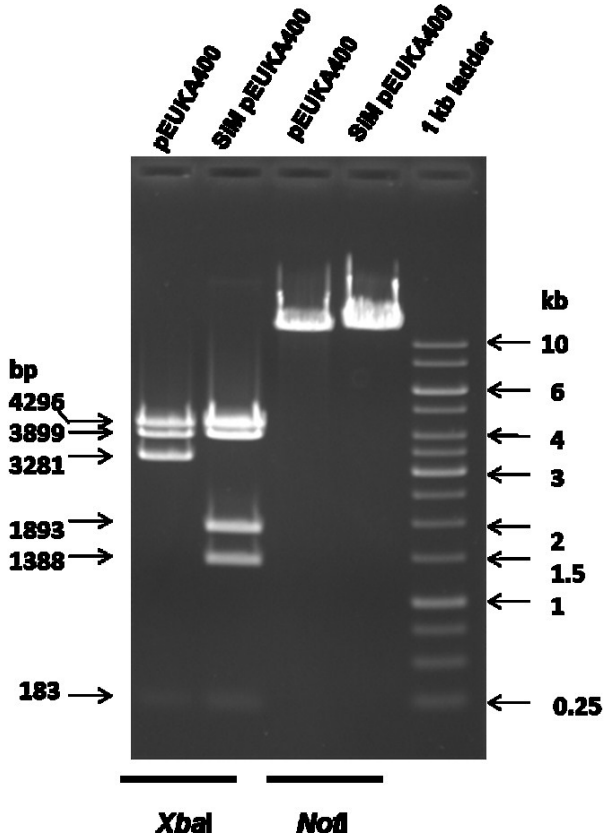

Figure 3.11: **Confirmation of the silent mutation in the *LeuA* gene. I.** DNA of the original plasmid pEUKA400 and the silently mutated SiM pEUKA400 after digestion with *Xba*I and *Not*I and separation by agarose gel electrophoresis. Ethidium-bromide stained agarose gel; fragment sizes are indicated on the left side.

Figure 3.11 shows the electrophoretic fragment pattern of the complete plasmid DNA of pEUKA400 and SiM pEUKA400 after digestion with two different restriction endonucleases. pEUKA400 contains only a single restriction site for *Not*I, and this was not changed by the vector modification. Both pEUKA400 and SiM pEUKA400 separated as a single fragment of the same size after digestion with *Not*I. Digestion with *Xba*I led to the formation of four fragments in pEUKA400 and 5 fragments in SiM pEUKA400, mirroring the existence of four and five restriction sites, respectively. The length of the individual fragments corresponds well with the predicted sizes (Table 3.4)

Table 3.4: **Digestion pattern of pEUKA400 and SiM pEUKA400**

Restriction enzyme	number of cuts	fragment length [bp]
pEUKA400		
*Not*I	1	11659
*Xba*I	4	183, 3281, 3899, 4296
SiM pEUKA400		
*Not*I	1	11659
*Xba*I	5	183, 1388, 1839, 3899, 4296

Vector analysis with the SIM Vector 4.5 program

When the coding sequence of the *LeuA* gene was amplified by PCR from the vectors and digested with *Xba*I, one band spanning the whole 2244 bp of the coding sequence was obtained from the pEUKA400 amplificated while the SiM pEUKA400 amplificate was cleaved into two fragments of 1252 and 992 bp, respectively, again proving the existence of a single *Xba*I restriction site within the *LeuA* gene sequence (Figure 3.12).

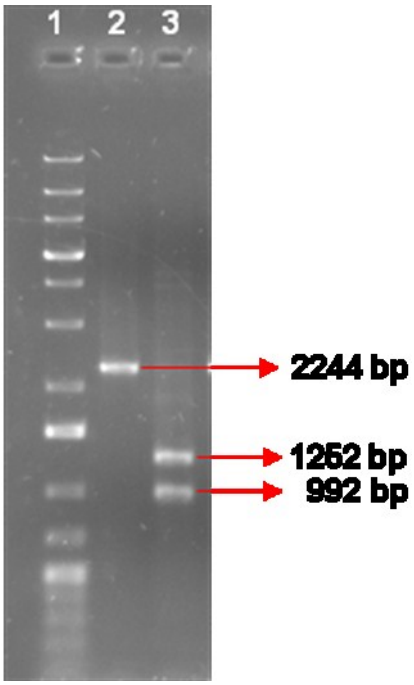

Figure 3.12: **Confirmation of the silent mutation in the *LeuA* gene. II.** Plasmid DNA was purified from the transformants and separated after *Xba*I digestion of the *LeuA* coding sequence. Ethidium bromide stained agarose gel, lane 1: 1 kb plus DNA ladder; lane 2: Wild type *LeuA* gene amplified by PCR from *E. coli* XL-1 Blue transformed with pEUKA400; lane 3: Silent mutated *LeuA* gene amplified by PCR SiM pEUKA400.

3.3.2 Transformation and isolation of transformants

The Ku70 MO was designed and used in addition to transformation of protoplasts of the *M. circinelloides* R7B leucine auxotroph with the vector SiM pEUKA400 carrying the silent mutated leucine A gene. Transformation was performed by electroporation in several experiments. The overall transformation efficiency was at approximately 25 - 30 transformants / µg DNA.

After regeneration, all protoplasts were transferred to stabilized minimal medium, where transformants now containing the plasmid copy of the *LeuA* gene were able to grow. The progeny of these colonies were tested for the presence of the *LeuA* gene. From control experiments, where the *M. circinelloides* R7B protoplasts were subjected to electroporation, but neither the plasmid nor Ku70 MO was added, no colonies were obtained on minimal medium, the mycelia remained auxotrophic for leucine.

Individual transformants were selected randomly in order to check the integration of the *LeuA* marker in *M. circinelloides* R7B and to characterize the different integrative events. Therefore, DNA was isolated from the selected transformants and subjected to molecular analysis by Southern hybridization.

3.3.3 Southern analysis of the transformants

For Southern blot analysis of the transformant DNA, two different probes were employed. The first, pTZ19R, hybridizes exclusively with the original phagemid part of the vector pEUKA400, comprising the origin of replication, the f1 origin, and the ampicillin resistance gene, but not with any of the later alterations incorporating the eukaryotic elements. Hybridization with this probe served to determine whether a transformation took place or not. The second probe was the 2.2 kb wild type *LeuA* gene amplified by PCR from the pEUKA400 vector (Figure 3.12). The genomic DNA of the transformants was digested with the *Xba*I restriction enzyme prior to hybridization to reveal the additional internal *Xba*I restriction site in the *LeuA* gene when transformed with SiM pEUKA400 when compared to the wild type gene. Figure 3.13A shows the result of the hybridization with pTZ19R. The blot was then probed again with the *LeuA* probe to reveal transfer and integration of the *LeuA* gene in the recipient genomic background (Figure 3.13B).

Probing with labeled pTZ19R should reveal a fragment of 4296 bp spanning the region between the *Xba*I restriction sites at positions 11489 and 4126 of both pEUKA400 and SiM pEUKA400. It shows at a size of 4.0 kb on the blot (Figure 3.13A). The 4296 bp fragment would also appear in transformants bearing the complete vector as autonomously replicating plasmid, in addition to a band with the size of the whole plasmid, at around 11000 kb. Incorporation as autonomously replicating plasmid occurred in 20 % of all cases. Ectopic integration with pTZ19R hybridizing with fragments of various length, was more common. On the other hand the pTZ19R probe should give no signal when hybridized with DNA isolated from wild type and R7B mutant of *M. circinelloides*. This was proven by the Southern blot: the pTZ19R probe hybridized to both

vector DNAs and to fragments obtained from three of the four types of transformants (Figure 3.13A).

When probed with the *LeuA* gene probe, wild type and mutant type DNA did give signals, as well as the pEUKA400 and SiM pEUKA400, which was to be expected (Figure 3.13B). In DNA from *M. circinelloides* wild type and the *leuA* defective mutant, the *Xba*I-fragment binding to the *LeuA* probe should be of the same size, as the defect consists in a few point mutations only, which would not interfere recognizably with hybridization. This fragment, at around 5.0 kb, was detected as well in T1, T2, T3, and T4, representing the defective gene of the recipient strain (Figure 3.13B).

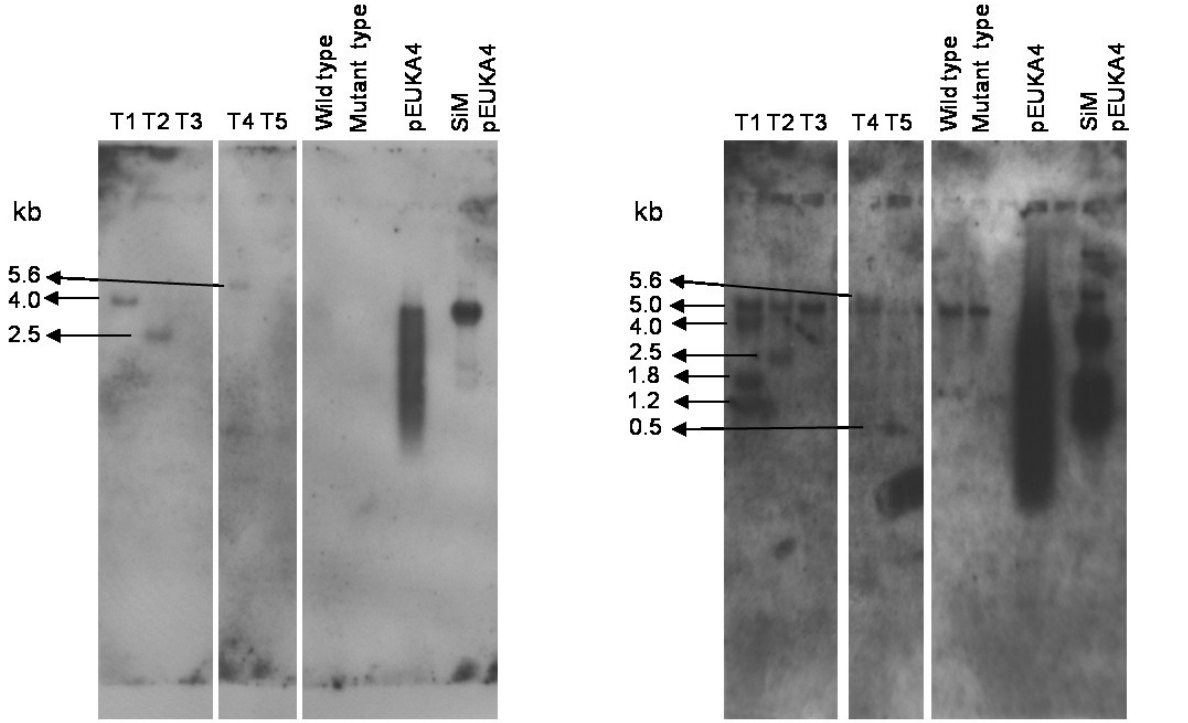

Figure 3.13: **Southern analysis of *LeuA* gene transformants.** Total DNA (6 µg) from wild type *M. circinelloides* (wild type) *M. circinelloides* R7B (mutant type), and *M. circinelloides* R7B transformed with SiM pEUKA400 (T1, T2, T3, T4 and T5), as well as plasmid DNA from pEUKA400 and SiM pEUKA400 was digested with *Xba*I, separated by agarose gel electrophoresis, blotted by capillary transfer to a nylon membrane and probed with either (A) DIG-labelled pTZ19R or (B) the DIG-labelled 2.2 kB *LeuA* gene amplified by PCR from the vector pEUKA400. The numbers on the left indicates the approximate size in kb.

The various transformants could all be grouped into one of the four distinct types, represented by T1 –T5 in Figure 3.13 A and B.

The band pattern of the T1 transformant shows a 4.0 kb band (Figure 3.13A), when hybridized with the vector probe and fragments of, 1.8 kb, 1.2 kb and at 5.0 kb (Figure 3.13B), when hybridized with the *LeuA* probe. The 1.8 kb and the 1.2 kb fragments are the result of cleavage at the novel *Xba*I restriction site in the *LeuA* coding sequence of the transformant DNA, which should appear when the plasmid SiM pEUKA400 is not integrated into the recipient genome. In general, the band pattern was identical to that of the SiM pEUKA400 plasmid strain (Figure 3.13B), and thus confirms this transformant to harbour the vector as autonomous replicating plasmid.

In contrast, the DNA of the T2/T4 type transformants revealed ectopically integrated copies of the *LeuA* gene as well as vector sequences, a majority of the integration events being attributable to non-homologous recombination.The band pattern of the shown T2 transformant DNA (Figure 3.13AB) is very interesting because it shows an intact 5.0 kb band when hybridised with the *LeuA* probe besides a novel 2.5 kb band (Figure 3.13B), and also a 2.5 kb fragment when hybridised with the vector probe. This reveals that the original *LeuA* gene has not been shifted and thus no homologous recombination has occurred. Nevertheless, there is a strong possibility of ectopic integration of SiM pEUKA400 at some other position. In the T4 transformant, the situation is similar, only here, the additional copy is found on a 5.6 kb fragment.

The band pattern of the T3 transformant (Figure 3.13A,B), is similar to the wild type and mutant type genomic DNA, showing the 5.0 kb fragment when hybridised with the *LeuA* probe. As no hybridization signal was seen with the vector probe, this was judged not to be a true transformant.

In the genomic digests of the T5 transformant DNA, the vector probe did not show up on the blot (Figure 3.13A), indicating that the plasmid was altered in a way that resulted in its subsequent loss. But also the parental 5.0 kb signal in the blot probed with the labelled *LeuA* fragment is very faint, indicating some alteration at this locus, too. A similarly weak band of approximately 4.0 kb and a

stronger one of 0.5 kb appear instead (Figure 3.13B). This hybridization pattern may be the result of an integration of the *LeuA* gene of SiM pEUKA400 into the *M. circinelloides LeuA* gene by homologous recombination.

As a conclusion, analysis of the transformants allowed the detection of three different integration events: 1) homologous recombination, 2) ectopic integration and 3) autonomous replication of the vector. Homologous recombination and ectopic integration occurred at higher frequencies among the transformants.

4. Discussion

4.1 Protoplast generation and regeneration

When organisms are encased within a thick and rigid cell wall, this provides considerable limitations to feasible experimental procedures. In research on plants and fungi, protoplast formation is therefore one of the basic necessities to obtain suitable material for experiments. This is especially true for any experiment where fusion of one cell with the next is required (Cocking 1979; Hinnen et al. 1978, Wöstemeyer and Brockhausen-Rohdemann 1987), and also for the delivery of macromolecules like DNA into cells when more individual methods, like microinjection, are not applicable. Protoplasts of fungi have also been used for the isolation of organelles such as mitochondria or vacuoles. Protoplast generation is a process that strongly depends on a combination of factors, among others the physical and chemical properties of the cell wall, the developmental age of the organism, temperature, the enzyme(s) used, and the duration of cell wall hydrolysation. The procedure needs to be adjusted according to the strain and experimental conditions. Optimized protocols for several zygomycete fungi already exist, so for *R. niveus* (Yanai et al. 1990), *A. glauca* (Wöstemeyer and Brockhausen-Rohdemann 1987), and several species of *Mucor*, among them *M. circinelloides* (van Heeswijck 1984). Conditions for *M. mucedo* have not been previously determined.

The first attempts at protoplasting were started with lytic enzymes purified from a variety of species (Peberdy 1979). Such preparations usually contain more than one protein species with different activities. With the zygomycetes, it was also soon found, that any single such enzyme sometimes had no effect, and a mixture of at least two different enzymes was necessary, reflecting the complex composition of the zygomycete cell walls with glucans, a small amount of chitin and 30 to 40 % chitosan (Bartnicki-Garcia and Nickerson 1962b). Without a chitinase activity, no lysis was observed in any species (van Heeswijck 1984; Yanai et al. 1990). For *A.*

glauca, a preparation from *Streptomyces* No. 6 proved sufficient for cell wall lysis (Wöstemeyer and Brockhausen-Rohdemann 1987), but not for *Mucor* (van Heeswijck 1984), and a purified chitosanase alone had also no effect on *R. niveus* (Yanai et al. 1990). When cultivated under inducing conditions, *Streptomyces* No. 6, as many related species, produces chitinase, chitosanase and cellulase (Gomes et al. 2000; Gupta et al. 1995; Taechowisan et al. 2003). The efficiency of cell wall lysis by the streptozyme mixture was observed to depend directly on the chitosanase content by van Heeswijck (1984) and on the specific activity of the chitosanase by Wöstemeyer and Brockhausen-Rohdemann (1987). They also determined a very high protease activity in the streptozyme mixture which might help with digestion of the cell walls. For the *Mucor* species and *Rhizopus*, the addition of Novozym 234, a glucanase-containing preparation from *Trichoderma harzianum*, proved necessary (van Heeswijck 1984; Yanai et al. 1990), and this was also the case for *M. mucedo*.

Enzyme treatment in *M. mucedo* provided much faster results than in the other species, with only 1 h until harvesting of the protoplasts. With *R. niveus* and different *Mucor* sp., incubation time was 4 – 6 and 4 hours, respectively. Although it is not possible to compare the absolute amounts of enzyme used in each treatment, this result cannot be caused by just using higher concentrations of the lytic enzymes. Here, as in all other studies, it was found that from an optimal amount, higher concentrations resulted in protoplast damage and reduced yields (van Heeswijck 1984; Wöstemeyer and Brockhausen-Rohdemann 1987; Yanai et al. 1990). Also, extended period of enzyme treatment usually result in deterioration of the protoplasts. In *Metarhizium anisopliae* and *Trichoderma harzianum,* this is ascribed to the detrimental effect of proteinase activities in the lytic enzyme mixtures (Kitamoto et al. 1988).

Similar to the other strains, it was found that very young germination stages and sporangiospores of *M. mucedo* were not lysed by the enzymes. The reason for that lies in the different composition of the spore wall compared to the hyphal wall of the germlings. Cultures older than 16 hours were also unsuitable for protoplast

formation, as the yield drastically dropped. This was also observed for *R. niveus*. One reason for this observation might be the increasing vacuolization, leading to altered turgor pressure inside the hyphae. Older mycelia of *A. glauca* and other *Mucor* species were found to be outright resistant to cell wall hydrolysis (van Heeswijck 1984; Wöstemeyer and Brockhausen-Rohdemann 1987). In other fungi, too, not only the formation, but also the regeneration and viability of protoplasts decreases with the age of the mycelium or tissue (Peng et al. 1993; Jain et al. 1992).

Another problem specific for *Mucor* sp. is the asynchronous spore germination. No trigger to manipulate the onset of germination is known, other than in *P. blakesleeanus*, where germination can be started reliably by heat shock treatment or acidification (Cerda-Olmedo and Lipson 1987). van Heeswijck (1984) alleviated this problem by pre-incubation for 4 hours. In the present study, the temperature and aeration regime, swelling of the spores at ice-bath temperature followed by gentle agitation only in the last phase, secured reasonable synchronicity of development.

As protoplasts are susceptible to osmolysis, they need to be suspended in a suitable stabilizer solution. Sorbitol is the most commonly used osmotic stabilizer for all fungal protoplasts with concentrations ranging from 0.8 M - 1.2 M. Other stabilizers reported are 0.8 M mannitol for *Coprinus cinereus* (Binninger et al. 1987), 0.6 - 0.7 M NaCl for various ascomycetes (Ballance and Turner 1985; Diez et al. 1987; Picard et al. 1987) and 1.2 M magnesium sulfate for *Aspergillus nidulans* (Tilburn et al. 1983). Magnesium sulfate or other inorganic salts proved generally unsuitable in all zygomycetes, as they either prevent protoplast formation or suppress the regeneration (van Heeswijck 1984; Wöstemeyer and Brockhausen-Rohdemann 1987; Yanai et al. 1990). With respect to stabilization by sugars or sugar alcohols, zygomycetes require generally lower concentrations than the ascomycetes mentioned above. With 0.6 M sorbitol, *M. mucedo* fits well into the range defined by *R. niveus* / *Mucor* sp. and *A. glauca*, from 0.35 – 0.5 M to 0.7 M, respectively. With all species, the concentration of the stabilizer was found to be more important than

the actual substance used (van Heeswijck 1984; Wöstemeyer and Brockhausen-Rohdemann 1987; Yanai et al. 1990).

The type of osmotic stabilizer also affects the shape and size of the protoplasts (Pfeifer and Khachatourians 1987; Hou and Jong 1985). Those released from *M. mucedo*, with 5 - 10 µm in diameter were in the same range as those reported for other *Mucor* species (van Heeswijck 1984), and smaller than those from *R. niveus* with 10 - 20 µm (Yanai et al. 1990). The diameter of the parent hypha seems to play no role, as the various *Mucor* species show marked differences in this aspect.

In the present study, the regeneration frequency was 70 % of the initial protoplast number for both *M. mucedo* and *M. circinelloides*. This regeneration frequency is rather good when compared to other studies and in about the same range as those observed for the ascomycetes such as *A. nidulans* and *Penicillium chrysogenum*, with 50 % and 60 %, respectively (Peng et al. 1993). For *M. circinelloides* it is also higher than the ≤ 40% reported by van Heeswijck (1984) and the ≥ 35% in *A. glauca* (Wöstemeyer and Brockhausen-Rohdemann 1987), and excellent compared to the 4 % regeneration rate seen in *R. niveus* (Yanai et al. 1990). This latter finding is somewhat surprising, as larger protoplasts should also contain more of the vesicular secretory apparatus for cell wall synthesis. However, low regeneration frequencies might also be due to unfavorable media or the use of inappropriately aged mycelia.

Regeneration of the protoplasts was also found to depend on their original location in the hyphae, and on the presence of nuclei or other organelles in *Fusarium culmorum, Glomerella cingulata* and *A. nidulans* (Garcia-Acha et al. 1966; Rodriguez and Yoder 1987; Peberdy and Gibson 1971), respectively. With the zygomycete germlings, this should not play any role, as the protoplasts emerge mainly from the growing tip of very young hyphae, were numerous nuclei are present (Figure 3.3).

4.2 *CrgA*-mediated regulation of sexual carotene production

Before the MO-mediated knockdown of gene expression could be used as tool for genetic analysis, a proof for their usefulness in zygomycetes was needed. In *Mucor* and related species, only very few of the already characterized genes are responsible for a measurable and obvious phenotype. We selected the *crgA*-mediated regulation of carotene production because this transcriptional repressor has already been characterized in detail (Navarro et al. 1995; 2000; 2001; Lorca-Pascual et al. 2004) and the intended knock-down should provide a measurable increase in β-carotene even under the restrictions posed by the experimental system. Light is a necessary inducer of β-carotene biosynthesis in *M. circinelloides* (Navarro et al. 1995) while in *crgA*-null mutants, a high amount of carotene is produced even in the dark (Navarro et al. 1995; 2001). *M. mucedo* also shows photocarotenogenesis, but carotene formation is also induced by a mating partner or the induction with trisporoids (Bu'Lock et al. 1972; Gooday et al. 1973).

One difficulty was with the time frame of the experiment (see below). In the very young cultures necessarily used in the experiment, light-induced carotene production is negligible. Therefore, sexual induction of carotene formation was tested and found to increase the carotene accumulation sufficiently. In that experiment, the cultures were between 36 and 48 h old when the TA was added, and were analyzed 24 h later. Cultures of that age are in a developmental stage where sexual reactions are already possible and zygophore formation will occur when cultivated on solid medium (Schimek et al. 2003, 2005; Wöstemeyer and Schimek 2007). Younger mycelia show no reactions on solid medium, but here it was found, that even germlings already react to stimulation with trisporic acid and produced measurable amounts of β-carotene. A possible explanation for the observed similarities in the level of carotene accumulation between the 24 h TA-treated undisturbed mycelia, and the 12 h TA-treated protoplasted cultures is, that the undisturbed mycelia grew in the same medium for the complete cultivation period and might have exhausted the available carbohydrate supply. The regenerated protoplasts were around 30 h

old at the time of analysis. With both procedures, only a very small amount of β-carotene accumulated when no TA was added.

In the cultures treated with the *crgA*-MO, an increase in carotene accumulation was observed. That result would have been the expected outcome for the light-regulated carotene production. The present study instead used the sexual induction pathway, therefore the result suggests that *crgA* is also involved in the regulation of sex-mediated carotenogenesis. This adds to the known cellular responses underlying regulation by that transcription factor. It is already known that *crg*A is also involved in the regulation of vegetative growth Quiles-Rosilo et al. (2003) and asexual sporulation (Nicolas et al. 2008), putting it at a par with other more general regulators, for example the FL transcription factor regulating macroconidiation in *N. crassa* (Springer, 1993). Based on the translated amino acid sequence, the CrgA protein contains a LON domain, characteristic for ATP-dependent protease activity and a RING-finger zinc-binding domain putting it in the E3 family of ubiquitin ligases that are involved in targeting proteins for degradation in proteasomes (Nicolas et al. 2008). This RING-finger motif plus one of the glutamine-rich stretches are essential for both regulatory CrgA effects, supporting the hypothesis that CrgA is a component of a pathway for ubiquitination and degradation of hitherto unknown target proteins involved in light-regulated responses of *M. circinelloides* (Lorca-Pasqual et a. 2004), and now also *M. mucedo*. Sequence comparison of the *crgA* fragment cloned from *M. mucedo* with the complete gene of *M. circinelloides* (Joint Genome Institute Genome database) shows, that most of the LON domain and part of the RING-zinc-binding-domain are present, also all of the glutamine-rich regions. This allows for full functionality of the protein in *M. mucedo*, too. In future steps, obtaining the complete sequence of the *M. mucedo* gene and expression analyses will help to define the actual function of the gene in this species. The shown regulation of the sex-regulated carotenogenesis already either enlarges the number of pathways regulated by CrgA, or puts both light- and sex-induction of carotene synthesis upstream of the target of *crgA*-mediated repression.

An overview of the effects of *crg*A in carotene regulation is shown in Figure 4.1.

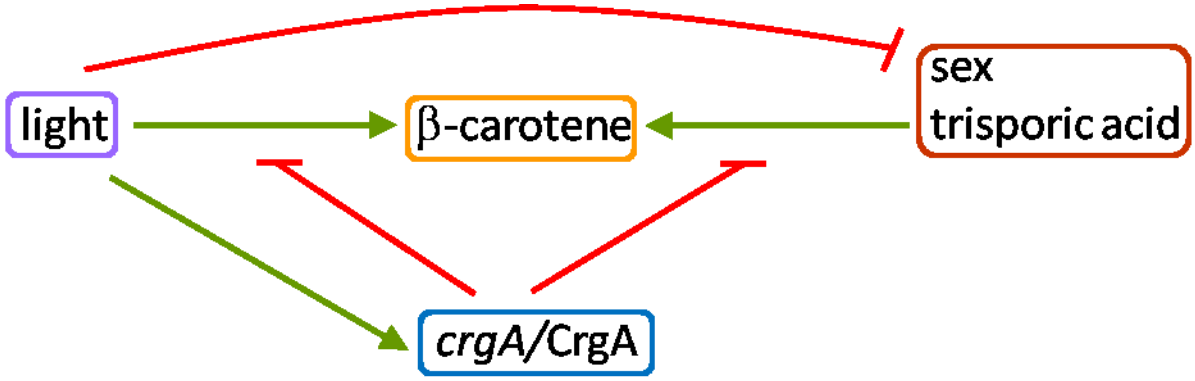

Figure 4.1: **Regulatory connections between light, trisporic acid, carotene production, and *crgA*.** Removal or blocking of the crgA input will lead to higher carotene accumulation. green: inducing connection; red: suppressing connection.

4.3 Gene silencing by Morpholino oligonucleotides

Although efficient and stable transformation systems are well established for certain ascomycetes, especially yeasts and *Aspergillus* sp., there are apparently large difficulties in developing such systems for basidiomycetes (Ulrich et al. 1985, Li et al. 2006), and also in zygomycetes. Apparently, specific mechanisms for the detection and elimination of foreign DNA exist in these groups. One of that mechanisms, siRNA-mediated RNA interference, has been studied in more detail (Nicolas et al. 2007; 2009; 2010). The authors transformed *M. circinelloides* with a self-replicating plasmid containing the wild type *carB* gene necessary for carotene synthesis. This was found to increase the number of transformants silenced in *carB* expression up to 30-fold, and all the resulting albino mutants were found to contain high copy numbers of the *carB*-carrying plasmid. As hypothesis, the introduced plasmid triggers gene silencing by inducing the RNA-dependent RNA polymerase to synthesize dsRNA to the plasmid template which then serves as target for dicer in the production of siRNA (Nicolas et al. 2009).

The silencing effect was also manufactured into a knockdown tool in manipulation of the *carRP* gene of *M. circinelloides* (Nicolas et al. 2008). This was done as described already, by transformation with sense *carRP* included in the vector. In 72 % of the transformants, post-transcriptional silencing occurred, and *carRP* mRNA was not detectable any longer. Instead, the transformants had accumulated 21 nt-sized siRNA.

In the present study, silencing was attempted not by RNAi, but by blocking translation of the mRNA with morpholino oligonucleotides designed to bind to the transcript. The design is important, as MO are especially effective when they bind to the 5' leader sequences or to the first 25 bp after the AUG translation start codon (Heasman 2002). The proposed technique, to bind the MO as close to the translation initiation site as possible and thus blocking the assembly of the initiation complex, was not possible, because the 5' part of the *M. mucedo* coding sequence could not be cloned. A MO based on the corresponding *M. circinelloides* region did not result in any effect on carotene production, indicating that the sequence identity near the translation start is not high enough between the two genes to ensure proper binding of MOs between the species. Database search reveals a short intron in the 5' region of the *M. circinelloides crgA* gene. The CrgA MO was therefore designed to hybridize to the 5' region of the identified *crgA* fragment from *M. mucedo* and the observed effect proves its functionality. It can be concluded, that this MO did block translation of the crgA mRNA either during elongation of the peptide chain, or by some other effect, possibly interfering with splicing of the pre-mRNA. This is another way MOs might act (Summerton 1999).

When using siRNA for gene silencing, in many cases only a transient effect is generated. With few exceptions, even autonomously replicating plasmids cannot be interminably maintained in the transformant strains. The siRNA is degraded together with the target mRNA, and new transcripts are being produced all the time and may overrun the effect of the siRNA. MO are safe from enzymatic degradation and thus can repeatedly silence newly made transcripts. Actually, they will also loose function

over time, as the nuclei divide and new transcripts are formed, so that in the end the MOs get diluted to a degree that makes efficient blocking impossible. The useful period for MO applications therefore depends on the transcription and translation characteristics of the target mRNA (Heasman 2002). In the experiment suppressing *crgA* expression in *M. mucedo*, the gene was supposed to be transcribed continuously, because the cultures were kept in the dark. CrgA was described first as repressor of carotene synthesis in the dark (Navarro et al. 1995). In that experiment, the dilution of the effect was well established, so that the analysis of carotene production had to be done already 12 h after delivery of the MOs. Longer incubation led to reduced carotene accumulation, presumably because more and more unblocked transcript was available.

4.4 Down-regulation of Ku70 by MO leads to a higher rate of integrative transformation events in *M. circinelloides*

The Ku70 knockdown strategy was used to promote the generation of integrative mutants. In that case, it was possible to use homologous sequence information in the MO design. Dilution effects leading to sub-functional concentrations of the MO could also be neglected, as the transformants were selected not for a physiological effect, but for a single event: integration or at least expression of the vector DNA, conferring prototrophy to the recipient. The transformation system consisted of a *M. circinelloides* mutant with defects in the single copy *LeuA* gene and a transformation vector manufactured to cure this defect. The vectors pEUKA400 and SiM pEUKA400 (Figure 2.1) are derivates of the minimal cloning vector pEUKA11 (J. Arnau, A. Burmester, personal communications; Appel et al. 2004). They are both suitable for autonomous replication in the *M. circinelloides* background, because they contain a hitherto unidentified ARS (Appel et al. 2004). Both plasmids contain a single *Not*I restriction site, and in SiM pEUKA400 the functional copy of the *LeuA* gene was silently mutated to display an internal *Xba*I restriction site. The fragments obtained after *Xba*I digestion of the intact plasmids and the amplified LeuA-fragments showed that this construct was indeed the only *Xba*I restriction site within the *LeuA* gene

fragment. This distinguishing feature could therefore be used to discriminate between the defective mutant copy and the vector copy after transformation.

The reversion frequency of the leucine auxotrophic strain *M. circinelloides* R7B is < 1.7 x 10^{-8} (van Heeswijck and Roncero 1984). This is due to the character of the mutation, which consists of several single nucleotide deletions and substitutions. The mutations responsible for the loss of function are proposed to be the substitution of a T to an A, leading to an amino acid substitution from Glu to Lys, and a frame shift towards the end of the coding sequence, resulting in an extension of the reading frame over the wild type stop codon (Appel et al. 2004). Reversing both alterations is supposedly a very rare event, but nevertheless the reversion frequency was re-checked prior to the transformation experiment. From a total number of 1.25 x 10^8 spores, no progeny was able to grow to full maturity on minimal medium. Six spores formed initially small colonies that were not able to maintain growth under selective conditions. The spores for that test were inoculated to 5 x 10^6 per plate, so that the few growing spores can be attributed to contamination of the minimal medium by deteriorating germlings and spores in their surrounding. This was considered to be of no effect for the outcome of the transformation experiment.

In transformation of zygomycetes, great attention needs to be paid to the conditions facilitating or enforcing the maintenance of the transformed plasmid or gene(s) in the recipient. Prolonged cultivation under selective conditions is generally necessary and will aid in stabilization of the transformants (Arnau et al. 1993), as loss of the plasmid will lead to death or stalled development. The high mitotic instabilities encountered by Anaya and Roncero (1991) and the other groups cited below, and also the marked segregation of transformed traits like GFP-expression (Schilde et al. 2001; Bartsch et al. 2002) are the result of the coenocytic nature of zygomycete mycelia and the fact that in most species the mitotic spores contain several nuclei.

In most cases, an auxotrophy marker was chosen, because they show advantages compared to dominant selection markers like antibiotic resistance genes. Development of resistance or effects leading to slow adaptation over time are thus excluded. As the present experiment was directed at identifying homologous recombination / integration at the *LeuA*-locus, the transformation was done without using the antibiotic resistances also transferred by the vector SiM pEUKA400 as selection marker. Integration of these genes at the *LeuA* site would have rendered the transformants incapable of surviving. On Neomycin/Kanamycin selection medium, surviving colonies could have masked the progeny of true homologous recombination events. Neomycin and other antibiotic resistances were already found to be of no great value for establishing positive transformants in *M. circinelloides* (van Heeswijck and Roncero 1984, Appel et al. 2004) and other zygomycetes (Wöstemeyer et al. 1987; Burmester et al. 1990).

Auxotrophies as single selection markers were employed in the strategy of the Roncero/Arnau groups (Anaya and Roncero 1991; Arnau et al. 1991; Arnau and Stroman 1993; Wada et al. 1996; Wolff and Arnau 2002; Appel et al. 2004) and others (Nicolas et al. 2008). The important factor in this strategy is the strength of the mutant. It should be impossible for the intended recipient to compensate the defect by alterations in other regulatory or metabolic pathways. Binary vectors combining auxotrophy and dominant markers have been found especially useful in *Agrobacterium*-mediated transformation (Michielse et al. 2004; Wei et al. 2010).

In ascomycetes, *Ku70* knockout mutants show an increase in homologous integration events. In *N. crassa* and *Aspergillus oryzae Ku* disruption strains yielded 100% transformants exhibiting integration at the homologous site, compared to only 10 – 30 % for a wild-type recipient (Ninomiya et al. 2004, Takahashi et al. 2006a). A *Ku70* mutant strain in *Sordaria macrospora* also produced 100 % homologous integration of exogenous DNA compared to 0.1 and 5 % in the wild type strains (Pöggeler and Kück 2006). The deletion of the *akuB*^{KU80} gene in *A. fumigatus* increased the frequency of homologous recombination (da Silva Ferreira et al.

2006). In *Botrytis cinerea*, deletions of the *Bcku70* gene in the B05.10 strain and the *Bcku80* gene in the T4 strain both affected the NHEJ DNA repair mechanism, and improved HR efficiency (Choquer et al. 2008). From these studies it appears that the creation of knockout mutants is easy in ascomycetes. From the above mentioned difficulties in stabilization of any mutant phenotype, arising from the possible coexistence of mutated and unaffected nuclei in the same compartment or spore, a similar efficiency was not to be expected for the Ku70-MO approach in *M. circinelloides*. Nevertheless, co-transfer of the Ku70-MO and the transformation vector resulted in a higher ratio of integrated transformants, both ectopically and by HR, and reduced the chance of autonomous replication of the vector. Similar ARS containing vectors were previously found not to promote any integration at all (Anaya and Roncero 1991). Expression of the *ku70*-gene of *M. circinelloides* was therefore sufficiently reduced by the Ku70-MO and the effect on the NHEJ repair apparatus allowed the observed integration events.

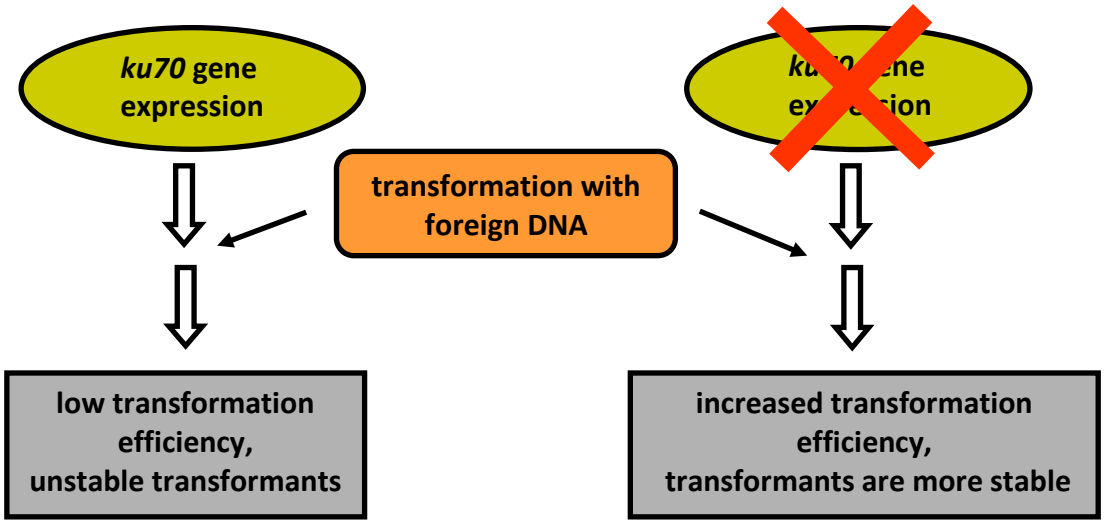

Figure 4.2: Model for the effect of down-regulation of *ku70* gene expression on transformation efficiency.

Previously, several hundred transformants had to be screened by Southern blot analysis in transformation experiments of *M. circinelloides* to obtain proof for integrative transformants among the numerous non-integrative progeny (Wolff and Arnau 2002). The most remarkable effect of the MO strategy was the high number of integrative events considering the small total number of transformants. Down-regulation of the *Ku70* gene in *M. circinelloides* resulted in roughly 50 – 60 % of integrative events when the MO was delivered together with the transformation vector. Around 20 % of the transformants were found to contain the autonomously replicating plasmid, indicating that the bias was shifted towards integration. It cannot be excluded that the residual progeny might also have harboured plasmid sequences, but in a very low copy number and way below the detection limit of the Southern analysis.

MO have been shown for the first time to be a functional tool in knockdown strategies in *Mucor*. This findings open up intriguing new avenues for all kinds of analyses requiring genetic manipulations in fungi and especially in zygomycetes. The necessary experiments to follow this first study are to determine the silencing frequency of the MOs in different transformation systems and the fate of the transformed gene(s) in the homologous or ectopic integration events.

References

Acs, K., Z. Kasza, et al. (2002). "Cloning and sequence analysis of Mucor circinelloides glyceraldehyde-3-phosphate dehydrogenase gene." Acta Microbiol Immunol Hung 49: 305-312.

Adams, P. R. and J. J. Deploey (1976). "Amylase production by Mucor miehei and M. pusillus." Mycologia 68: 934-893.

Anaya, N. and M. I. Roncero (1991). "Transformation of a methionine auxotrophic mutant of Mucor circinelloides by direct cloning of the corresponding wild type gene." Mol Gen Genet 230: 449-455.

Appel, K. F. A. M. Wolff, et al. (2004). "A multicopy vector system for genetic studies in Mucor circinelloides and other zygomycetes." Mol Genet Genomics 271: 595-602.

Arnau, J., L. P. Jepsen, et al. (1991). "Integrative transformation by homologous recombination in the zygomycete Mucor circinelloides." Mol Gen Genet 225: 193-198.

Arnau, J., F. J. Murillo, et al. (1988). "Expression of Tn5-derived kanamycin resistance in the fungus Phycomyces blakesleeanus." Mol Gen Genet 212: 375-377.

Arnau, J. and P. Stroman (1993). "Gene replacement and ectopic integration in the zygomycete Mucor circinelloides." Curr Genet 23: 542-546.

Austin, D. G., J. D. Bu'Lock, et al. (1969). "Trisporic acid biosynthesis and carotenogenesis in Blakeslea trispora." Biochem J 113: 34.

Ballance, D. J. (1986). "Sequences important for gene expression in filamentous fungi." Yeast 2: 229-236.

Ballance, D. J., F. P. Buxton, et al. (1983). "Transformation of Aspergillus nidulans by the orotidine-5'-phosphate decarboxylase gene of Neurospora crassa." Biochem Biophys Res Commun 112: 284-289.

Ballance, D. J. and G. Turner (1985). "Development of a high-frequency transforming vector for Aspergillis nidulans." Gene 36: 321-331.

Barber, A.J. (1988). "Evening primrose oil: A panacea." Pharm J 240: 723-725.

Barnett, H. L., V. G. Lilly, et al. (1956). "Increased production of carotene by mixed + and - cultures of Choanephora cucurbitarum." Science 123: 141-142.

Bartnicki-Garcia, S. and E. Lippman (1969). "Fungal morphogenesis: cell wall construction in Mucor rouxii." Science 165: 302-304.

Bartnicki-Garcia, S. and W. J. Nickerson (1961). "Thiamine and Nicotinic Acid: Anaerobic Growth Factors for Mucor Rouxii." J Bacteriol 82: 142-148.

Bartnicki-Garcia, S. and W. J. Nickerson (1962a). "Nutrition, growth, and morphogenesis of Mucor rouxii." J Bacteriol 84: 841-858.

Bartnicki-Garcia, S. and W. J. Nickerson (1962b). "Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of Mucor rouxii." Biochim Biophys Acta 58: 102-119.

Bartsch, S., C. Schimek, et al. (2002) "Microprojectile bombardment as a reliable method for transformation of the mucoralean fungus Absidia glauca." Mycoscience 43:213-217.

Beach, D. and P. Nurse (1981). “High-frequency transformation of the fission yeast Schizosaccharomyces pombe.” Nature (London) 290:140-142.

Beggs, J. D. (1978). “Transformation of yeast by a replicating hybrid plasmid.” Nature (London) 275: 104-109.

Benito, E. P., V. Campuzano, et al. (1995). "Isolation, characterization and transformation, by autonomous replication, of Mucor circinelloides OMPdecase-deficient mutants." Mol Gen Genet 248: 126-135.

Benito, E. P., J. M. Diaz-Minguez, et al. (1992). "Cloning and sequence analysis of the Mucor circinelloides pyrG gene encoding orotidine-5'-monophosphate decarboxylase: use of pyrG for homologous transformation." Gene 116: 59-67.

Benny, G. L., R. A. Humber, et al. (2001). "Zygomycota: Zygomycetes." In: The Mycota VII. Systematics and Evolution. Part A. (McLaughlin, D. J., McLaughlin, E. G. and Lemke, P. A., eds.). Springer-Verlag, New York, pp. 113-146.

Bertani, G. (1951). "Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli." J Bacteriol 62: 293-300.

Biebl, H., and N. Pfennig (1978). "Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate-reducing bacteria." Arch Microbiol 117: 9-16.

Binninger, D. M., C. Skrzynia, et al. (1987). "DNA-mediated transformation of the basidiomycete Coprinus cinereus." Embo J 6: 835-840.

Birnboim, H. C. and J. Doly (1979). "A rapid alkaline extraction procedure for screening recombinant plasmid DNA." Nucleic Acids Res 7: 1513-1523.

Blakeslee, A. F. (1906). "Zygospores and sexual strains in the common bread mould, Rhizopus nigricans." Science 24: 118-122.

Boulton, S. J. and S. P. Jackson (1998). "Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing." Embo J 17(6): 1819-28.

Britton, G., S. Liaaen-Jensen, et al. (1995). "Carotenoids." Vols I and II. Birkhäuser Verlag. Basel.

Bronstein, I., R. R. Juo, et al. (1991). "Novel chemiluminescent adamantyl 1,2-dioxetane enzyme substrates." In: Stanley P. and Kricka L. J. (eds.), Bioluminescence and Chemiluminescence, John Wiley, Chichester England, pp. 73-82.

Bu'Lock, J. D., D. Drake, et al. (1972). "Specificity and transformations of the trisporic acid series of fungal sex hormones." Phytochemistry 11: 2011-2018.

Bu'Lock, J. D., B. E. Jones, et al. (1976). "The apocarotenoid system of sex hormones and prohormones in Mucorales." Pure Appl Chem 47: 191– 202.

Bullock, W.O., Fernandez, J.M. and Stuart, J.M. (1987). "XL 1-Blue: A high efficiency plasmid transforming *recA Escherichia coli* strain with beta galactosidase selection." BioTechniques 5: 376-379.

Burmester, A., A. Wöstemeyer, et al. (1990). "Integrative transformation of a zygomycete, Absidia glauca, with vectors containing repetitive DNA." Curr Genet 17: 155–161.

Burmester, A. (1992). "Transformation of the mycoparasite Parasitella simplex to neomycin resistance." Curr Genet 21: 121-124.

Burmester, A., M. Richter, et al. (2007). "Cleavage of beta-carotene as the first step in sexual hormone synthesis in zygomycetes is mediated by a trisporic acid regulated beta-carotene oxygenase." Fungal Genet Biol 44: 1096-1108.

Burmester, A., A. Wöstemeyer, et al. (1992). "The SEG1 element: a new DNA region promoting stable mitotic segregation of plasmids in the zygomycete Absidia glauca." Mol Gen Genet 235: 166-172.

Burmester, A. and J. Wostemeyer (1987). "DNA sequence and functional analysis of an ARS-element from the zygomycete Absidia glauca." Curr Genet 12: 599-603.

Buxton, F. P., and A. Radford (1984). "The transformation of mycelial spheroplasts of Neurospora crassa and the attempted isolation of an autonomous replicator." Mol Gen Genet 196: 337-344.

Caglioti, L., G. Cainelli, et al. (1966). "The structure of trisporic-C acid." Tetrahedron Lett 7: 175-177.

Case, M. E., M. Schweizer, et al. (1979). "Efficient transformation of Neurospora crassa by utilizing hybrid plasmid DNA." Proc Natl Acad Sci USA 76: 5259-5263.

Cerda-Olmedo, E. (2001). "Phycomyces and the biology of light and color". FEMS Microbiol Rev 25: 503–512.

Cerda-Olmedo, E., E. D. Lipson (1987). "Phycomyces". Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 337-339.

Cerda, G. A., J. E. Thomas, et al. (2006). "Electroporation of DNA, RNA, and morpholinos into zebrafish embryos." Methods 39: 207-211.

Chang, P. K. (2008). "A highly efficient gene-targeting system for Aspergillus parasiticus." Lett Appl Microbiol 46: 587-592.

Choquer, M., G. Robin, et al. (2008). "Ku70 or Ku80 deficiencies in the fungus Botrytis cinerea facilitate targeting of genes that are hard to knock out in a wild-type context." FEMS Microbiol Lett 289: 225-232.

Ciegler, A., M. Arnold, et al. (1959). "Microbiological production of carotenoids. IV. Effect of various grains on production of beta-carotene by mated strains of Blakeslea trispora." Appl Microbiol 7: 94-98.

Cocking, E. C. (1979). "All sorts of protoplasts." Nature 281: 180-181.

Corey, D. R. and Abrams, J. M. (2001). "Morpholino antisense oligonucleotides: tools for investigating vertebrate development." Genome Biol 2:reviews 1015.1–1015.3

Corrochano, L. M., P. Galland, et al. (1988). "Photomorphogenesis in Phycomyces: fluence-response curves and action spectra." Planta 174: 315-320.

Cullen, D., G. L. Gray, et al. (1988). "Molecular cloning vectors for Aspergillus and Neurospora." Biotechnol 10: 419-433.

da Silva Ferreira, M. E., M. R. Kress, et al. (2006). "The akuB(KU80) mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in Aspergillus fumigatus." Eukaryot Cell 5: 207-211.

Diez, B., E. Alvarez, et al. (1987). "Selection and characterization of pyrG mutants of Penicillium chrysogenum lacking orotidine-5'-phosphate decarboxylase and complementation by the pyr4 gene of Neurospora crassa." Curr Genet 12: 277-282.

Dorsett, Y. and T. Tuschl (2004). "siRNAs: applications in functional genomics and potential as therapeutics." Nat Rev Drug Discov 3: 318-329.

Draper, B. W., P. A. Morcos, et al. (2001). "Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown." Genesis 30: 154-156.

Edwards, J. A., V. Schwarz, et al. (1971). "Fungal sex hormones. The synthesis of 7, 9 -trisporic acid B methyl ester. The stereochemistry at C-9 of the trisporic acids." Chem Commun 292-293.

Eslava, A. P., M. I. Alvarez, et al. (1975). "Genetic recombination in sexual crosses of Phycomyces." Genetics 80: 445-462.

Gadau, M. (1992). "Protoplast fusion in fungi." in Handbook of Applied Mycology, Vol. 4: Fungal Biotechnology, Arora, D.K., Elander, R.P. and Mukerji, K.G., eds., Marcel Dekker, Inc., New York, pp. 101-128.

Garcia-Acha, I., F. Lopez-Belmonte, et al. (1966). “Regeneration of mycelial protoplasts of Fusarium culmorum.” J Gen Microbiol 45: 515-523.

Goins, C. L., K. J. Gerik, et al. (2006). "Improvements to gene deletion in the fungal pathogen Cryptococcus neoformans: Absence of Ku proteins increases homologous recombination, and co-transformation of independent DNA molecules allows rapid complementation of deletion phenotypes." Fungal Genet Biol 43: 531-544.

Gomes, R. C., L. T. Semedo, et al. (2000). "Chitinolytic activity of actinomycetes from a cerrado soil and their potential in biocontrol." Lett Appl Microbiol 30: 146-150.

Gooday, G. W., Fawcett P. et al. (1973). "Formation of fungal sporopollenin in zygospore wall of Mucor mucedo – role for sexual carotenogenesis in Mucorales." J Gen Microbiol 74: 233-239.

Gooday, G. W. (1994). “Hormones in mycelial fungi.” In: Wessels, J. G. H., and Meinhardt, F. (eds). The Mycota. Springer-Verlag, New York, pp 401-411.

Gonzalez-Hernandez, G. A., L. Herrera-Estrella, et al. (1997). "Biolistic transformation of Mucor circinelloides." Mycol Res 101: 953–956.

Govind, N. S. and E. Cerda-Olmedo (1986). "Sexual activation of carotenogenesis in phycomyces blakesleeanus." Gen Microbiol 132: 2775-2780.

Guangtao, Z. L. Hartl, et al. (2009). "Gene targeting in a nonhomologous end joining deficient Hypocrea jecorina." J Biotechnol 139: 146–151.

Gupta, R., R. K. Saxena, et al. (1995). "Chitinase production by Streptomyces viridificans: its potential in fungal cell wall lysis." J Appl Bacteriol 78: 378-383.

Gonzales-Hernanadez, G. A., L. Herrera-Estrella, et al. (1997). "Biolistic transformation of Mucor circinelloides." Mycol Res 101: 953-956.

Gutierrez, A., S. Lopez-Garcia, et al. (2011). "High reliability transformation of the basal fungus Mucor circinelloides by electroporation." J Microbiol Methods 84: 442-446.

Haarmann, T., N. Lorenz, et al. (2008). "Use of a nonhomologous end joining deficient strain (Deltaku70) of the ergot fungus Claviceps purpurea for identification of a nonribosomal peptide synthetase gene involved in ergotamine biosynthesis." Fungal Genet Biol 45: 35–44.

Hall, T. A. (1999). "BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT." Nucleic Acids Symp Ser 41: 95–98.

Heasman, J. (2002). "Morpholino oligos: making sense of antisense?" Dev Biol 243: 209-214.

Hinnen, A., J. B. Hicks, et al. (1978). "Transformation of yeast." Proc Natl Acad Sci USA 75: 1929-1933.

Hoff, B., J. Kamerewerd, et al. (2010). "Homologous recombination in the antibiotic producer Penicillium chrysogenum: strain DeltaPcku70 shows up-regulation of genes from the HOG pathway." Appl Microbiol Biotechnol 85:1081–1094.

Horiuchi, H., N. Takaya, et al. (1995). "Cloning of the Rhizopus niveus pyr4 gene and its use for the transformation of Rhizopus delemar." Curr Genet 27: 472-478.

Hou, H.H. and S. C. Jong (1985). “Protoplast formation from mycelia of Penicillium digitatum by cell wall-lytic enzymes of Trichoderma harzianum.” J Fermen Technol 63: 189-192.

Hsiao, C. L. and J. Carbon (1979). “High-frequency transformation of yeast by plasmids containing the cloned yeast ARG4 gene.” Proc Natl Acad Sci USA 76: 3829-3833.

Hudziak, R. M., E. Barofsky, et al. (1996). "Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation." Antisense Nucleic Acid Drug Dev 6: 267-272.

Hutchinson, H. T. and L. H. Hartwell (1967). “Macromolecule synthesis in yeast spheroplasts.” J Bacteriol 94:1697-1705.

Hynes, M. J. (1986). "Transformation in filamentous fungi." Exp Mycol 10: 1-8.

Ingold, C.T. (1978). "The biology of Mucor and its allies." Edward Arnold Ltd., London, pp. 1-17.

Iturriaga, E. A., J. M. Diaz-Minguez, et al. (1992). "Heterologous transformation of Mucor circinelloides with the Phycomyces blakesleeanus leu1 gene." Curr Genet 21: 215-223.

Jain, S. M., E. A. Shahin, et al. (1988). "Interspecific fusion for the transfer of atrazine resistance from solanum nigrum to tomato (Lycopersicon esculentum)." Plant Cell Tiss Org Cult 12: 189-192.

Jantti, J., T. Nikkari, et al. (1989). "Evening primrose oil in rheumatoid arthritis: changes in serum lipids and fatty acids." Ann Rheum Dis 48: 124-127.

Jones, D., J. S. D. Bacon, et al. (1968). "Lysis of cell walls of Mucor ramannianus Moller by a Streptomyces sp." J Microbiol Serol 83: 173-182

Jubin R. (2005). "Optimizing electroporation conditions for intracellular delivery of morpholino antisense oligonucleotides directed against the hepatitis C virus internal ribosome entry site." Methods Mol Med 106: 309-322.

Kawai, S., W. Hashimoto, et al. (2010). "Transformation of *Saccharomyces cerevisiae* and other fungi. Methods and possible underlying mechanism." Bioeng Bugs 1: 395-403.

Kitamoto, Y., N. Mori, et al. (1988). "A simple method for protoplast formation and improvement of protoplast regeneration from vaarious fungi using an enzyme from Trichoderma harzianum." Appl Microbiol Biotechnol 28: 445-450.

Krappmann, S. (2007). "Gene targeting in filamentous fungi: the benefits of impaired repair." Fungal Biol Rev 21: 25–29

Krappmann, S., C. Sasse, et al. (2006). "Gene targeting in Aspergillus fumigatus by homologous recombination is facilitated in a nonhomologous end- joining-deficient genetic background." Eukaryot Cell 5: 212-215

Kitamoto, Y., N. Mori, et al. (1988). “A simple method for protoplast formation and improvement of protoplast regeneration from various fungi using an enzyme from Trichoderma harzianum.” Appl Microbiol Biotechnol 28: 445-450.

Kuzina, V. and E. Cerda-Olmedo (2006). "Modification of sexual development and carotene production by acetate and other small carboxylic acids in Blakeslea trispora and Phycomyces blakesleeanus." Appl Environ Microbiol 72: 4917-4922.

Levy, M., A. Erental, et al. (2008). "Efficient gene replacement and direct hyphal transformation in Sclerotinia sclerotiorum." Mol Plant Pathol 9: 719–725.

Li, G., R. Li, et al. (2006). "A highly efficient polyethylene glycol-mediated transformation method for mushrooms." FEMS Microbiol Lett. 256: 203-208.

Linden, H., P. Ballario, et al. (1997). "Blue light regulation in Neurospora crassa." Fungal Genet Biol 22: 141-150.

Linnemanstöns, P., M. M. Prado, et al. (2002). "A carotenoid biosynthesis gene cluster in Fusarium fujikuroi: the genes carB and carRA." Mol Genet Genomics 267: 593-602.

Liou, C. M., K. Yanai, et al. (1992). "Transformation of a Leu- mutant of Rhizopus niveus with the leuA gene of Mucor circinelloides." Biosci Biotechnol Biochem 56: 1503-1504.

Lorca-Pascual, J. M., L. Murcia-Flores, et al. (2004). "The RING-finger domain of the fungal repressor crgA is essential for accurate light regulation of carotenogenesis." Mol Microbiol 52: 1463-1474.

Lübbehüsen, T. L., J. Nielsen, et al. (2003). "Characterization of the Mucor circinelloides life cycle by on-line image analysis." J Appl Microbiol 95: 1152-1160.

Machlis, L. (1972). "The coming of age of sex hormones in plants." Mycologia 64: 235-247.

Meister, G., and T. Tuschl (2004). "Mechanisms of gene silencing by double-stranded RNA." Nature. 431: 343-349.

Meyer, V., M. Arentshorst, et al. (2007). "Highly efficient gene targeting in the Aspergillus niger kusA mutant." J Biotechnol 128: 770–775

Michielse, C. B., K. Salim, et al. (2004). "Development of a system for integrative and stable transformation of the zygomycete Rhizopus oryzae by Agrobacterium-mediated DNA transfer." Mol Genet Genomics 271: 499-510.

Mishra, N. C., G. Szabo, et al. (1973). “Nucleic acid induced genetic changes in Neurospora.” In: M. C. Niu and S. J. Segal (ed.), The role of RNA in reproduction and development. Elsevier/North Holland Publishing Co.. Amsterdam, pp. 259-268.

Mishra, N. C. and E. L. Tatum (1973). “Non-mendelian inheritance of DNA-induced inositol independence in Neurospora.” Proc. Natl. Acad. Sci. USA 70: 3875-3879.

Morcos, P. A. (2001). "Achieving efficient delivery of morpholino oligos in cultured cells." Genesis 30: 94-102.

Munoz-Rivas, A., C. A. Specht, et al. (1986). "Transformation of the basidiomycete Schizophyllum commune." Mol Gen Genet 205:103-106.

Murillo, F. J. and E. Cerda-Olmedo (1976). "Regulation of carotene synthesis in Phycomyces." Mol Gen Genet 148: 19-24.

Nakayashiki, H., Q. B. Nguyen et al. (2008). "RNA interferece: role in fungal biology." Curr Opin Microbiol 11: 494-502.

Navarro, E., J. M. Lorca-Pascual, et al. (2001). "A negative regulator of light-inducible carotenogenesis in Mucor circinelloides." Mol Genet Genomics 266: 463-470.

Navarro, E., V. L. Ruiz-Perez, et al. (2000). "Overexpression of the crgA gene abolishes light requirement for carotenoid biosynthesis in Mucor circinelloides." Eur J Biochem 267: 800-807.

Navarro, E., G. Sandmann, et al. (1995). "Mutants of the carotenoid biosynthetic pathway of Mucor circinelloides." Exp Mycol 19: 186-190.

Nayak, T., E. Szewczyk, et al. (2006). "A versatile and efficient gene-targeting system for Aspergillus nidulans." Genet 172: 1557–1566.

Nelson, M. A., G. Morelli, et al. (1989). "Molecular cloning of a Neurospora crassa carotenoid biosynthetic gene (albino-3) regulated by blue light and the products of the white collar genes." Mol Cell Biol 9: 1271-1276.

Nelson, P. T., A. G. Hatzigeorgiou, et. al. (2004). "miRNP:mRNA association in polyribosomes in a human neuronal cell line." RNA 10: 387-394.

Nicolas, F. E., J. P. de Haro, et al. (2007). "Mutants defective in a Mucor circinelloides dicer-like gene are not compromised in siRNA silencing but display developmental defects." Fungal Genet Biol 44: 504-516.

Nicolas, F. E., S. Moxon, et al. (2010). " Endogenous short RNAs generated by Dicer 2 and RNA-dependent RNA polymerase 1 regulate mRNAs in the basal fungus Mucor circinelloides." Nucleic Acids Res 38: 5535-5541.

Nicolas, F. E., S. Calo, et al. (2008). "A RING-finger photocarotenogenic repressor involved in asexual sporulation in Mucor circinelloides." FEMS Microbiol Lett 280: 81-88.

Nicolas, F.E., S. Torres-Martinez, et al. (2003). "Two classes of small antisense RNAs in fungal RNA silencing triggered by non-integrative transgenes." EMBO J 22: 3983–3991.

Nicolas, F. E., S. Torres-Martínez, et al. (2009). "Transcriptional activation increases RNA silencing efficiency and stability in the fungus Mucor circinelloides." J Biotechnol 142: 123-126.

Nieuwenhuis, M. (1975). "Sex specificity of hormone synthesis in Mucor mucedo." Arch Microbiol 102: 167-169.

Ninomiya, Y., K. Suzuki, et al. (2004). "Highly efficient gene replacements in Neurospora strains deficient for nonhomologous end-joining." Proc Natl Acad Sci USA 101: 12248-12253.

Nyilasi, I., K. Acs, et al. (2005). "Agrobacterium tumefaciens-mediated transformation of Mucor circinelloides." Folia Microbiol (Praha) 50: 415-420.

Nyilasim I., T. Papp, et al. (2008). "Agrobacterium tumefaciens-mediated transformation of the zygomycete fungus Backusella lamprospora." J Basic Microbiol 48: 59-64.

Ochi, T., B. L. Shibanda, et al. (2010). "Structural biology of DNA repair: spatial organisation of the multicomponent complexes of nonhomologous end joining." J Nucleic Acids, Vol. 2010, Article ID 621695, doi:10.4061/2010/621695

Partridge, M., A. Vincent, et al. (1996). "A simple method for delivering Morpholino antisense oligos into the cytoplasm of cells." Antisense Nucleic acid Drug Dev 6: 169-175.

Pastwa, E. and J. Blasiak (2003). "Non-homologous DNA end joining." Acta Biochem. Pol. 50: 891–908.

Peberdy, J. F. (1979). "Fungal protoplasts: isolation, reversion, and fusion." Annu Rev Microbiol 33: 21-39.

Peberdy, J. F. and R. K. Gibson (1971). "Regeneration of Aspergillus nidulans protoplasts." J Gen Microbiol 69: 325-330.

Peng, M., P. A. Lemke, et al. (1993). “Improved conditions for protoplast formation and transformation of Pleurotus ostreatus.” Appl Microbiol Biotechnol 40: 101-106.

Pfeifer, T. A. and G. G. Khachatourians (1987). “The formation of protoplasts from Beauveria bassiana." Appl Microbiol Biotechnol 26: 248-253.

Picard, M., R. Debuchy, et al. (1987). "Transformation by integration in Podospota aniser-inati. 11. Targeting to the resident locus with cosmids and instability of the transformants." Mol Gen Genet 210: 129-134.

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

Quiles-Rosillo, M. D., R. M. Ruiz-Vazquez, et al. (2005). "Light induction of the carotenoid biosynthesis pathway in Blakeslea trispora." Fungal Genet Biol 42: 141-153.

Quiles-Rosillo, M. D., S. Torres-Martínez S, et al. (2003). "cigA, a light-inducible gene involved in vegetative growth in Mucor circinelloides is regulated by the carotenogenic repressor crgA." Fungal Genet Biol 38: 122-132.

Rambosek, J. and J. Leach (1987). "Recombinant DNA in filamentous fungi: progress and prospects." Crit Rev Biotechnol 6: 357-393.

Revuelta, J. L. and M. Jayaram (1986). "Transformation of Phycomyces blakesleeanus to G-418 resistance by an autonomously replicating plasmid." Proc Natl Acad Sci U S A 83: 7344-7347.

Ribes, J. A., C. L. Vanover-Sams, et al. (2000). "Zygomycetes in human disease." Clin Microbiol Rev 13: 236-301.

Ripley, G. J. and P. M. Bramley (1976). "The subcellular distribution of carotenoids in Phycomyces blakesleeanus C115 car-42 mad-107 (-)." Biochem Biophys Acta 450: 429-440.

Ripley, G. J. and P. M. Bramley (1982). "The biosynthesis of carotenes in cell organelles of Phycomyces blakesleeanus C115 carS42 mad-107 (-)." Cytobios 34: 97-104.

Rodriguez, R. J. and O. C. Yoder (1987). "Selectable genes for transformation of the fungal plant pathogen Glomerella cingulata f. sp. phaseoli (Colletotrichum lindemuthianum)." Gene 54: 73-81.

Roncero, M. I., L. P. Jepsen, et al. (1989). "Characterization of a leuA gene and an ARS element from Mucor circinelloides." Gene 84: 335-343.

Rosen, J. N., M. F. Sweeney, et al. (2009). "Microinjection of zebrafish embryos to analyze gene function." J Vis Exp 25: DOI: 10.3791/1115

Rossier, C., A. Pugin, et al. (1985). "Genetic analysis of transformation in a micro-conidiating strain of Neurospora crassa." Curr Genet 10: 313-320.

Ruiz-Diez, B. (2002). "Strategies for the transformation of filamentous fungi." J Appl Microbiol 92: 189-195.

Ruiz-Hidalgo, M. J., E. P. Benito, et al. (1997). "The phytoene dehydrogenase gene of Phycomyces: Regulation of its expression by blue light and vitamin A." Mol Gen Genet 253: 734-744.

Sambrook, J. and D. W. Russell (2001). "Molecular cloning: a laboratory manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sambrook, J., J. Fritsch, et al. (1989). "Molecular Cloning: a laboratory manual." 2nd ed. N.Y., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, 1659 p. ISBN 0-87969-309-6.

Schachtschabel, D., A. David, et al. (2008). "Cooperative biosynthesis of Trisporoids by the (+) and (-) mating types of the zygomycete Blakeslea trispora." Chembiochem 9: 3004-3012.

Scherer, L. and J. J. Rossi (2004). "RNAi applications in mammalian cells." Biotechniques 36: 557-561.

Schilde, C., J. Wöstemeyer, et al. (2001). "Green fluorescent protein as a reporter for gene expression in the mucoralean fungus Absidia glauca." Arch Microbiol 175:1-7.

Schimek, C., K. Kleppe, et al. (2003). "Sexual reactions in Mortierellales are mediated by the trisporic acid system." Mycol Res 107: 736-747.

Schimek, C., A. Petzold, et al. (2005). "4-Dihydromethyltrisporate dehydrogenase, an enzyme of the sex hormone pathway in Mucor mucedo, is constitutively transcribed but its activity is differently regulated in (+) and (-) mating types." Fungal Genet Biol 42: 804-812.

Schimek, C., and J. Wöstemeyer (2006). "Pheromone actions in the fungal groups Chytridiomycota, and Zygomycota, and in the Oomycota." In: The Mycota I; Kües, U. and Fischer R. eds., pp 215-231.

Schimek, C. and J. Wöstemeyer (2009). "Carotene derivatives in sexual communication of zygomycete fungi." Phytochem 70: 1867-1875.

Schmidhauser, T. J., F. R. Lauter, et al. (1990). "Cloning, sequence, and photoregulation of al-1, a carotenoid biosynthetic gene of Neurospora crassa." Mol Cell Biol 10: 5064-5070.

Schmidhauser, T. J., F. R. Lauter, et al. (1994). "Characterization of al-2, the phytoene synthase gene of Neurospora crassa. Cloning, sequence analysis, and photoregulation." J Biol Chem 269: 12060-12066.

Schultze, K., C. Schimek, et al. (2005). "Sexuality and parasitism share common regulatory pathways in the fungus Parasitella parasitica." Gene 348: 33-44.

Skory, C. D. (2002). "Homologous recombination and double-strand break repair in the transformation of Rhizopus oryzae." Mol Genet Genomics 268: 397-406.

Smith, P. K., R. I. Krohn, et al. (1985). "Measurement of protein using bicinchoninic acid." Anal Biochem 150: 76–85.

Snoek, I. S., Z. A. van der Krogt, et al. (2009). "Construction of an hdfA Penicillium chrysogenum strain impaired in nonhomologous end-joining and analysis of its potential for functional analysis studies." Fungal Genet Biol 46: 418-426

Somkuti, G. A. (1974). "Synthesis of cellulase by Mucor pusillus and Mucor miehei." J Gen Microbiol 81: 1-6.

Somkuti, G. A. and F. J. Babel (1968). "Lipase activity of Mucor pusillus." Appl Microbiol 16: 617-619.

Southern, E. (1975). "Detection of specific sequences among DNA fragments separated by gel electrophoresis." J Mol Biol 98: 503-517.

Springer, M. L. (1993). "Genetic control of fungal differentiation: the three sporulation pathways of Neurospora crassa." Bioessays 15: 365-374.

Struhl, K., D. T. Stinchcomb, et al. (1979). "High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules." Proc Natl Acad Sci USA 76: 1035-1039.

Suarez, T. and A. P. Eslava (1988). "Transformation of Phycomyces with a bacterial gene for kanamycin resistance." Mol Gen Genet 212: 120-123.

Summerton, J. (1999). "Morpholino antisense oligomers: the case for an RNase H-independent structural type." Biochem Biophys Acta 1489: 141-158.

Summerton, J., D. Stein, et al. (1997). "Morpholino and phosphorothioate antisense oligomers compared in cell-free and in-cell systems." Antisense Nucleic Acid Drug Dev 7: 63-70.

Summerton, J. and D. Weller (1997). "Morpholino antisense oligomers: design, preparation, and properties." Antisense Nucleic Acid Drug Dev 7: 187-195.

Sutter, R. P. (1970). "Trisporic acid synthesis in Blakeslea trispora." Science 168: 1590-1592.

Sutter, R. P., D. A. Capage, et al. (1973). "Trisporic acid biosynthesis in separate plus and minus cultures of Blakeslea trispora: identification by Mucor assay of two mating-type-specific components." J Bacteriol 114: 1074-1082.

Sutter, R. P., A. B. Grandin, et al. (1996). "(-) mating type-specific mutants of Phycomyces defective in sex pheromone biosynthesis." Fungal Genet Biol 20: 268-279.

Sutter, R. P. and J. P. Whitaker (1981). "Zygophore-stimulating precursors (pheromones) of trisporic acids active in (-)-Phycomyces blakesleeanus. Acid-catalyzed anhydro derivatives of methyl 4-dihydrotrisporate-C and 4-dihydrotrisporate-C." J Biol Chem 256: 2334-2341.

Taechowisan, T., J. F. Peberdy, et al. (2003). "Chitinase production by endophytic Streptomyces aureofaciens CMUAc130 and its antagonism against phytopathogenic fungi." Ann Microbiol 53: 447-461.

Takahashi, T., T. Masuda, et al. (2006a). "Enhanced gene targeting frequency in ku70 and ku80 disruption mutants of Aspergillus sojae and Aspergillus oryzae." Mol Genet Genomics 275: 460-470.

Takahashi, T., T. Masuda, et al. (2006b). "Identification and analysis of Ku70 and Ku80 homologs in the koji molds Aspergillus sojae and Aspergillus oryzae." Biosci Biotechnol Biochem 70: 135-143.

Takaya, N., K. Yanai, et al. (1996). "Cloning and characterization of the Rhizopus niveus leu1 gene and its use for homologous transformation." Biosci Biotechnol Biochem 60: 448-452.

Tako, M. and A. Csernetics (2005). "Genotypic analysis of variability in Zygomycetes. A mini-review." Acta Biol Hung 56: 345-357.

Tilburn, J., C. Scazzocchio, et al. (1983). “Transformation by integration in Aspergillus nidulans.” Gene 26: 205-221.

Tonouchi, N., H. Shoun, et al. (1986). "Cloning and sequencing of a gene for Mucor rennin, an aspartate protease from Mucor pusillus." Nucleic Acids Res 14: 7557-7568.

Ulrich, R. C., C. P. Novotny, et al. (1985). "Transforming basidiomycetes." Mol Genet of Filamentous Fungi. Alan R. Liss, Inc. pp. 39–57.

Valentina, C., V. Mariarosaria, et al. (2011). "Use of a non-homologous end-joining-deficient strain (delta-ku70) of the biocontrol fungus Trichoderma virens to investigate the function of the laccase gene lcc1 in sclerotia degradation." Curr Genet 57:13–23.

van den Ende, H. (1967). "Sexual factor of the Mucorales." Nature 215: 211-212.

van den Ende, H. (1968). "Relationship between sexuality and carotene synthesis in Blakeslea trispora." J Bacteriol 96: 1298-1303.

van den Ende, H., and D. Stegwee (1971). "Physiology of sex in mucorales." Botanical Rev 37: 22-36.

van den Ende, H., A. H. Wiechmann, et al. (1970). "Hormonal interactions in Mucor mucedo and Blakeslea trispora." J Bacteriol 101(2): 423-428.

van Heeswijck, R. (1984). "The formation of protoplasts from Mucor species." Carlsberg Res Commun 49: 597-609.

van Heeswijck, R. (1986). "Autonomous replication of plasmids in Mucor transformants." Carlsberg Res Commun 51: 433-443.

van Heeswijk, R. and M. I. G. Roncero (1984). "High frequency transformation of Mucor with recombinant plasmid DNA." Carlsberg Res Commun 48: 691-702.

Verdel, A., S. Jia, et al. (2004). "RNAi-mediated targeting of heterochromatin by the RITS complex." Science 303: 672-676.

Velayos, A., J. L. Blasco, et al. (2000). "Blue-light regulation of phytoene dehydrogenase (carB) gene expression in Mucor circinelloides." Planta 210(6): 938-946.

Vogelstein, B. and D. Gillespie (1979). "Preparative and analytical purification of DNA from agarose." Proc Natl Acad Sci USA 76: 615-619.

Wada, M., T. Beppu, et al. (1996). "Integrative transformation of the zygomycete Rhizomucor pusillus by homologous recombination." Appl Microbiol Biotechnol 45: 652-657.

Wei, D. S., Y. H. Zhang, et al. (2010). "Agrobacterium rhizogenes-mediated transformation of a high oil-producing filamentour fungus Umbelopsis isabellina." J Appl Genet 51:225-232.

Weiner, M. P., G.L. Costa, et al. (1994). "Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction." Gene 151: 119–123.

Werkman, B. A. (1976). "Localization and partial characterization of a sex-specific enzyme in homothallic and heterothallic Mucorales." Arch Microbiol 109: 209–213.

Wetzel, J., O. Scheibner, et al. (2009). "4-dihydrotrisporin-dehydrogenase, an enzyme of the sex hormone pathway of Mucor mucedo: purification, cloning of the corresponding gene, and developmental analysis." Eukaryot Cell 8: 88-95.

Wöstemeyer, J. (1985). "Strain-dependent variation in ribosomal DNA arrangement in Absidia glauca." Eur J Biochem 146: 443-448.

Wöstemeyer, J., A. Burmester, et al. (1987). "Neomycin resistance as a dominantly selectable marker for transformation of the zygomycete Absidia glauca." Curr Genet 12: 625-627.

Wöstemeyer, J., A. Burmester, et al. (2002). "Gene transfer in the fungal host-parasite system Absidia glauca-Parasitella parasitica depends on infection." In: Syvanen M, Cado CI eds. Horizontal Gene Transfer, Academic Press, New York, pp. 237-247.

Wöstemeyer, J. and E. Brockhausen-Rohdemann (1987). "Inter-mating type protoplast fusion in the zygomycete Absidia glauca." Curr Genet 12: 435-441.

Wöstemeyer, J., and C. Schimek (2009). "Trisporic acid and mating in zygomycetes." In: Sex in Fungi. Molecular Determination and Evolutionary Implications. Heitman J et al. eds. pp 431-443.

Wolff, A. M., and J. Arnau (2002). "Cloning of glyceraldehyde-3-phosphate dehydrogenase-encoding genes in Mucor circinelloides (Syn. racemosus) and use of the gpd1 promoter for recombinant protein production." Fungal Genet Biol 35: 21-29.

Yamada, T., K. Makimura, et al. (2009). "Enhanced gene replacements in Ku80 disruption mutants of the dermatophyte, Trichophyton mentagrophytes." FEMS Microbiol Lett 298:208–217.

Yanai, K., H. Horiuchi, et al. (1990). "Preperation of protoplasts of Rhizopus niveus and their transformation with plasmid DNA." Agric Biol Chem 54: 2689–2696.

Yelton, M. M., J. E. Hamer, et al. (1984). "Transformation of Aspergillus nidulans by using a trpC plasmid." Proc Natl Acad Sci USA 81: 1470-1474.

Zoberi, M. H. (1985). "Liberation of asexual propagules in the Mucorales." Bot J Linn Soc 91: 167-173.

Declaration

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of the Friedrich Schiller University, Jena that the submitted thesis was written only with the assistance and literature cited in the text.

The thesis has not been previously submitted either to the Friedrich Schiller University, Jena or to any other University.

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